Supplementary Material

1. Human embryonic stem cells culture maintain culture

Human embryonic stem cells (hESCs-H9, WiCell) were used as the primary cell source for motor neuron differentiation. Cells were maintained in mTeSR medium (STEMCELL Technologies, #85850) on Matrigel-coated plates (1:100, Corning, #354277) at 37°C with 5% CO_2. Daily media changes were performed, and cells were passaged at ~70% confluency using ReLeSR (STEMCELL, #05872).

2. Motor neuron differentiation protocol

Motor neurons were differentiated from hESCs-H9 over a 29-day period following established protocols[1] with some modifications. Specific modifications were introduced to enhance differentiation efficiency and consistency. Using optimized seeding densities of 0.5×10^5 cells/cm² (day 0) and 7.5×10^4 cells/cm² (day 8), natural sedimentation method for media changing at first 8 days, frozen MNPs-D8 for motor neuron differentiation, the protocol ensured consistent and reproducible results in motor neuron development and maturation.

hESCs-H9 were dissociated using 1 mg/mL collagenase type IV (Gibco, #17104019) for at least 5 minutes at 37 °C. Initial seeding density was 0.5×10^5/cm²in 6-well plate hESCs were cultured as spheroids in neuronal basic medium (50% Neurobasal medium (Gibco, #21103049), 50% DMEM/F12 (Gibco, #11320033), 1% N2 (Gibco, #17502048), 2% B27 without vitamin A (Gibco, #12587010)). Spheroid differentiation was induced in three stages: day 0-2 (Npneural induction): Medium supplemented with 5 µM ROCK inhibitor (RI, Y-27632 dihydrochloride, Sigma, #Y0503), 40 µM SB431524 (New England Biolabs, #14775S), 0.2 µM LDN193189 (LDN, Abcam, #ab278073), and 3 µM CHIR99021 (GSK-3 inhibitor, Sigma, #SML1046); day 2-6 (motor neuron patterning): Medium changed to include 0.1 µM retinoic acid (RA, Sigma, #R2625) and 500 nM smoothened agonist (SAG, Sigma, #SML1314) ;day 6-8 (early maturation): Additional 10 ng/mL Brain-derived Neurotrophic Factor (BDNF, Sigma, #B379) and 10 ng/mL Glial Cell-Derived Neurotrophic Factor (GDNF, Sigma, #G1777) were added alongside RA and SAG to support motor neuron progenitors (MNPs) formation.

On day 8, MNP-D8 spheroids were dissociated using Accutase (Gibco A1110501), centrifuged at 300g for 3 minutes, and seeded at 7.5×10^4 cells/cm² on coverslips or hydrogel discs. The coverslips were pre-coated with PLL (Sigma, P5899, 100 µg/mL) and laminin (Gibco™ 23017015, 20 µg/mL). For laminin coating, a freshly prepared 20 µg/mL laminin solution in DMEM/F12 (Sigma, #23017015) was added to cover the entire surface of the coverslips and incubated at 37°C for at least 30 minutes.

Differentiation progressed through defined stages. Motor neuron differentiation takes place on days 8-13 by supplementing medium with 0.1 µM retinoic acid (RA, Sigma R2625-50MG), 500 nM smoothened agonis (SAG, Sigma SML1314-1MG), 10 ng/mL brain derived neurotrophic factor (BDNF, Sigma B3795-10UG), 10 ng/mL glial derived neurotrophic factor (GDNF, Sigma G1777-10UG), and 10 µM of Inhibitor of γ-secretase (DAPT, Chem-Supply, GK0567). On Days 13-15 Post-mitotic differentiation is induced with BDNF, GDNF, and increased DAPT (20 µM). In Days 15-29 culture medium consists of BDNF, GDNF, and 10 ng/mL ciliary neurotrophic factor (CNTF, Sigma C3710-10UG) to promote neuronal maturation.

During the entire differentiation process, the medium was partially changed every two days by replacing half of the medium volume to maintain the concentration of differentiation factors while minimizing cellular stress.

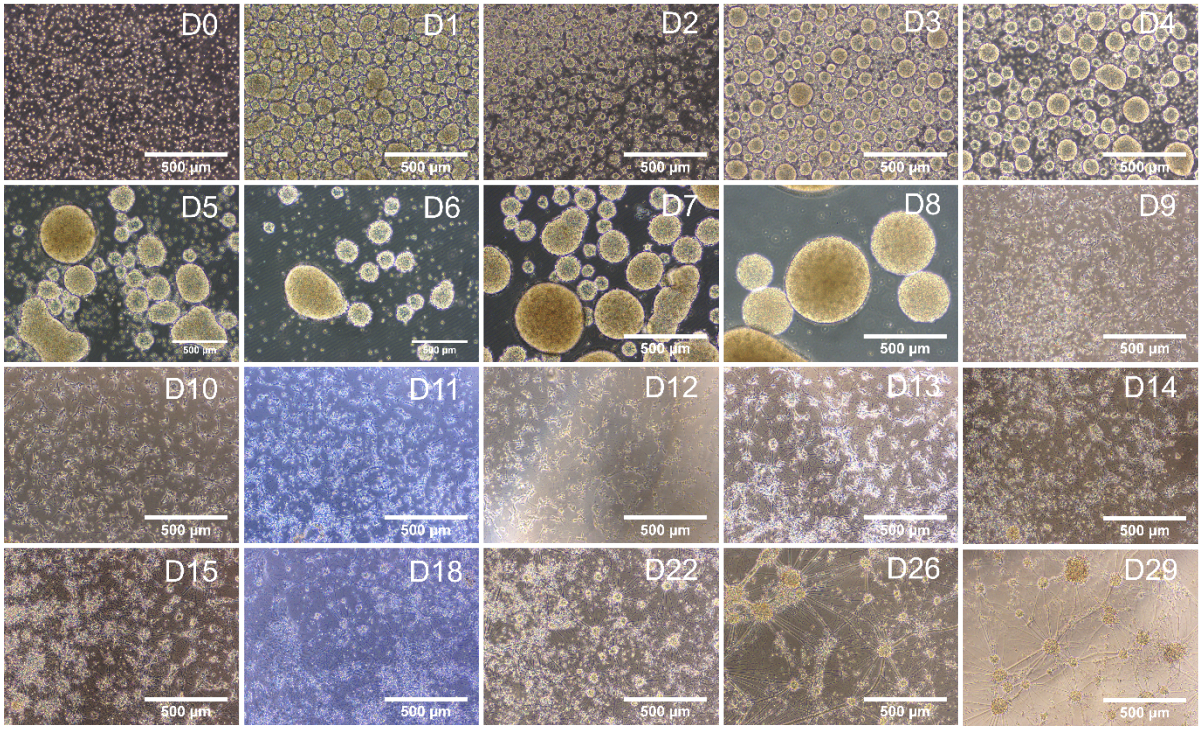

Figure S1. Morphological progression during hESC-H9-derived motor neuron differentiation. Phase-contrast images showing morphological changes throughout the differentiation process on petrol dish (D0-D8) and, PLL and Laminin coated coverslips (D9-D29), scale bar = 500 µm.

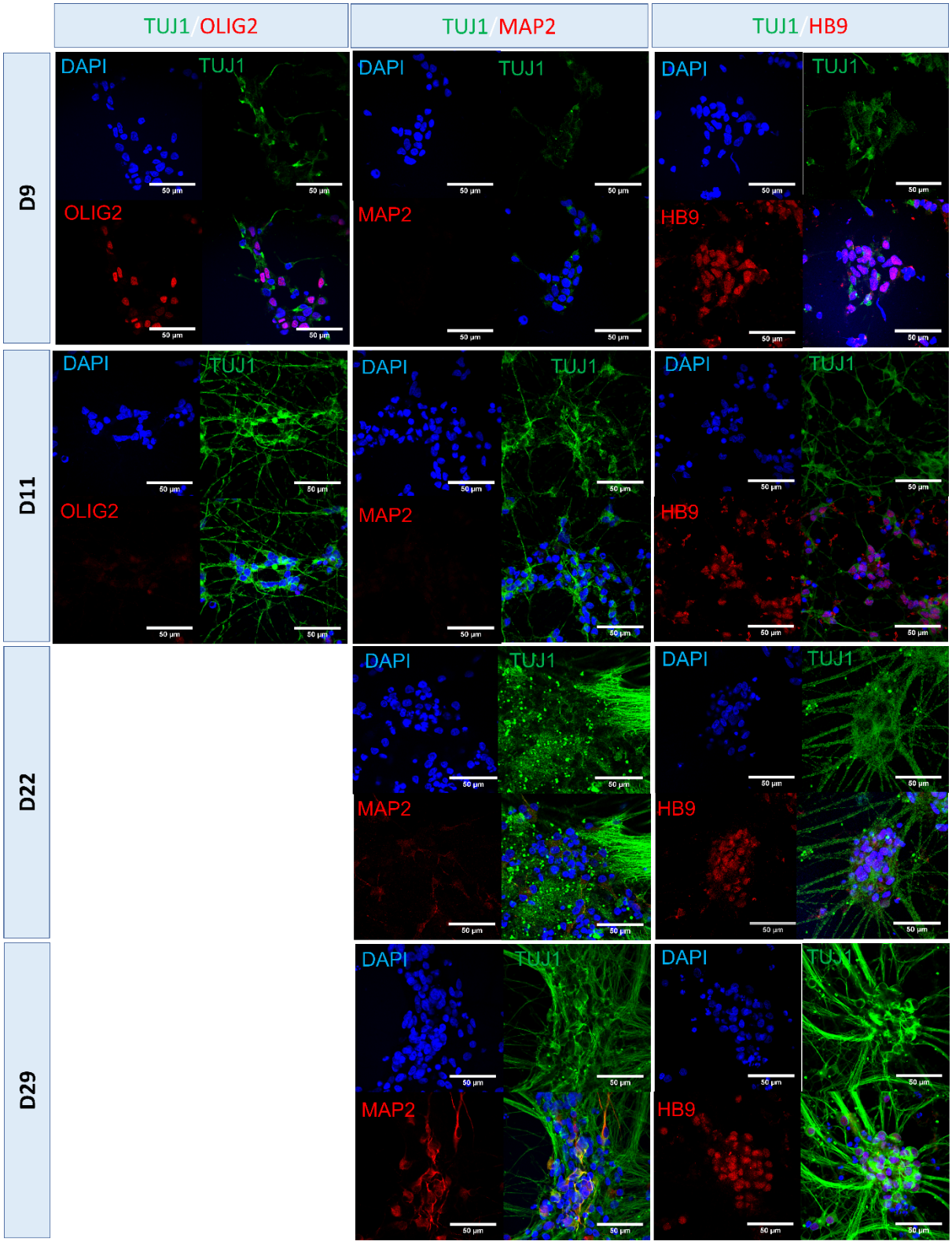

Figure S2. Representative immunostaining images at days 9, 11, 22, and 29 highlighting sequential expression of motor neuron markers. TUJ1 (green) labels neuronal axons, Olig2 (red) marks motor neuron progenitors, HB9 (red) identifies post-mitotic motor neurons, MAP2 (red) indicates mature dendritic structures, and DAPI (blue) stains nuclei; scale bar = 50 µm.

3. Effect of PLL-integration and sterilization on mechanical properties.

Mechanical testing was performed on a separate set of hydrogels using unconfined static compression within a strain range of 15–20%. Hydrogels were evaluated under swelling equilibrium conditions and following steam autoclave sterilization. Representative stress-strain curves for each experimental condition are shown in Figure S3.

As shown in Figure S4, the mean compressive modulus of base PVA-MA hydrogels under swelling equilibrium conditions was 272.7 ± 46.2 kPa. A comparable modulus was observed for blended PVA-PLL hydrogels (263.4 ± 15.7 kPa). In contrast, hydrogels incorporating PLL via covalent crosslinking exhibited a 31.4% increase in compressive modulus (353.0 ± 27.9 kPa). Following sterilization, the compressive modulus of both blended and covalently modified PVA-PLL hydrogels decreased to values comparable to PVA-MA controls (245 ± 38.7 kPa).

The mechanical properties of PVA/PLL hydrogels were expected to approximate those of the base PVA-MA formulation. Mechanical behavior in polymer networks is primarily governed by crosslink density[2]. Given the substantial disparity in composition between PVA (15 wt%) and PLL (0.002 wt%), corresponding to a 7500:1 ratio, PLL incorporation was anticipated to exert minimal influence on bulk mechanical properties.

The observed increase in compressive modulus following covalent incorporation of PLL suggests that chemical integration may induce localized alterations in network architecture. One possible explanation is that covalently tethered PLL-MA promotes secondary molecular rearrangements within the PVA network, like chain compaction or constrained chain mobility, thereby increasing effective network stiffness. Such rearrangements are less likely in blended systems, where PLL is incorporated primarily through physical interactions rather than chemical crosslinking.

Interestingly, steam sterilization eliminated the stiffness increase while bioactivity remained detectable, confirming the continued presence of PLL. This finding suggests that the elevated temperature and pressure associated with autoclaving may facilitate relaxation or reconfiguration of polymer chains, reducing the mechanically reinforcing effects of covalent PLL integration without disrupting its biological functionality.

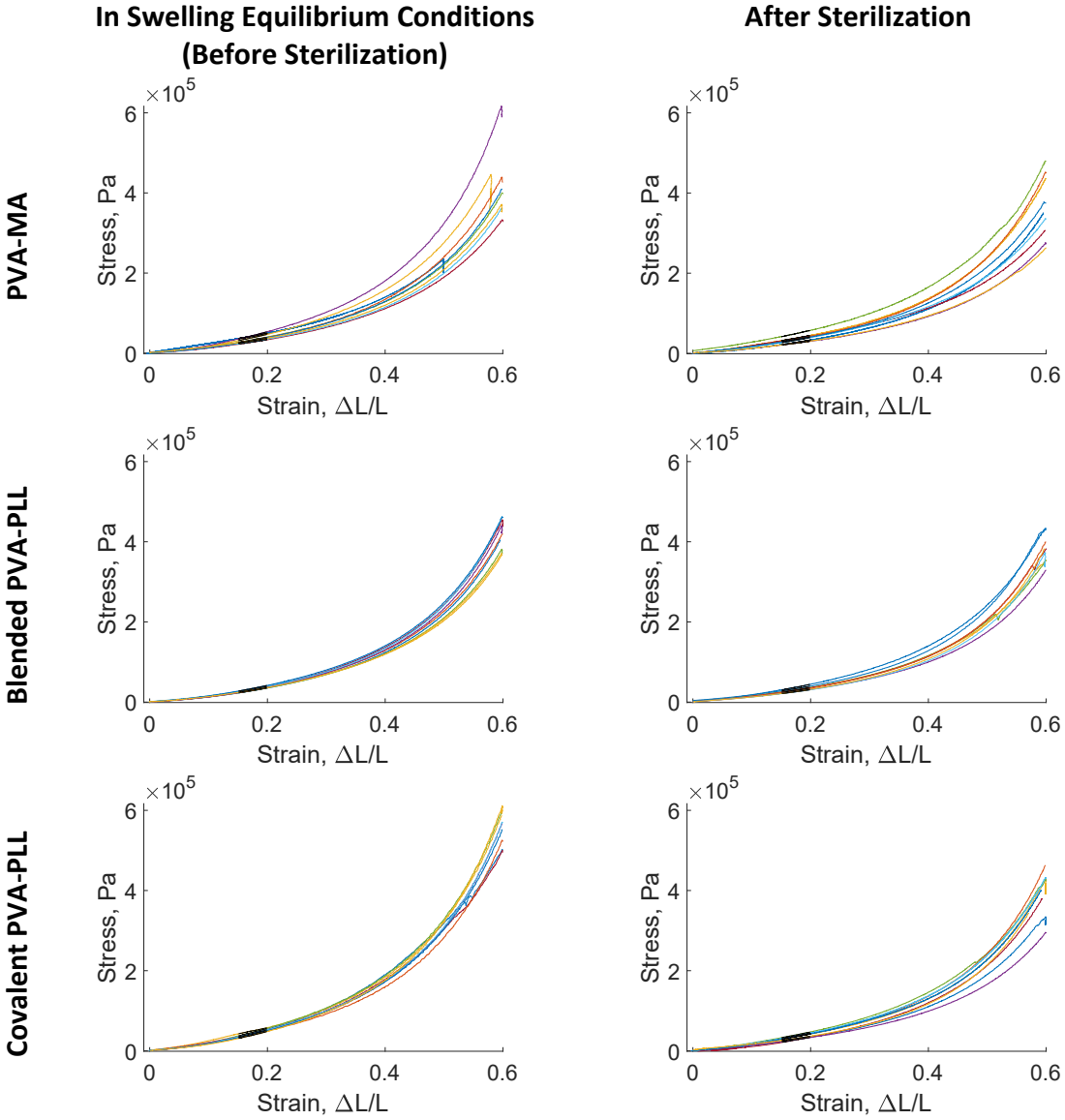

Figure S3. Stress-strain curves of hydrogels with and without PLL tested under swelling equilibrium conditions (24 hours in PBS at 37 °C) and following steam-sterilization (121 °C, 200 kPa, 15 mins)

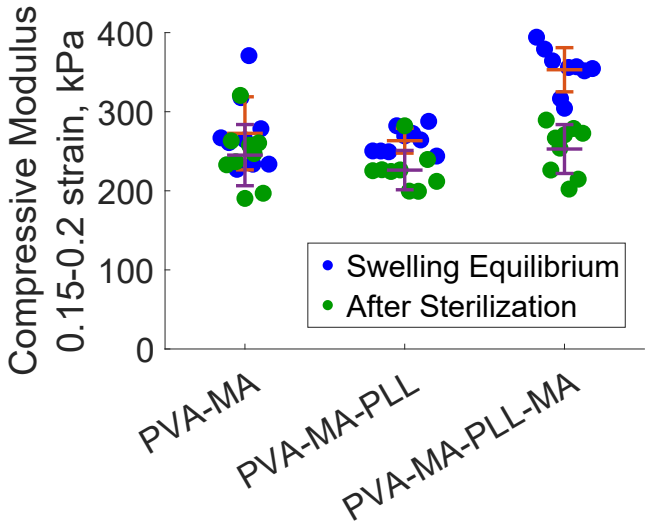

Figure S4. Compressive moduli of hydrogels with and without PLL tested under swelling equilibrium conditions and following steam-sterilization

4. Differentiation of MNP-D8 spheroids on PVA-PLL hydrogel substrates

Feasibility studies were conducted to evaluate the ability of the PVA-PLL (15/0.002 wt%) hydrogel substrate to support motor neuron differentiation over a 21-day period (total 29 days). These studies using MNP-D8 spheroids rather than dissociated cells formed the basis for assessing the extent of differentiation in the culture and determining the appropriate endpoint for analysis. MNP-D8 spheroids were cultured on flat hydrogel discs and analysed by immunostaining for nuclei (DAPI), the motor neuron transcription factor HB9, and the neuronal marker TUJ1 (Figure S5).

At day 1, DAPI staining confirmed the structural integrity of the spheroids following seeding onto the hydrogel surface. HB9 expression was minimal, indicating limited motor neuron differentiation at this early stage. TUJ1 staining was detectable but weak, with short neurite projections extending from the spheroid periphery, suggesting the initial onset of neuronal differentiation and early substrate interaction.

By day 3, HB9 expression increased markedly and was predominantly localized around the spheroid core, consistent with the early stages of motor neuron specification. Concurrently, TUJ1 staining revealed more prominent neurite extensions projecting radially from the spheroids, indicating progressive neuronal differentiation and neurite initiation.

At day 7, HB9 expression further intensified and extended towards the spheroid periphery, consistent with continued maturation of motor neuron populations. TUJ1-positive neurites formed extensive projections across the hydrogel surface, generating a dense network of interconnected processes surrounding the spheroid. The increasing neurite density and length indicated robust neuronal differentiation and active network formation within the culture.

By day 14, individual spheroids had begun to aggregate into larger structures, reflecting increased cellular cohesion during culture progression. At this stage, TUJ1-positive axons extended radially in multiple directions across the hydrogel surface, reaching lengths of approximately 2 mm, indicative of active neurite elongation and early neural network organization.

At day 21, overall morphology and marker distribution were comparable to those observed at day 14. HB9 and TUJ1 staining remained prominent, and the established neurite networks appeared stable, suggesting that the cultures had reached a relatively mature state with limited further expansion of axonal networks over this period. Of note, the formation of large spheroid aggregates resulted in detachment of neuronal cultures from the hydrogel surface, overall complicating reliable morphometric quantification. Consequently, the 21-day time point was selected as the optimal endpoint for analysis, enabling robust assessment of motor neuron differentiation and neurite outgrowth while maintaining stable substrate attachment.

Overall, the progressive increase in HB9 expression and the expansion of TUJ1-positive neurite networks over time demonstrate that the PVA-PLL hydrogel substrate supports the differentiation of MNP-D8 spheroids into motor neurons and promotes extensive neurite outgrowth and network formation under 3D culture conditions.

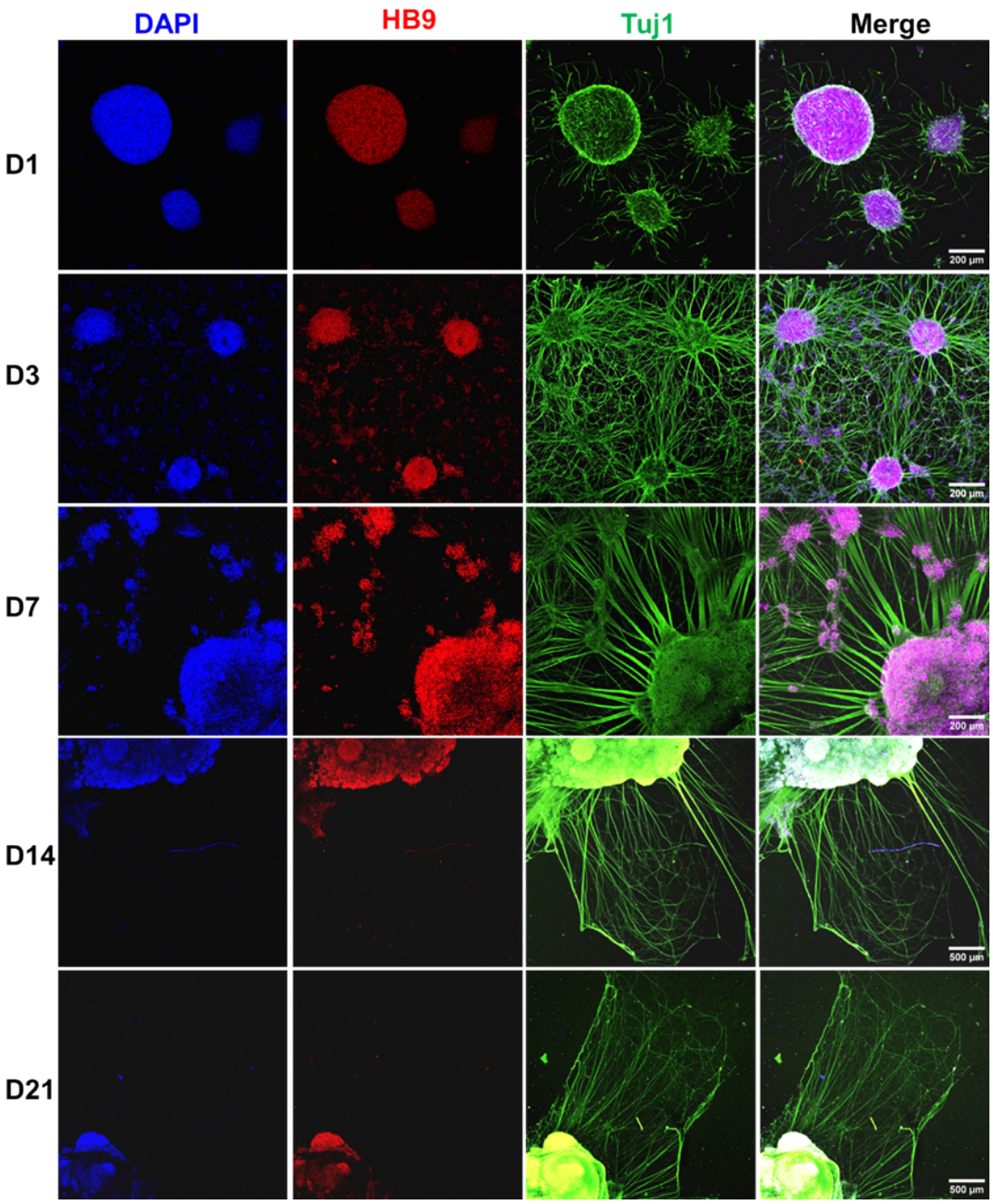

Figure S5. Immunostaining analysis of MNP-D8 spheroids differentiated on 15/0.002 wt% PVA-PLL hydrogel (flat disc) for 21 days. DAPI (blue) stains nuclei, HB9 (red) marks motor neuron differentiation, and TUJ1 (green) highlights neurite outgrowth. Scale bars: 200 µm (D1, D3, D7), 500 µm (D14, D21).

Reference

1. W. Guo, M. Naujock, L. Fumagalli, T. Vandoorne, P. Baatsen, R. Boon, L. Ordovás, A. Patel, M. Welters, T. Vanwelden, N. Geens, T. Tricot, V. Benoy, J. Steyaert, C. Lefebvre-Omar, W. Boesmans, M. Jarpe, J. Sterneckert, F. Wegner, S. Petri, D. Bohl, P. Vanden Berghe, W. Robberecht, P. Van Damme, C. Verfaillie and L. Van Den Bosch, *Nat Commun*, 2017, **8**, 861.

2. U. A. Aregueta-Robles, P. J. Martens, L. A. Poole-Warren and R. A. Green, *Journal of Polymer Science, Part B: Polymer Physics*, 2018, **56**, 273-287.