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

Application Note

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Introduction

Human neural stem cells (hNSCs) are multipotent stem cells of the nervous system that can self-renew and give rise to neurons, astrocytes, and oligodendrocytes. These cells have tremendous potential in basic neuroscience research, tissue engineering, and cell-based therapies for neurodegenerative diseases. These applications require robust culture conditions that allow for efficient large-scale expansion and differentiation of hNSCs. Previously, we have shown that hNSCs can be cultured on Corning rLaminin-521 (Human), which supports the self-renewal of hNSCs in a serum-free environment[1]. Here, we developed rLaminin-521 precoated ready-to-use cultureware and demonstrated undifferentiated expansion of hNSCs (H9 hESCs-derived) on the surface. Corning® PureCoat™ rLaminin-521 cultureware utilizes the rLaminin-521 (Human) matrix in a ready-to-use form, minimizing time and variability from self-coating. Human NSCs cultured on PureCoat rLaminin-521 cultureware exhibited doubling time and viability comparable to cells cultured on user-coated rLaminin-521 and Poly-L-Ornithine with Laminin (PLO/Laminin). Human NSCs expressed undifferentiated NSC markers Nestin and SOX2 during the course of the culture. Post-expansion, cells were successfully differentiated into astrocytes, oligodendrocytes, and neurons on all three surfaces. Corning PureCoat rLaminin-521 cultureware offers an animal-free ready-to-use platform for hNSC expansion and differentiation.

Materials and 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) were 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). PLO/Laminin coated plates were stored at room temperature with 3 mL DPBS without calcium and magnesium that was aspirated just prior to use. Corning PureCoat rLaminin-521 cultureware (Corning Cat. No. 356290) were removed from storage at 4°C 30 minutes prior to use to equilibrate to room temperature. 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 of StemPro® NSC SFM (Thermo Fisher Cat. No. A1050901). Media changes were performed every other day, and cells 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 (Human) user-coated and PureCoat rLaminin-521 cultureware surfaces. 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 were added to each sample (Table 1). Samples were incubated at room 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 by 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).

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

Results and Discussion

In order to assess the ability of Corning PureCoat™ rLaminin-521 cultureware to support hNSC expansion, hNSCs were cultured on PureCoat rLaminin-521 6-well plates and compared to 6-well plates coated with Corning rLaminin-521 (Human), and 6-well plates coated with PLO/Laminin. During the study, typical hNSC morphology was observed and similar confluence was achieved on all three of the surfaces after 96 hours of culture (Figure 1). Throughout the 3-passage study, hNSCs maintained a consistent

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

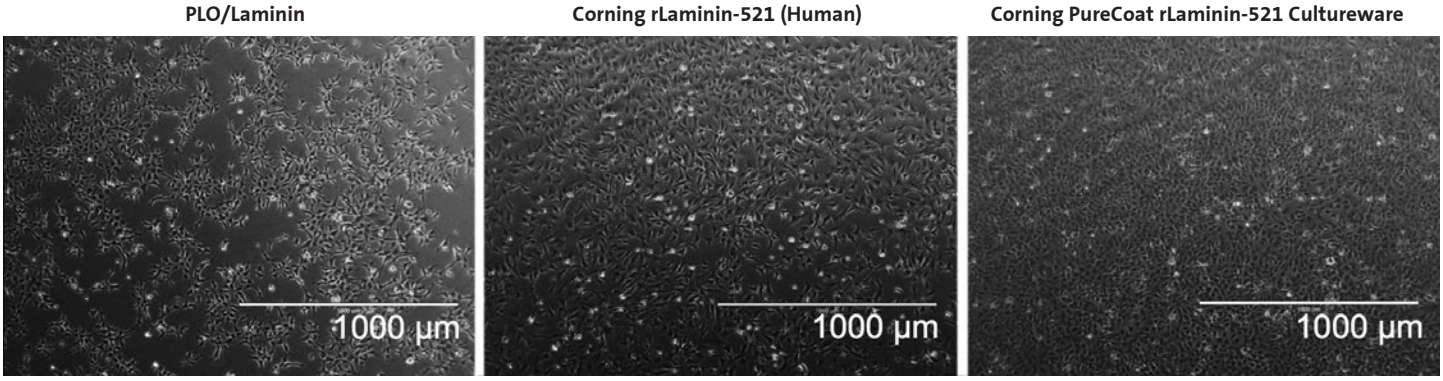

Figure 1. Human NSC morphology and confluence were comparable across the Corning PureCoat rLaminin-521 cultureware, user-coated rLaminin-521 (Human), and PLO/Laminin surfaces. Images show 4X representations of hNSCs after 72 hours in culture on the PureCoat rLaminin-521 cultureware and 6-well plates coated with PLO/Laminin or rLaminin-521 (Human).

doubling time of 41.1 ± 5.5 hours on all three surfaces without significant differences (Figure 2). Human NSCs also maintained high cell viability (>96%) on all three 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

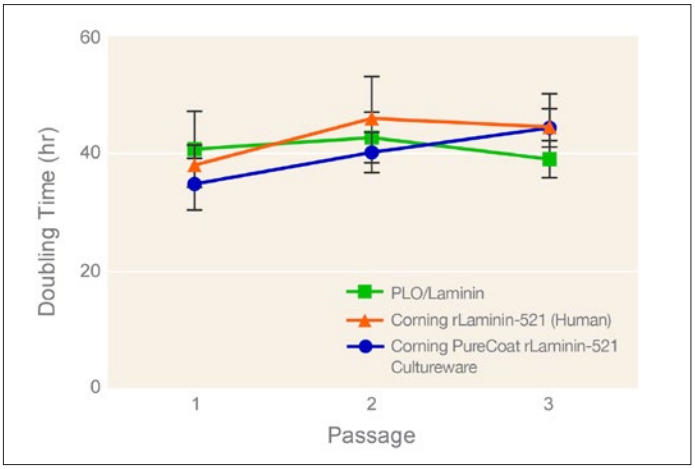

Figure 2. Doubling time of hNSCs was consistent across three passages and comparable between the Corning PureCoat rLaminin-521 cultureware, user-coated rLaminin-521 (Human), and PLO/Laminin surfaces. Average hNSC doubling time on each surface was 41.1 ± 5.5 hours across 3 passages. No significant difference was observed in doubling time across the three surfaces tested using ANOVA with Bonferroni post-test. N = 18 wells for each surface.

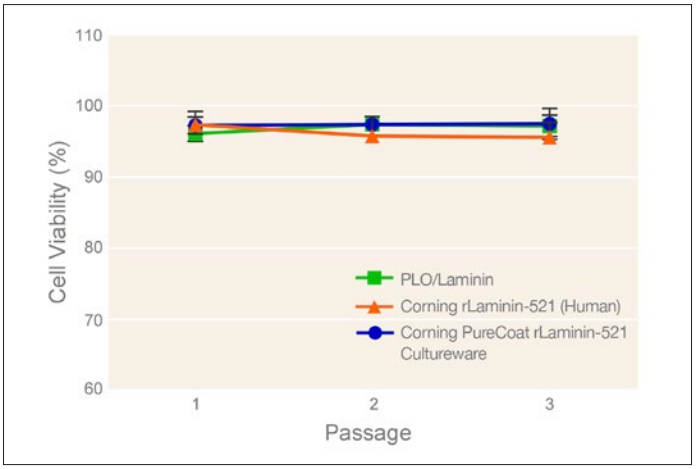

Figure 3. Viability of hNSCs was maintained on the Corning PureCoat rLaminin-521 cultureware, user-coated rLaminin-521 (Human), and PLO/Laminin surfaces. Average viability of >96 % was maintained on each surface across 3 passages without significant differences using ANOVA with Bonferroni post-test. N = 18 wells for each surface.

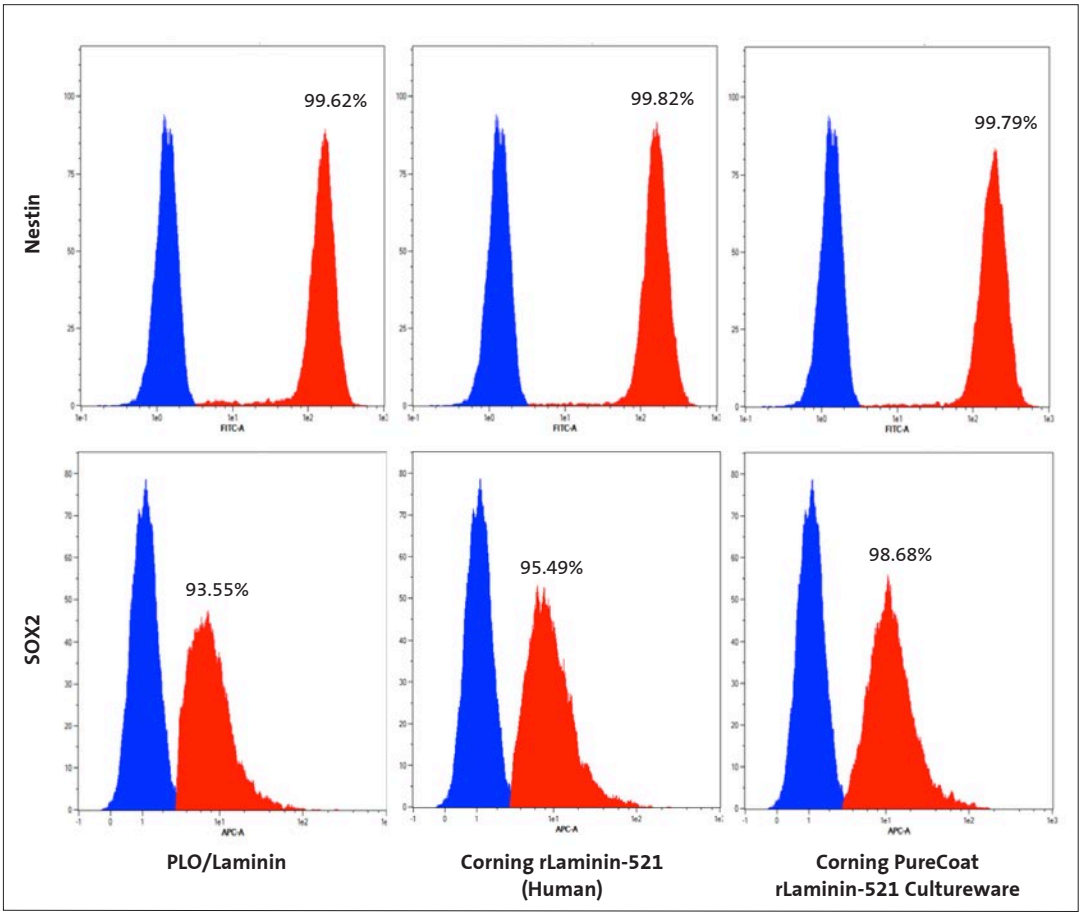

Figure 4. Expression of multipotency markers on the Corning PureCoat rLaminin-521 cultureware, user-coated rLaminin-521 (Human), and PLO/Laminin coated surfaces. Representative histograms of Nestin and SOX2 expression from passage 1 hNSCs cultured on the PureCoat rLaminin-521 cultureware and 6-well plates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

the 3-passage study (Figure 5) demonstrate the undifferentiated state was maintained on Corning® PureCoat™ rLaminin-521 cultureware, user-coated rLaminin-521 (Human), and PLO/Laminin. There were no significant differences in the expression levels of Nestin and SOX2 between the three surfaces.

After hNSCs were cultured on the PureCoat rLaminin-521 cultureware 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

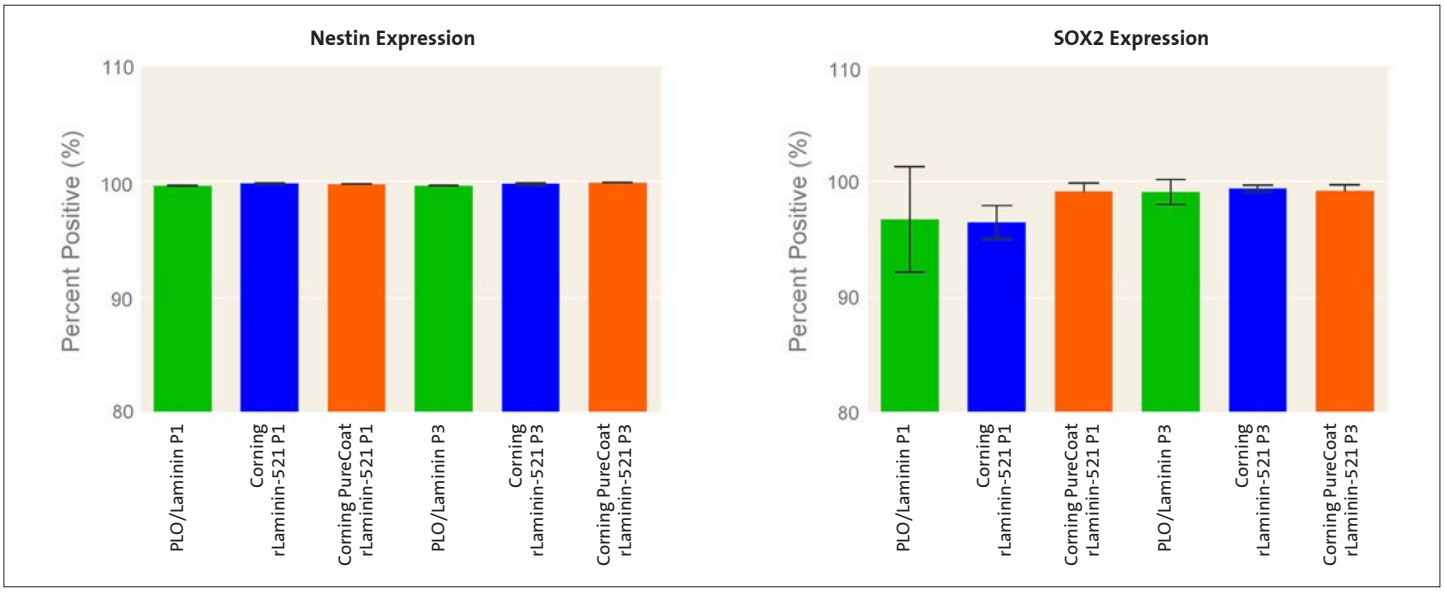

Figure 5. Expression levels of multipotency markers Nestin and SOX2 were maintained on the Corning PureCoat rLaminin-521 cultureware, user-coated rLaminin-521 (Human), and PLO/Laminin surfaces. SOX2 expression was >93.5% on each surface at both passage 1 (P1) and passage 3 (P3) without significant differences 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.

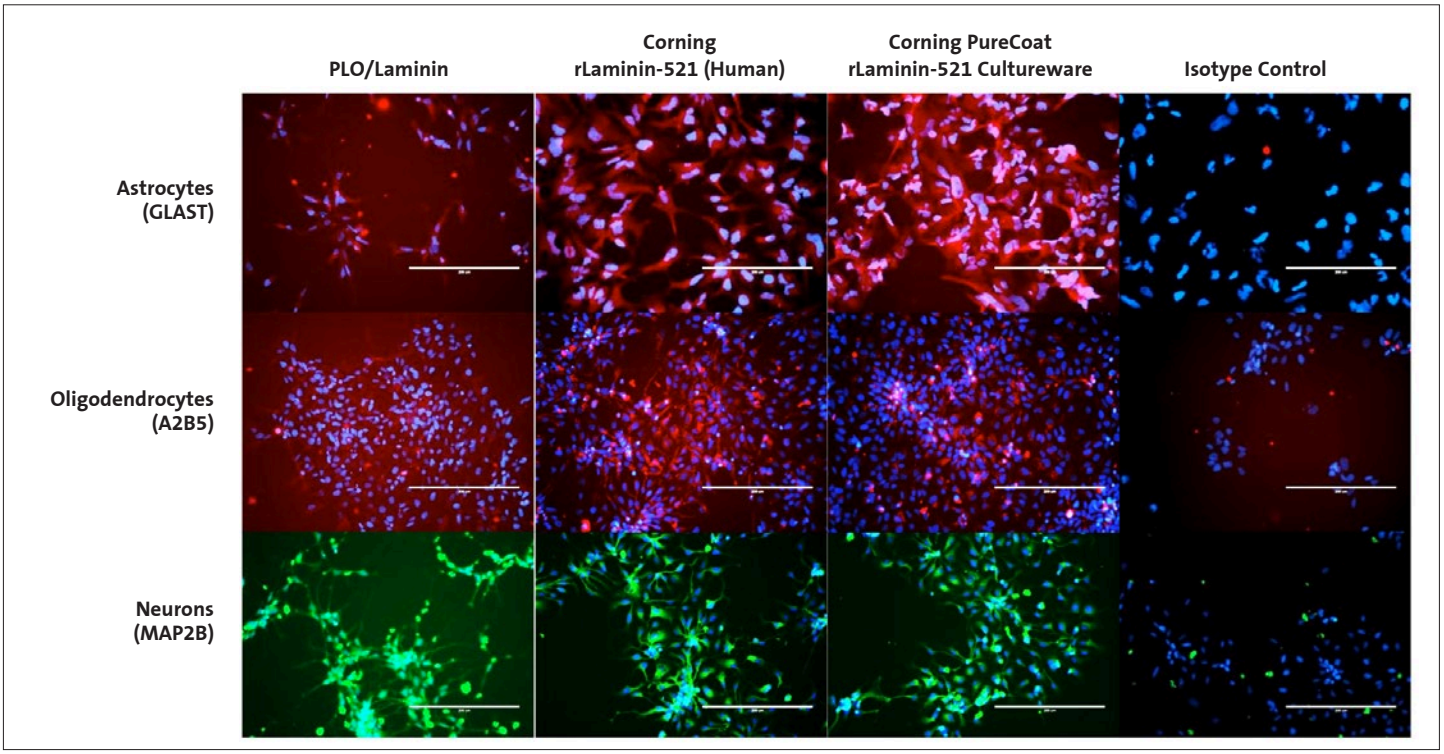

Figure 6. Human NSCs differentiated into astrocytes, oligodendrocytes, and neurons on the Corning PureCoat rLaminin-521 cultureware, user-coated rLaminin-521(Human), and PLO/Laminin surfaces. Representative 20X images of astrocytes (GLAST), oligodendrocytes (A2B5), and neurons (MAP2B) with Hoechst 34580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

differentiation markers. Figure 6 shows representative immunocytochemistry images of the differentiated cells on Corning® PureCoat™ rLaminin-521 cultureware, as well as rLaminin-521 (Human), and PLO/Laminin coated surfaces for the different lineages. The data demonstrates the ability of hNSCs cultured for three passages on the PureCoat rLaminin-521 cultureware to differentiate into the three lineages examined.

Conclusions

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

Reference

Corning Application Note CLS-DL-AN-339.

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