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# Fibrin hydrogels induce mixed dorsal/ventral spinal neuron identities during differentiation of human induced pluripotent stem cells

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ABSTRACT

We hypothesized that generating spinal motor neurons (sMNs) from human induced pluripotent stem cell (hiPSC)-derived neural aggregates (NAs) using a chemically-defined differentiation protocol would be more effective inside of 3D fibrin hydrogels compared to 2D poly-L-ornithine(PLO)/laminin-coated tissue culture plastic surfaces. We performed targeted RNA-Seq using next generation sequencing to determine the substrate-specific differences in gene expression that regulate cell phenotype. Cells cultured on both substrates expressed sMN genes *CHAT* and *MNX1*, though persistent WNT signaling contributed to a higher expression of genes associated with interneurons in NAs cultured in 3D fibrin scaffolds. Cells in fibrin also expressed lower levels of astrocyte progenitor genes and higher levels of the neuronal-specific gene *TUBB3*, suggesting a purer population of neurons compared to 2D cultures.

**Statement of Significance**

Fibrin scaffolds can support the neuronal differentiation of pluripotent stem cells. This study provides insight into how fibrin hydrogels affect neuronal induction by analyzing of the signaling pathways activated during the differentiation process. These insights can then be used to tailor the properties of these hydrogels to optimize the generation of sMNs for regenerative medicine applications.


## 1. Introduction

Deriving neurons from human induced pluripotent stem cells (hiPSCs) provides a strategy for modeling neurological diseases and it could potentially supply patient-specific cells for regenerative medicine applications [1,2]. Numerous protocols can convert hiPSCs into several neuronal cell-types *in vitro* through the administration of small molecules, growth factors, and neurotrophic proteins [3–5]. These protocols typically generate heterogeneous populations of neural cells—including astrocytes and oligodendrocytes—in addition to neurons. Deriving pure populations of neurons remains challenging [6,7]. Furthermore, an ideal protocol would efficiently specify the neuronal subtype during differentiation as numerous subclasses of neurons exist with different functions. Two small molecule morphogens, retinoic acid (RA) and purmorphamine (puro), promote the differentiation of hiPSCs into spinal motor neurons (sMNs) [5,8,9]. RA acts as a caudalizing factor in the neural plate and neural tube during vertebrae development, plays a role in cell patterning, and induces axonal outgrowth [10,11]. RA along with the sonic hedgehog (SHH) protein establish a ventral cell-fate that generates sMNs [12]. Puro is a small molecule agonist of the Hedgehog (HH) pathway that can replace the SHH protein during differentiation [13].

Cell culture substrates also influence stem cell differentiation [14,15]. The compliance of a cell culture substrate can cause stem cells to preferentially differentiate into specific cell lineages [16]. Cells cultured in high compliance (soft) substrates are typically neurogenic while cells cultured in medium compliance substrates are myogenic, and those cells cultured in low compliance (stiff) substrates are osteogenic [17,18]. Furthermore, the presence of extracellular matrix (ECM) proteins during differentiation can also influence cell patterning and development through integrin-mediated signaling [19]. The glycoprotein laminin-1, for example, plays an important role in axonal guidance and cell migration in the developing nervous system and serves as a potent stimulator of axonal outgrowth *in vitro* [20–22]. Laminin-1 binds to at least

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three integrins expressed by neuronal cell-types and integrin function blocking assays have confirmed laminin-1's role in neuronal development [19,23,24].

Deriving sMNs from hiPSCs inside of 3D biomaterial scaffolds presents a unique challenge. Biomaterial niches must recapitulate some of the signals present during development. These cues need to induce differentiation to the proper cell-type, while directing the proper cell-patterning to create functional tissue. Recent studies have focused on 3D fibrin scaffolds for supporting the neuronal induction of pluripotent stem cells. Fibrin, a blood-derived hydrogel formed when fibrinogen is polymerized by thrombin [25], supports neural differentiation of human neural stem cells (NSCs) and embryonic stem cells (ESCs), and engraftment of neurons at the lesion site in murine models of spinal cord injury followed by axonal outgrowth [26–29].

Based on these studies, it is hypothesized that 3D fibrin hydrogels will provide a microenvironment that more efficiently generates spinal neuron from hiPSCs than traditional techniques using laminin-coated 2D tissue culture plastic surfaces. Here, we compared hiPSC-derived neural aggregates (NAs) cultured in 3D fibrin hydrogels to those cultured on 2D poly-L-ornithine and laminin (PLO/laminin)-coated tissue-culture plastic surfaces in defined, serum-free, conditions supplemented with RA and puro to induce sMN differentiation. We compared the cell phenotypes generated on each substrate using flow cytometry, immunocytochemistry and performed transcriptome analysis using next generation sequencing. For the transcriptome analysis, we examined the expression of 232 target genes relevant to neural development and differentiation. We showed that NAs in fibrin maintain a spherical morphology while those on PLO/laminin spread extensively. Cells differentiated into neurons on each substrate with cultures on PLO/laminin expressing higher levels of genes associated with sMNs as well as early expression of astrocyte progenitor genes. Cells cultured in fibrin differentiated into a mixed population of sMNs and spinal interneurons and had lower expression of astrocyte progenitor genes, suggesting a purer neuronal population.

## 2. Materials and methods

### 2.1. hiPSC culture

All cells were cultured under standard conditions (37 °C and 5% $CO_2$) using reagents supplied by STEMCELL Technologies unless otherwise stated. Experiments using hiPSCs were conducted with the approval of the University of Victoria's Human Ethics Committee (protocol number: 12–187). hiPSCs (iPS(Foreskin)-1, Lot 1-DL-01, WiCell) were cultured as previously described [30]. The media was changed daily and cells were passaged approximately every 5 days using ReLeSR™ reagent to dissociate the colonies. Dissociated colonies were either diluted and plated on freshly prepared Vitronectin-XF-coated plates or used for further experiments.

### 2.2. Formation of NAs from hiPSCs

NAs were formed by adding approximately $1.5 \times 10^6$ dissociated hiPSCs in 2 mL Neural Induction Medium (NIM) to AggreWell™ 800 plates as previously described [31]. On day 5, the neural aggregates that had formed were removed from the AggreWell™ by gentle pipetting and seeded on the appropriate substrate.

### 2.3. Seeding and culture of NAs on 2D PLO/laminin surfaces and in 3D fibrin scaffolds

NAs were plated on PLO/laminin-coated (Sigma) 24-well plates at a density of one aggregate per well. They were incubated for 7 or 15 additional days (12 or 20 days total) in NIM supplemented with 10 μg/mL aprotinin (bovine lung, Sigma), 1 μM RA (⩾98% (HPLC), powder, Sigma) and 1 μM puro (Cayman Chemical Company). NAs were seeded in fibrin as previously described [32]. Briefly, 11.1 mg/mL sterile fibrinogen (plasminogen-depleted, human plasma, Millipore) was polymerized with 40units/mL thrombin and 50nM $CaCl_2$ in a 24-well plate to create a 10 mg/mL (1% w/v) fibrin gel with a final volume of 400 μL. One NA per well was placed on top of the fibrin and an additional 100 μL of fibrinogen/thrombin/$CaCl_2$ was placed over the cells and allowed to polymerize. Then, 1 mL of NIM supplemented with 10 μg/mL aprotinin, 1 μM RA, and 1 μM puro was added to each well. Aprotinin, a protease inhibitor, was used to slow the degradation of fibrin by the cells. Aprotinin was also included in the PLO/laminin sample group to ensure the media conditions were identical, leaving the substrate as the major variable. Phenotype and gene expression were measured at day 12 and day 20 using immunocytochemistry and transcriptome analysis.

### 2.4. Flow cytometry of pluripotency markers

Flow cytometry was used to characterize pluripotency markers in undifferentiated hiPSCs (day 0) and NAs (day 5). Undifferentiated hiPSCs were selected using ReLeSR™ reagent and neural aggregates were dissociated with 0.05% trypsin (Life Technologies) in phosphate buffer saline (PBS, Life Technologies). Dissociated cells were stained using the Human/Mouse Pluripotent Stem Cell Multi-Color Flow Cytometry Kit (R&D Systems) according to the manufacturer's protocol. Cells were stained with a 1:50 dilution of OCT3/4-PE (Rat $IgG_{2A}$, Clone 240408), SOX2-PE (Mouse $IgG_{2A}$, Clone 245610), SSEA-1-PerCP (Mouse IgM, Clone MC-480), or SSEA-4-CFS (Mouse $IgG_3$, Clone MC-813-70). Flow cytometry was performed using a Guava EasyCyte HT flow cytometer and GuavaSoft EasyCyte software (Millipore) and analysis of the collected data was performed using FlowJo software (vX.0.7). Positive expression of pluripotency markers was calculated using the Overton percent (%) positive method [33].

### 2.5. Immunocytochemistry and image analysis

Cells were fixed and stained on day 12 and day 20 as previously described [30]. Briefly, cells were fixed and permeabilized then stained with primary antibodies for TUJ1 (β-III tubulin, mouse host, clone TU-20, Millipore), HB9 (MNX1, rabbit host, Millipore), GFAP (rabbit host, Abcam), or O4 (mouse host, clone 81, Millipore) overnight at 4 °C, followed by Alexa Fluor 405 (goat anti-rabbit), Alexa Fluor 488 (goat anti-mouse), or Alexa Fluor 568 (goat anti-rabbit) secondary antibodies (all from Life Technologies) for 4 h at room temperature. The cells were then washed and imaged using a Leica DMI3000B inverted microscope, Lumen Dynamics X-Cite 120Q LED fluorescence light source, and QImaging camera and software. 3D aggregates were imaged in multiple focal planes and the images merged using ImageJ software (v1.49) and the complex wavelet-based method [34].

### 2.6. RNA-Seq

Cells were collected on day 0 (undifferentiated hiPSCs), day 5 (NAs), and day 12 and day 20 from PLO/laminin and fibrin sample groups. Total RNA (tRNA) was isolated from the cells using the Single Cell RNA Purification Kit (Norgen). The sequencing library was constructed using a TruSeq Targeted RNA Expression Stem Cell Panel with an additional 132-gene Supplement Panel (Illumina). The workflow was performed according to the TruSeq Targeted RNA Expression Guide (Illumina #15034665 Rev. C). Briefly, cDNA was reverse transcribed RNA using ProtoScript II (New England Biolabs). Targeted cDNA was then hybridized with oligomers and