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A robust vitronectin-derived peptide for the scalable long-term expansion and neuronal differentiation of human pluripotent stem cell (hPSC)-derived neural progenitor cells (hNPCs)

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ABSTRACT

Despite therapeutic advances, neurodegenerative diseases and disorders remain some of the leading causes of mortality and morbidity in the United States. Therefore, cell-based therapies to replace lost or damaged neurons and supporting cells of the central nervous system (CNS) are of great therapeutic interest. To that end, human pluripotent stem cell (hPSC) derived neural progenitor cells (hNPCs) and their neuronal derivatives could provide the cellular 'raw material' needed for regenerative medicine therapies for a variety of CNS disorders. In addition, hNPCs derived from patient-specific hPSCs could be used to elucidate the underlying mechanisms of neurodegenerative diseases and identify potential drug candidates. However, the scientific and clinical application of hNPCs requires the development of robust, defined, and scalable substrates for their long-term expansion and neuronal differentiation. In this study, we rationally designed a vitronectin-derived peptide (VDP) that served as an adhesive growth substrate for the long-term expansion of several hNPC lines. Moreover, VDP-coated surfaces allowed for the directed neuronal differentiation of hNPC at levels similar to cells differentiated on traditional extracellular matrix protein-based substrates. Overall, the ability of VDP to support the long-term expansion and directed neuronal differentiation of hNPCs will significantly advance the future translational application of these cells in treating injuries, disorders, and diseases of the CNS.

1. Introduction

The lack of effective therapies for neurological injuries, disorders, and diseases of the central nervous system (CNS) creates an enormous burden on society. Current pharmacological-based treatments of these diseases are inadequate as they only treat symptoms and not the underlying disease etiology—the damage, degeneration, and death of the neurons and supporting cell types of the CNS. Stem-cell based technologies have emerged as a promising approach for the study and treatment of these diseases [1–3]. Specifically, human pluripotent stem cell (hPSC)-derived neural progenitor cells (hNPCs), a multipotent cell population that is capable of extensive *in vitro* expansion and subsequent differentiation into the various cell types that comprise the CNS, could provide an unlimited source of cells for such cell-based therapies [2,4–6]. In fact, recent research supports the use of these cells as the basis for regenerative medicine therapies to reverse or arrest neurodegeneration or replace dead or diseased neural cells [2,3,6–8]. In addition, generating neural cells from human disease specific hPSCs is of particular interest because animal models of neurodegenerative diseases do not display important pathological hallmarks and do not adequately model the complex genetics associated with human neurodegenerative diseases [9–12]. Furthermore, such hPSC-based 'disease-in-a-dish' models can be used to discover new drug targets and develop efficacious therapeutic compounds [9]. However, to realize the full potential of hNPCs in these applications the development of defined, robust, and scalable culture conditions for their expansion and neuronal differentiation are needed.

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The extracellular matrix (ECM) is a dynamic component of the cell microenvironment that not only functions to support cell attachment and growth but also regulates cell differentiation and fate [13,14]. To that end, we and others have investigated the effects of various extracellular matrix proteins (ECMPs) on the self-renewal and differentiation of hPSCs [15–18]. As it relates to the culture of hNPCs, the most common ECMP-based culture substrates, such as Matrigel™ and laminin [19,20], are difficult to isolate, expensive, biochemically undefined, subject to batch-to-batch inconsistencies, and contain potentially hazardous xenogeneic components, thereby limiting the scientific and clinical application of cells cultured with these substrates. In addition, ECMPs are structurally complex molecules that contain several receptor binding motifs, making it difficult to elucidate and control their biological function. By comparison, peptide-based materials consisting of short amino acid sequences derived from the cell binding domains of ECMPs are inexpensive, completely defined, and easily produced. As such, several peptide-based substrates have been developed for the long-term culture and directed differentiation of hPSCs [21–27]. However, completely defined peptide-based substrates that support the adhesion, growth, and differentiation of hNPCs have not been previously developed.

In this study, we characterized the ECM and cell surface integrin profile of hNPCs to rationally design peptide-based substrates for the growth and differentiation of hNPCs. Of the peptides tested, we identified one 14 amino acid long peptide derived from the cell-binding domain of vitronectin [28] that provides for the expansion and neuronal differentiation of hNPCs. Moreover, this peptide, referred to as vitronectin-derived peptide (VDP), is easily coated onto tissue-culture treated polystyrene (TCPS) plates and supports the long-term propagation and directed neuronal differentiation of multiple hNPC lines in completely defined medium conditions. Overall, VDP is a completely defined and scalable substrate that supports the long-term expansion and directed neuronal differentiation of hNPCs in quantities necessary for their scientific and clinical applications.

2. Materials and methods

2.1. Human pluripotent stem cell (hPSC) culture

All media components were from Life Technologies unless otherwise noted. For hPSC culture on mouse embryonic fibroblast (MEF) feeders, the following media were used: MEF (1X high glucose DMEM, 10% fetal bovine serum, 1% (v/v) L-glutamine penicillin/streptomycin). H9/HES3/RiPSC hPSCs (1X DMEM-F12, 20% (v/v) Knockout Serum Replacement, 1% (v/v) non-essential amino acids, 0.5% (v/v) glutamine, 120 μM 2-mercaptoethanol [Sigma]). HSF4 (1X high glucose DMEM + L-Glutamine, 20% (v/v) Knockout Serum Replacement, 1% (v/v) non-essential amino acids, 100 μM 2-mercaptoethanol). All hPSC lines were maintained on feeder layers of mitotically inactivated MEFs (Millipore). All hPSC cultures were supplemented with 30 ng/ml FGF2 (Life Technologies). For culture of hPSCs in the absence of feeders, hPSCs were grown on Matrigel (BD Biosciences) or Geltrex (Life Technologies) in the presence of MEF-conditioned media (MEF-CM; produced by culturing hPSC medium on MEFs for 24 h followed by sterile filtering), mTeSR2 (Stem Cell Technologies), or Essential 8 (Life Technologies). Cells were routinely passaged every 4–5 days with Accutase and 5 μM Rho kinase inhibitor (Y-27632) (Stemgent) to aid in cell survival. HPSCs were differentiated to early endoderm (EN), mesoderm (ME), and ectoderm (EC) cell populations as previously described [29].

2.2. Human neural progenitor cell (hNPC) generation, expansion, and differentiation

H9-, HES3-, and RiPSC-hNPCs were derived as previously described [30]. Briefly, to initiate neural differentiation hPSCs were cultured in feeder-free conditions for a minimum of 2 passages. Cells were then detached with Accutase and resuspended in neural induction media [1X DMEM-F12, 1% (v/v) N2 supplement (Life Technologies), 1% (v/v) B27 supplement (Life Technologies)] supplemented with 5 μM Rho kinase inhibitor (Y-27632), 50 ng/ml recombinant mouse Noggin (R&D Systems), 0.5 μM Dorsomorphin (Tocris Bioscience). Next, 1–2 × 10^6 cells were pipetted to each well of a 6-well ultra-low attachment plate (Corning). The plates were then placed on an orbital shaker set at 95 rpm in a 37 °C/5% CO_2 tissue culture incubator. The next day, the cells formed spherical cultures (embryoid bodies [EBs]) and the media was changed to neural induction media with 50 ng/ml recombinant mouse Noggin and 0.5 μM Dorsomorphin. Half of the media was subsequently changed every day. After 5 days in suspension culture, the EBs were then transferred to a 10 cm dish (1–2 6 wells per 10 cm dish) coated with Matrigel™. The plated EBs were cultured in neural induction media supplemented with 50 ng/ml recombinant mouse Noggin and 0.5 μM Dorsomorphin for an additional 5–7 days. Neural rosettes were cut out by dissection under an EVOS (Life Technologies) microscope. Rosettes were then plated on surfaces that had been coated first with poly-L-ornithine (PLO) and then with mouse laminin (LN; 5 μg/mL) as described as follows: Tissue culture plates were coated with 10 μg/mL PLO at 37 °C for 4 h. After 4 h of incubation, the PLO solution was aspirated and the plates were washed 3 times with PBS. The plates were then coated with 5 μg/mL LN at at 37 °C overnight and washed 3 times with PBS prior to use. In the manuscript and figures these plates are simply referred to as LN-coated. Plated neural rosettes were cultured in LN-coated dishes in neural induction media supplemented with 30 ng/ml mouse FGF2 and 30 ng/ml mouse EGF (R&D systems). HSF4-hNPCs were generated as previously described [31]. For routine maintenance, hNPCs were passaged onto LN-coated plates at a density of 1–5 × 10^4 cells/cm^2 in neural induction media supplemented with 10 ng/ml mouse FGF2 and 10 ng/ml mouse EGF2. For neuronal differentiation, hNPCs were grown to confluence and the media was changed to neuronal differentiation media [1X DMEM-F12, 0.5% (v/v) N2 supplement (Life Technologies), 0.5% (v/v) B27 supplement (Life Technologies)] with 20 ng/ml BDNF (R&D Systems), 20 ng/ml GDNF (R&D Systems), 1 μM DAPT (Tocris Bioscience), and 0.1 mM dibutyryl-cAMP (db-cAMP).

2.3. HNPC culture and neuronal differentiation on peptide substrates

All peptides were custom synthesized by AnaSpec. The linear peptide sequences were synthesized on a resin using standard Fmoc chemistries. Analysis of the peptides by analytical HPLC and MALDI-TOF confirmed that the peptides had the correct expected masses. The peptides were then subjected to HPLC using C18 columns to remove any impurities. Analytical HPLC and ESI-MS were used to confirm the purity and mass, respectively. The solvents used to dissolve the peptide were Buffer A (0.1% TFA in water) and Buffer B (0.1% TFA in acetonitrile). Over a run time of 8.5 min, the step-wise gradient increased the percentage of Buffer B from 1% to 30%. The analytical HPLC was monitored at 220 nm. Peptide sequences are listed in Supplementary Table 1. Peptide surfaces were prepared by reconstituting lyophilized peptide in sterile water and coating multi-well plates overnight at 37 °C. Peptide-coated plates were washed twice with PBS prior to culture. HNPC culture and neuronal differentiation was performed in a similar manner as described for PLO/LN-coated surfaces. For conjugation of Fluorescein-5-maleimide (F5M) to VDP coated surfaces,