

WJSC 6th Anniversary Special Issues (3): Embryonic stem cells

Neural differentiation from pluripotent stem cells: The role of natural and synthetic extracellular matrix

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Author contributions: Li Y prepared the original draft; Liu M and Yan Y contributed to writing and editing some sections in the manuscript; Yang ST revised and finalized the manuscript.

Supported by FSU start up fund and FSU Research Foundation GAP award; partial support from National Science Foundation, No.1342192

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Received: October 1, 2013 **Revised:** October 23, 2013

Accepted: November 2, 2013

Published online: January 26, 2014

Abstract

Neural cells differentiated from pluripotent stem cells (PSCs), including both embryonic stem cells and induced pluripotent stem cells, provide a powerful tool for drug screening, disease modeling and regenerative medicine. High-purity oligodendrocyte progenitor cells (OPCs) and neural progenitor cells (NPCs) have been derived from PSCs recently due to the advancements in understanding the developmental signaling pathways. Extracellular matrices (ECM) have been shown to play important roles in regulating the survival, proliferation, and differentiation of neural cells. To improve the function and maturation of the derived neural cells from PSCs, understanding the effects of ECM over the course of neural differentiation of PSCs is critical. During neural differentiation of PSCs, the cells are sensitive to the properties of natural or synthetic ECMs, including biochemical composition, biomechanical properties, and structural/topographical features. This review summarizes recent advances in neural differentiation of human

PSCs into OPCs and NPCs, focusing on the role of ECM in modulating the composition and function of the differentiated cells. Especially, the importance of using three-dimensional ECM scaffolds to simulate the *in vivo* microenvironment for neural differentiation of PSCs is highlighted. Future perspectives including the immediate applications of PSC-derived neural cells in drug screening and disease modeling are also discussed.

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Key words: Pluripotent stem cells; Neural differentiation; Extracellular matrix; Three-dimensional; Drug screening

Core tip: Neural cells derived from human pluripotent stem cells (hPSCs), including oligodendrocyte progenitor cells and neural progenitor cells, emerge as an unlimited and physiologically relevant cell source for drug screening, disease modeling, and regenerative medicine. Natural and synthetic extracellular matrices play an important role in regulating neural differentiation, cell migration, and the derived neural cell maturation. Recent advances in neural differentiation of hPSCs on extracellular matrices in 2-D and 3-D systems are reviewed in this paper. The immediate applications of the derived neural cells in drug screening and disease modeling are also discussed.

Li Y, Liu M, Yan Y, Yang ST. Neural differentiation from pluripotent stem cells: The role of natural and synthetic extracellular matrix. *World J Stem Cells* 2014; 6(1): 11-23 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i1/11.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i1.11>

INTRODUCTION

Human pluripotent stem cells (hPSCs), including human

embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have extensive proliferation potential and the unique ability to produce any type of somatic cells^[1,2]. Due to their self-renewal ability, hPSCs potentially can provide unlimited numbers of neural cells for cell therapy and drug discovery^[3,4]. For example, oligodendrocyte progenitor cells (OPCs) derived from hESCs have been tested in Geron's Phase I clinical trial approved by Food and Drug Administration in 2010 to treat spinal cord injury (SCI)^[5,6]. OPCs derived from hiPSCs have also been shown to myelinate and rescue a mouse model of congenital hypomyelination^[7]. Compared to other animal cells and tissues, hPSCs can provide physiologically relevant cells to deliver more efficacious medicines and to provide accurate models for drug screening^[8,9]. For example, *in vitro* model of amyotrophic lateral sclerosis (ALS) can be established from the motor neurons differentiated from hPSCs, which are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation^[10]. Compared to hESCs, hiPSCs can be derived from individual patients, providing "personalized" medicine and the *in vitro* models to study pathological neural development and disease progression^[11]. For neurological diseases where somatic neural cells are limited in number, hPSCs emerge as a powerful tool for drug screening, disease modeling, and regenerative medicine.

The ability to obtain high-purity and functionally mature neural cells is the pre-requisite to fulfill the potential of hPSCs in neurological disease treatments. Differentiating hPSCs into OPCs or neural progenitor cells (NPCs) with a high purity has been demonstrated, but their function and maturation are still under investigation^[12-14]. Extracellular matrix (ECM) plays an important role in neural differentiation of hPSCs and the maturation of the derived neural cells^[15]. ECM proteins, through the interaction with integrins expressed on the neural cells, modulate cell survival, migration, proliferation, and the differentiated cell function^[16]. Besides ECM composition, the mechanical property of ECMs is also found to regulate neural lineage commitment of hPSCs recently. To better understand the *in vivo* development and the "niches", *i.e.*, microenvironment, of neural tissue development^[15], three-dimensional (3-D) ECMs, both natural and synthetic, have been investigated for efficient neural differentiation of hPSCs. 3-D ECM scaffolds provide not only physical support for cell adhesion, but also the structural and biomechanical cues that can be transduced into biochemical signals, affecting cellular composition during neural differentiation^[17,18]. By regulating biochemical composition, biomechanical properties, and physical structure of 3-D ECMs, neural differentiation of hPSCs can be effectively controlled.

This review summarizes recent advances and the development of protocols for *in vitro* differentiation of hPSCs to OPCs and NPCs with high purity and desired function. To provide the 3-D microenvironment that more resembles *in vivo* tissues than traditional 2-D cultures, 3-D neural differentiation systems based on various natural and synthetic ECMs have been extensively

studied and are discussed in this review, with an emphasis on the effects of ECMs on neural lineage commitment of hPSCs. Current progress in the application of hPSC-derived neural cells for drug screening is also discussed and highlighted.

PLURIPOTENT STEM CELL-DERIVED OLIGODENDROCYTE PROGENITOR CELLS

Oligodendrocytes derived from OPCs can remyelinate axons upon maturation. However, transplanting OPCs instead of mature oligodendrocytes is a better strategy to restore neural function^[19]. While OPCs from somatic tissues are limited in cell number, OPCs derived from hPSCs provide novel alternative autologous or allogeneic cell sources. There are two types of OPC differentiation protocols from hPSCs in general: epithelial growth factor (EGF)-dependent protocol and platelet-derived growth factor (PDGF)-dependent protocol (Table 1)^[20]. OPCs were initially derived from hESCs through embryoid body (EB) formation in the presence of fibroblast growth factor (FGF)-2, retinoic acid (RA), and EGF for 4 wk followed by attaching the neural spheres onto Matrigel-coated surface for another 2 wk (6-wk protocol)^[21]. A high-purity population of OPCs was achieved with the expression of more than 70% NG2, oligodendrocyte transcription factor 1 (OLIG1), OLIG2, and (sex determining region Y)-box 10 (SOX10) using this EGF-dependent protocol. When culturing the cells on human laminin in the absence of mitogen EGF, the derived OPCs displayed minimal neuronal and astrocyte markers, and could mature into oligodendrocytes, which expressed Gal C, O4, and myelin basic protein. Transplanting hESC-OPCs was shown to remyelinate axons and restore the locomotor function in a rat contusion model^[19]. The transplanted OPCs phenotypically replaced lost oligodendrocytes, remyelinated axons, and also secreted neurotrophic factors to establish a repair environment in the lesion^[22,23]. This EGF-dependent OPC differentiation protocol has been successfully used in a manufacturing process in a cGMP (*i.e.*, current Good Manufacturing Practices) facility to produce OPCs for treating SCI patients in Phase I clinical trials^[6]. Although the preliminary safety data were obtained, additional trials are required to demonstrate the efficacy of the hESC-derived OPCs. Different protocols have been developed later using different induction factors, including sonic hedgehog (Shh) protein, PDGF, insulin-like growth factor (IGF)-1, bone morphogenetic protein antagonists such as noggin, neurotrophic factors such as neurotrophin (NT)-3 and ciliary neurotrophic factor, with or without EGF^[24-28]. High-purity OPCs were obtained, and they also showed remyelination capacity in animal study^[29]. The main drawback of these PDGF-dependent protocols is their lengthy (10-14 wk) and complicated procedures with multiple growth factors and multiple steps of suspension and adherent cultures, which are difficult and expensive to scale up for

Table 1 Protocols and *in vivo* studies for oligodendrocyte progenitor cells differentiation from human pluripotent stem cells

Growth factors	Cell source	Cell characteristics	Ref.
EGF-dependent protocol			
RA/EGF (short FGF-2 exposure)	hESC lines: H1 and H7	Olig1 (80%-90%), Sox10 (76%-84%), NG2 (95%); remyelinated in a rat thoracic contusion model	Nistor <i>et al</i> ^[21] , 2005; Keirstead <i>et al</i> ^[19] , 2006; Li <i>et al</i> ^[13] , 2013
RA/EGF (short FGF-2 exposure)	hiPSC lines: Royan hiPSC1, hiPSC8	> 90% Olig2, Sox 10, > 80% NG2 and PDGFR α ; tested in a rat model of optic chiasm demyelination	Pouya <i>et al</i> ^[33] , 2011
RA/EGF (short FGF-2 exposure)	hESC H7 line	OPCs remyelinated in a rat cervical contusion model	Sharp <i>et al</i> ^[94] , 2010
RA/EGF (short FGF-2 exposure)	hiPSC lines: 201B7, 253G1	O4 ⁺ cells were observed in EGF-dependent protocol	Ogawa <i>et al</i> ^[20] , 2011
PDGF-dependent protocol			
PDGF/FGF-2 (short EGF exposure)	SNUhES1 line	PDGFR α (81%), A2B5 (90.4%), NG2 (91.3%) and O1 (81%); myelinate axons in co-cultures with fetus hippocampal neurons.	Kang <i>et al</i> ^[24] , 2007
RA/Shh/FGF-2/PDGF	hESC lines: H1, H9 H14	> 80% PDGFR α ⁺ , also co-express Olig2, Nkx2.2, Sox10 and NG2	Hu <i>et al</i> ^[26] , 2009
RA/Shh/FGF-2/PDGF	hiPSC lines: K04, C14, and C27	70%-90% Olig2 ⁺ /Nkx2.2 ⁺ , OPCs myelinated the brains of myelin-deficient shiverer mice	Wang <i>et al</i> ^[7] , 2013
FGF-2/EGF/PDGF/CNTF plus laminin;	hESC lines: HS360 and HS362;	> 90% NG2 ⁺ , > 80% PDGFR α ⁺ ; multilayered myelin sheet formation around axons was detected in co-culture with neuronal cells	Sundberg <i>et al</i> ^[28] , 2010
Shh, PDGF, IGF-1, EGF, FGF-2 and CNTF plus RA and laminin	Regea 06/040 and Regea 08/023		Sundberg <i>et al</i> ^[27] , 2011
Noggin/FGF-2/FGF-4/PDGF/EGF	hESC H1 line	> 95% cells expressing Sox10, A2B5, PDGFR α , NG2, O4, O1; increased neurological response in a rat contusion model	Kerr <i>et al</i> ^[29] , 2010

RA: Retinoic acid; EGF: Epidermal growth factor; FGF-2: Fibroblast growth factor 2; hESC: Human embryonic stem cell; hiPSC: Human induced pluripotent stem cell; PDGFR: Platelet-derived growth factor receptor; PDGF: Platelet-derived growth factor; Shh: Sonic hedgehog; CNTF: Ciliary neurotrophic factor; IGF-1: Insulin-like growth factor-1.

generating cells needed for clinical studies.

The differentiation of iPSCs to oligodendrocytes was initially performed with mouse iPSCs for the possible application in SCI^[30]. A lower percentage of O4⁺ cells was obtained compared to the differentiation from mouse ESCs^[31]. However, the variability of iPSC lines due to different tissues of origin and reprogramming methods may account for the difference^[32]. The differentiation of hiPSCs to oligodendrocytes was performed using two types of hESC-OPC protocols based on PDGF- or EGF-induced differentiation^[20]. The O4⁺ oligodendrocytes were only observed in the EGF-dependent protocol with a low induction efficiency (< 0.01%). Later, the protocol developed by Nistor *et al*^[21] was tested for hiPSC differentiation, and more than 90% of the differentiated cells expressed OPC markers (OLIG2, NG2, and O4), similar to that obtained with hESC-OPCs. The derived OPCs were transplanted into a demyelinated rat model and showed maturation into oligodendrocytes and the ability of remyelination^[33]. An *OLIG* gene targeting protocol was also developed for hiPSCs, providing the possibility of genetic correction of patient-specific hiPSCs for cell therapy^[34]. High-purity (70%-90%) Olig2⁺/Nkx2.2⁺ OPCs were obtained from hiPSCs treated with RA, Shh, FGF-2 and PDGF, and these OPCs were shown to myelinate the brains of myelin-deficient shiverer mice^[7]. Given the progress made for OPC differentiation from hiPSCs, there is an urgent need for a clinical relevant system to generate a large amount of hiPSC-OPCs for drug screening and autologous transplantation. ECM is an important component during OPC differentiation, affecting both the differentiation efficiency and the derived cell function (Table 2). Thus, understanding the cell-ECM interactions

and development of defined ECM substrates are critical steps for future clinical applications^[13].

EFFECTS OF ECM ON OPC DIFFERENTIATION FROM PSCS

For various types of OPC differentiation protocols, replating the neural progenitors on ECM-coated surface is always part of the procedure^[21,26]. The most common ECMs that have been used for OPC differentiation include laminin, fibronectin, alone or with poly-D-lysine, and Matrigel, which comprises mostly of laminin (Table 2). Oligodendrocytes were reported to express the laminin receptor $\alpha 6 \beta 1$ integrin^[25]. Laminin is thus a potent promoter of oligodendrocyte survival and myelination. Direct comparison of various ECM proteins including fibronectin, laminin, and Matrigel was performed on OPCs isolated from embryonic day 15 rat spinal cords. All three ECMs were found to promote OPC survival, proliferation, migration, and maturation as compared to poly-D-lysine^[35]. Recently, another ECM protein, vitronectin, was shown to promote oligodendrocyte differentiation from hESCs by synergistically interacting with Shh protein^[36]. Besides $\alpha 6 \beta 1$, vitronectin receptors $\alpha v \beta 1$, $\alpha v \beta 3$, and $\alpha v \beta 5$ are also differentially expressed at different OPC developmental stages and play an important role in modulating OPC migration, proliferation, and differentiation^[37]. Especially, vitronectin-derived synthetic peptide acrylate surface (VN-PAS), which contains the active binding site of vitronectin, has been shown to support high-purity OPC derivation from hPSCs (Figure 1)^[13]. Compared to Matrigel-coated surface, VN-PAS supported higher NG2 expression with similar expressions of nestin and

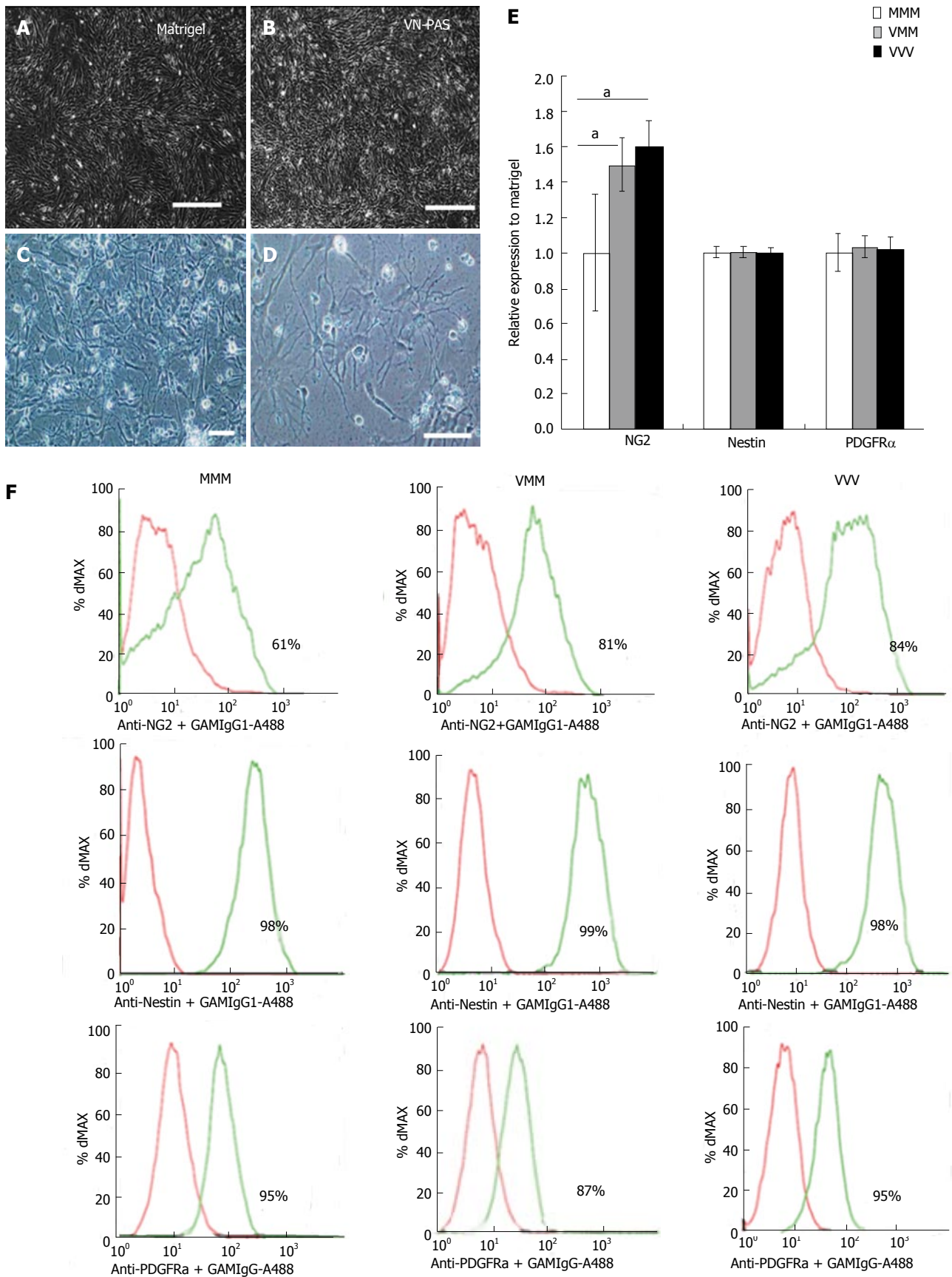


Figure 1 Oligodendrocyte progenitor cells derived from human pluripotent stem cells. A: Morphology of day 41 Oligodendrocyte progenitor cells (OPCs) derived from cells grown on Matrigel; B: Morphology of day 41 OPCs derived from cells grown on vitronectin-derived synthetic peptide acrylate surface (VN-PAS); scale bar: 200 μ m; C and D: Oligodendroglial morphology after OPC maturation; C: Low magnification; D: High magnification, scale bar: 100 μ m; E: OPC marker expression; MMM: all the steps of human pluripotent stem cell (hPSC) expansion and differentiation were performed on Matrigel; MVV: hPSC expansion on Matrigel and differentiation on VN-PAS; VVV: All the steps of hPSC expansion and differentiation were performed on VN-PAS. ^a $P < 0.05$ vs MMM. F: Flow cytometry histograms of OPC markers; PDGFR α : Platelet-derived growth factor receptor alpha. This figure is adapted from Li *et al.*^[13]

Table 2 Effects of extracellular matrices proteins on neural differentiation of pluripotent stem cells

ECM protein	Integrins	Cell source	Neural differentiation	Ref.
Laminin	$\alpha 6\beta 1$	HESC lines: TE03 and TE06	Neuronal generation and neurite outgrowth were significantly greater on laminin and laminin-rich Matrigel than fibronectin, poly-D-lysine, and collagen I	Ma <i>et al</i> ^[16] , 2008
Matrigel (rich in laminin)	$\alpha 3\beta 1$			
Fibronectin	$\alpha 5\beta 1$	Oligodendrocyte progenitor cells from rats	Promoted OPC survival, proliferation, migration, process extension, and OPC purity	Hu <i>et al</i> ^[35] , 2009
Vitronectin	$\alpha v\beta 1, \alpha v\beta 3, \alpha v\beta 5$	hESC lines: Miz-hES4, Miz-hES6	Promoted oligodendrocyte differentiation in the presence of RA/Shh/Noggin	Gil <i>et al</i> ^[36] , 2009
Collagen IV	$\alpha 1\beta 1, \alpha 10\beta 1$	Rabbit neural progenitor cells	Conducive for both neuronal and glial cell differentiation	Raghavan <i>et al</i> ^[61] , 2013
Collagen I	$\alpha 2\beta 1$	Rabbit neural progenitor cells	Conducive for both neuronal and glial cell differentiation	Raghavan <i>et al</i> ^[61] , 2013
Vitronectin-derived synthetic peptide acrylate surface	$\alpha v\beta 5$	hESC line: H1	Promoted oligodendrocyte progenitor differentiation; higher NG2 expression compared to Matrigel	Li <i>et al</i> ^[13] , 2013
Heparan sulfate	Binding heparin	Rabbit neural progenitor cells	Addition of heparan sulfate to collagen mixtures improved neuronal differentiation	Raghavan <i>et al</i> ^[61] , 2013

ECM: Extracellular matrix; hESC: Human embryonic stem cells; RA: Retinoic acid; Shh: Sonic hedgehog; OPC: Oligodendrocyte progenitor cells.

platelet-derived growth factor receptor alpha, demonstrating the active role of ECM-integrin interactions in OPC differentiation. In addition to the single ECM protein, decellularized ECM derived from bone marrow mesenchymal stem cells (MSCs) has also been evaluated for its ability to support neural cell growth^[38]. Compared to poly-D-lysine, MSC-ECM enhanced the differentiation into astrocytes and oligodendrocytes beside neurons, prolonged survival, and better protected the cells from nutrient and growth factor deprivation.

Besides natural ECM proteins, synthetic ECMs have also been developed to better control biochemical and biomechanical microenvironments. Synthetic ECMs such as chitosan and poly (lactic) acid have been used to promote myelination by providing suitable environment to activate Schwann cell function post SCI^[39]. OPCs have been shown to be mechanosensitive; the survival, proliferation, and migration of OPCs in polyacrylamide gels were optimal on intermediate stiffness (0.7-1 kPa) while differentiation efficiency increased with the substrate stiffness^[40]. Chitosan was tested as the substrate for oligodendrocyte differentiation from neural stem cells (NSCs), where stiff surface (> 7 kPa) promoted NSC differentiation into oligodendrocyte while soft surface (< 1 kPa) promoted oligodendrocyte maturation and myelination^[41]. Hybrid-scaffolds combining synthetic ECMs with cell-derived ECMs would be a better strategy as they could provide both biomechanical stability and the large amount of neurotropic factors in treating spinal cord^[42]. ECMs not only modulate the late-stage OPC differentiation, but also provide a cell delivery strategy to enhance the *in vivo* remyelination and tissue regeneration^[42]. However, the effects of ECMs during differentiation of hiPSC into OPCs and using ECM in cell delivery of hiPSC-derived OPCs have not been well studied.

PLURIPOTENT STEM CELL-DERIVED NEURAL PROGENITOR CELLS

NPCs and NSCs are able to differentiate into neurons, astrocytes, and oligodendrocytes, with neuronal lineage

as the dominant population in most cases. Robust neural differentiation has been observed from various hESC and hiPSC lines, although variations among cell lines exist (Table 3)^[12,43,44]. The differentiation of hPSCs into NPCs has been performed either by monolayer induction or by the formation of EBs in suspension, with inducing factors including RA, FGF-2, EGF and Shh, *etc*^[45-49]. Recently, the synergistic induction using two inhibitors of SMAD signaling, noggin and SB431642, resulted in efficient neural differentiation for various hPSC lines^[12,50]. SMADs are intracellular proteins that transduce extracellular signals from TGF- β ligands to the nucleus where they activate downstream gene transcription. The derived neural progenitors demonstrated the ability to further differentiate into dopaminergic neurons, when treated with Shh and FGF8, and motor neurons, when treated with brain-derived neurotrophic factor, ascorbic acid, Shh and RA^[12]. Both monolayer induction and EB formation methods produced high-purity (> 80%) NSCs or NPCs. However, the populations obtained in different studies had different potential to differentiate into mature neuronal types. For example, FGF-2/EGF expanded hiPSC-derived NSCs showed a high tendency to differentiate into γ -aminobutyric acid neurons while RA/FGF-2 induced hESC-derived NPCs differentiated easily into motor neurons^[46,47].

Specific neuronal cell types are required for treating particular neurological diseases. For example, protocols of motor neuron differentiation have been developed by several groups due to their potentials to treat SCI, ALS, and muscular atrophy, *etc*^[11,51]. For the application in treating Alzheimer's disease, the hiPSC-derived neuronal cells were shown to express amyloid precursor protein and capable of secreting A β protein^[52]. To treat stroke-damaged brain, early-stage neural progenitors expressing nestin, Pax6, and Musashi have been used in several studies^[45,53,54]. Human ESC-derived NPCs were transplanted into the cortex rats after permanent distal middle cerebral artery occlusion. Some improvements in sensorimotor functions were observed but more complicated functions were not restored^[45]. HiPSC-derived NPCs have

Table 3 Protocols and *in vivo* studies for neural progenitor cells differentiation from human pluripotent stem cells

Growth factors	Cell source	Cell characteristics	Ref.
EB-based protocol			
FGF-2/RA/ascorbic acid, db-cAMP, HA _g	16 hiPSC lines	13 of the cell lines produced functional motor neurons. Treat with BDNF, GDNF, CNTF to produce motor neurons (4%-15% ISL ⁺ neurons)	Boulting <i>et al</i> ^[43] , 2011
RA/FGF-2	hESC lines: H7, hCSC14, hCSC14-CL1	Produce neuronal progenitors (> 95% nestin and Musashi-1), can become cholinergic neurons, GABA neurons, <i>etc</i>	Nistor <i>et al</i> ^[47] , 2011
Isolated Rosette expanded with EGF/FGF-2	hiPSC lines	Generate long-term expandable neuro-epithelial like stem cells (Lt-NES); in stoke model, the cells improved recovery of fine forelimb movements	Oki <i>et al</i> ^[53] , 2012
RA/Shh	hESC lines H1 and H9	To generate motor neurons (50% HB9 ⁺ motor neurons)	Hu <i>et al</i> ^[51] , 2009
Monolayer-based protocol			
Noggin and SB431542	hESC line: H9	> 80% PAX6 ⁺ cells; Shh/FGF8 for midbrain dopamine neurons; BDNF, Shh, RA, ascorbic acid for motor neurons	Chambers <i>et al</i> ^[12] , 2009
	hiPSC lines: iPS-14, iPS-27		
Noggin and SB431542	hESC line: H9	Treat BDNF, GDNF, NT-3 for forebrain neurons which secrete Aβ for drug screening.	Yahata <i>et al</i> ^[52] , 2011
	hiPSC line: 253G4		
Noggin only; FGF-2 may be added at later stage	hESC line: H1, H7, H9	> 90% nestin, musashi, and PSA-NCAM; For TH neurons, add Shh/FGF8/ascorbic acid; followed by BDNF, GDNF, ascorbic acid and laminin	Gerrard <i>et al</i> ^[48] , 2005
FGF-2	hESC line: SA002 and AS034	> 90% nestin, NCAM; For neuronal lineage, add Shh/FGF8; after differentiation TH ⁺ cells, MAPab ⁺ cells and astrocytes existed	Axell <i>et al</i> ^[49] , 2009

EB: Embryoid body; FGF-2: Fibroblast growth factor-2; RA: Retinoic acid; db-cAMP: Dibutyl- α -cAMP; HA_g: A small molecule agonist of the sonic hedgehog pathway; hESC: Human embryonic stem cell; hiPSC: Human induced pluripotent stem cell; BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell-derived neurotrophic factor; CNTF: Ciliary neurotrophic factor; GABA: Gamma-Aminobutyric acid; Shh: Sonic hedgehog; NT-3: Neurotrophin-3; PSA-NCAM: Polysialylated-neural cell adhesion molecule; TH: Tyrosine hydroxylase; NCAM: Neural cell adhesion molecule.

also been shown to engraft with little neuroblasts or morphologically mature neurons in a rat model^[55,56]. Recently, transplantation of hiPSC-derived NSCs exhibited functional recovery and electrophysiological properties of mature neurons, and was proved to be a safe approach for neuron replacement in stroke-damaged brain^[53]. However, the cell engraftment and *in vivo* maturation are yet to be improved. Transplantations of NPCs derived from hiPSCs for treating other neurological diseases such as ALS and muscular atrophy have also been demonstrated in proof-of-principle studies^[47,57]. The neural progenitors survived and engrafted *in vivo*, and the nestin-positive cells differentiated into neuronal phenotype and motoneuron-like structure in both wild-type rats and the ALS rats harboring a mutated human *SOD1* (*G93A*) gene^[57]. To eliminate the risk of tumorigenicity of the residual undifferentiated hPSCs, intermediate NPC and NSC lines were established from hPSCs, which can be maintained for more than 100 passages^[46]. There are growing interests in functional NPC differentiation from hPSCs to generate neural cells with clinically relevant quality and quantity for preclinical and potential clinical studies^[58]. Current challenges include the functional maturation of NSCs and NPCs both *in vitro* and *in vivo*^[58]. Large-scale generation of a specific neural subtype also remains a major challenge for neuronal differentiation of hPSCs. Recreating the stem cell niches enriched with ECMs is being pursued to address these challenges^[15].

EFFECTS OF ECM ON NPC DIFFERENTIATION FROM PSCS

ECM proteins have been shown to regulate the survival,

proliferation, and neurite outgrowth of hESC-derived NPCs in a dose-dependent manner through integrin-ECM signaling (Table 2)^[16]. Similar to OPCs, NPCs also express integrin $\alpha 6 \beta 1$ and its ligand laminin is a major ECM protein that regulates NPC differentiation. Neuronal generation and neurite outgrowth were significantly greater on laminin and laminin-rich Matrigel substrates than other substrates including fibronectin, poly-D-lysine, and collagen I^[16]. Delivering NPCs in laminin- or fibronectin-based constructs into injured brain showed the improved survival, migration, and behavioral recovery at 8 wk post-transplant^[59]. Endogenous ECMs derived from the RA-treated EBs also accelerated neural differentiation, demonstrating the signaling capacity of ECM environment associated with the lineage commitment^[60]. The native ECMs derived from PSC aggregates had a high content of fibronectin, laminin, collagen IV and vitronectin (Figure 2), which after decellularization can be used as 3-D scaffolds to promote stem cell adhesion, proliferation and differentiation. Such ECM scaffolds contain the balanced composition with the sequestered biological factors which provide the unique signaling to mediate the coordinated cellular events of stem cells. The composition of ECM proteins consisting of laminin, collagen IV, and heparan sulfate was found to regulate the balance of neuronal and glial cell differentiation; the ECM containing a higher portion of laminin and heparan sulfate induced more neuronal differentiation^[61]. Neural differentiation of PSCs is associated with the switch from E-cadherin expression to N-cadherin expression. Hence, recombinant ECM components based on E-cadherin and N-cadherin hybrid substratum were also shown to support neural differentiation of ESCs and iPSCs^[62].

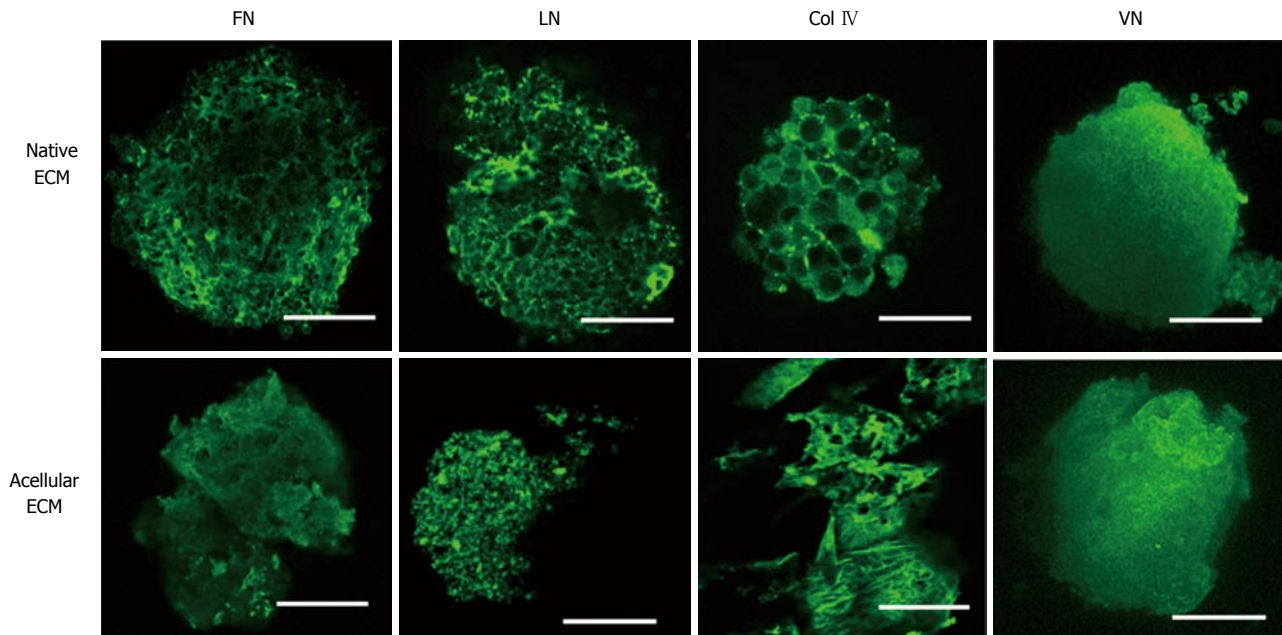


Figure 2 Three-dimensional extracellular matrix scaffolds derived from pluripotent stem cell aggregates. Confocal images of fibronectin (FN), laminin (LN), Collagen IV (Col IV), and vitronectin (VN) expression pre- and post-decellularization [acellular extracellular matrix (ECM) and native ECM, respectively]. Scale bar: 100 μm . For native Col IV, scale bar: 50 μm . The ECM scaffolds can be used for neural differentiation. Images are adapted from Sart *et al.*^[60].

Besides the ECM composition, the mechanical property of ECMs such as stiffness also affects neural differentiation. HPSCs are sensitive to biomechanical cues of the microenvironment^[63-65] and respond quickly to stiffness change^[65,66]. For hPSCs, a stiff surface was found to promote cell attachment and proliferation with dense F-actin expression while a soft surface led to cell detachment^[67]. For neural lineage, soft hydrogels (100-500 Pa) promoted neuronal lineage while hard hydrogels (1-10 kPa) promoted glial differentiation^[68-70]. Similarly, soft ECMs with a stiffness similar to that of the neural tissue (100-700 Pa) promoted the generation of early neural ectoderm from hPSCs, while this effect was less pronounced for hard ECMs (7.5 kPa)^[71]. In studies simulating the biomechanical environment in each germ-layer, the scaffolds with high (1.5-6 MPa), intermediate (0.1-1 MPa), and low elastic moduli (< 0.1 MPa) were found to promote mesodermal, endodermal, and ectodermal differentiation of hPSCs, respectively^[66,72]. ECMs may function as force sensors and transduce the biomechanical signals through the ECM-integrin-cytoskeleton pathway^[73]. Therefore, the biomechanical elasticity of ECMs is a potent regulator for neural lineage commitment of hPSCs.

THREE-DIMENSIONAL NEURAL DIFFERENTIATION OF PSCS

Because cells *in vivo* are exposed to a 3-D ECM environment, 3-D neural differentiation in natural or synthetic ECM scaffolds has been studied to mimic the architecture and biological role of the ECM in modulating stem cell fate decision^[17,74]. Different 3-D synthetic ECM scaffolds

including hydrogels, microfibrillar, and nanofibrillar matrices have been used for neural differentiation from PSCs or PSC-derived neural precursors (Table 4)^[75-78]. For example, using chitin-alginate 3-D microfibrillar scaffolds together with RA and noggin, nestin-expressing neural progenitors were derived from three independent hiPSC and hESC lines^[75]. Neuron growth factor-grafted poly(ϵ -caprolactone)-poly(γ -hydroxybutyrate) scaffolds were demonstrated to improve iPSC differentiation into neurons while inhibiting differentiation into other lineages^[79]. In another example of a 3-D synthetic hydrogel-based system, PuraMatrixTM, hESC-derived neuronal cells developed more branched neurite structures and formed more electrically active networks as compared to 2-D differentiation, better resembling the *in vivo* tissues^[76]. Electrospun polyurethane fibrous scaffolds have been shown to preferably differentiate hESCs into the neuronal lineage over the glial lineage^[80]. A 3-D system involving an air-liquid interface was shown to generate a self-organized three-dimensional neural tissue guided by endogenous developmental cues on hydrophilic polytetrafluoroethylene membrane^[81]. Tissue-engineered fibrin scaffolds were developed to enhance PSC-derived NPC survival and direct differentiation into neurons^[82]. All these studies demonstrated that 3-D scaffolds physically influenced neural lineage commitment from PSCs.

The contact guidance and topography effects of 3-D scaffolds on neural differentiation were revealed in several studies recently^[77,83]. The 3-D microfibrillar poly(ethylene terephthalate) (PET) scaffolds have been shown to support neural differentiation of PSCs induced in an astrocyte-conditioned medium^[84,85]. Compared to 2-D differentiation, 3-D differentiation in microfibrillar matri-

Table 4 Three-dimensional natural and synthetic extracellular matrices scaffolds for neural differentiation of pluripotent stem cells

Scaffolds	Cell source	Neural differentiation	Ref.
Poly(lactic-co-glycolic acid) and poly(L-lactic acid) scaffolds	hESC	Enhanced numbers of neural structures and staining of nestin and β -tubulin III were observed	Levenberg <i>et al</i> ^[74] , 2005
Synthetic hydrogel matrix PuraMatrix	hESC-derived neuronal cells	HESC-derived neurons, astrocytes, and oligodendrocytes grew, matured and migrated in hydrogel; neuronal cells had electrically active connections.	Ylä-Outinen <i>et al</i> ^[76] , 2012
Poly(epsilon-caprolactone)-poly(beta-hydroxybutyrate) scaffolds	Mouse iPSCs	Improved iPSCs to differentiate into neurons and inhibited other differentiations.	Kuo <i>et al</i> ^[79] , 2012
Polycaprolactone nanofiber matrices	HESC-derived neural precursors	Aligned fibrous matrices showed higher rate of neuronal differentiation compared to random micro- and nano-fibers (62%-86% <i>vs</i> 27%-32%).	Mahairaki <i>et al</i> ^[77] , 2011
Polyurethane nanofibrous scaffolds	hESC line SA002	Neuronal differentiation was preferred over astrocyte differentiation.	Carlberg <i>et al</i> ^[80] , 2009
Tissue-engineered fibrin scaffolds	Mouse ESC-derived NPCs	Enhanced NPC survival and directed differentiation into neurons.	Johnson <i>et al</i> ^[82] , 2010
PET microfibrous scaffolds	Mouse ESC D3 line	Enhanced neuronal differentiation indicated by nestin, Nurr1, and tyrosine hydroxylase compared to 2-D culture.	Liu <i>et al</i> ^[84,85] , 2013, 2013
Multiwalled carbon nanotube modified PET microfibrous scaffolds	Mouse ESC D3 line	Enhanced neuronal differentiation compared to unmodified scaffolds	Zang <i>et al</i> ^[83] , 2013
Chitin-alginate 3-D microfibrous scaffolds	hESC line: HUES 7 hiPSC lines: PD-iPS5 and hFib2-iPS4	Efficient neuronal differentiation: > 95% nestin ⁺ ; able to mature into neurons (> 90% β -tubulin III ⁺)	Lu <i>et al</i> ^[75] , 2012
3-D ECM scaffolds derived from ESC aggregates	Mouse ESC D3 line	ECM scaffolds derived from RA-treated EBs enhanced nestin and β -tubulin III expressions	Sart <i>et al</i> ^[60] , 2013
Inverted colloidal crystal (ICC) scaffolds containing alginate, poly(gamma-glutamic acid), and surface peptide; or chitin-chitosan-gelatin ICC scaffolds	Mouse iPSCs	Accelerated neuronal differentiation (β -tubulin III expression) of iPSCs.	Kuo <i>et al</i> ^[86,87] , 2013, 2013

hESCs: Human embryonic stem cells; iPSCs: Induced pluripotent stem cells; NPC: Neural progenitor cells; PET: Poly(ethylene terephthalate); ECM: Extracellular matrix; RA: Retinoic acid; EB: Embryoid body.

ces resulted in a higher percentage of nestin-positive cells (68% *vs* 54%) and upregulated the expressions of nestin, Nurr1, and tyrosine hydroxylase. Multiwalled carbon nanotubes (MWCNTs) were used to coat and provide nano-features on the surface of 3-D PET fibers, which significantly enhanced neuronal differentiation of ESCs compared to the surface without MWCNTs (Figure 3)^[83]. Without MWCNTs, cells were flatly spread out on the PET membrane with few neurites formed. In contrast, with MWCNT, more neurons were observed across the surfaces of carbon nanotubes, forming a neural network with extensive neurite bridges between adjacent cells both on 2-D PET membrane and 3-D PET matrices. The 3-D differentiation in PET scaffolds was also demonstrated in stirred bioreactors for potential scale up^[85]. The effects of fiber diameter and fiber orientation of polycaprolactone fiber matrices were evaluated for hESC-derived neural precursors^[77]. The NPCs adhered on the aligned fibers showed a higher rate of neuronal differentiation as compared to cells cultured on random micro- and nano-fibers (62%-86% *vs* 27%-32%). The alginate, poly(-glutamic acid), and surface peptide based inverted colloidal crystal (ICC) scaffolds were shown to provide hexagonal crystals of polystyrene microspheres with interconnected pores, in which topography together with the surface peptide improved the differentiation of iPSCs into neuron cells^[86]. Chitin-chitosan-gelatin scaffolds with ICC geometry were also found to accelerate neuronal differentia-

tion of iPSCs compared to free-form constructs^[87]. The topography with different surface gratings can increase the rate of neural differentiation of hPSCs, although the mechanisms that transduce the topographical signals into cell phenotype remain unknown^[88]. By ingenious design of novel 3-D scaffolds, the neural differentiation from PSCs or the derived NPCs can be promoted.

DRUG SCREENING BASED ON HPSC-DERIVED NEURAL CELLS

Current drug screening methods using immortalized human lines or rodent models cannot accurately represent how various drugs would initiate the response in humans due to the physiological differences between animal and human as well as the lack of native metabolic and biological functions^[89]. Although the sensitivity of human primary cells (*e.g.*, human cardiomyocytes) may give better response, these somatic cells are often limited by the available cell numbers. Estimates indicate that every 1% increase in predictability of toxicity in human would save up to \$100 million in the pharmaceutical industry^[90]. A human cell-based drug screening platform is thus desirable for drug discovery and mechanistic studies of various neurological diseases. Human PSCs, especially iPSCs, provide a great platform to generate allogeneic or patient-specific neural cells that are physiologically relevant for drug screening and disease modeling^[8]. For example, A β -

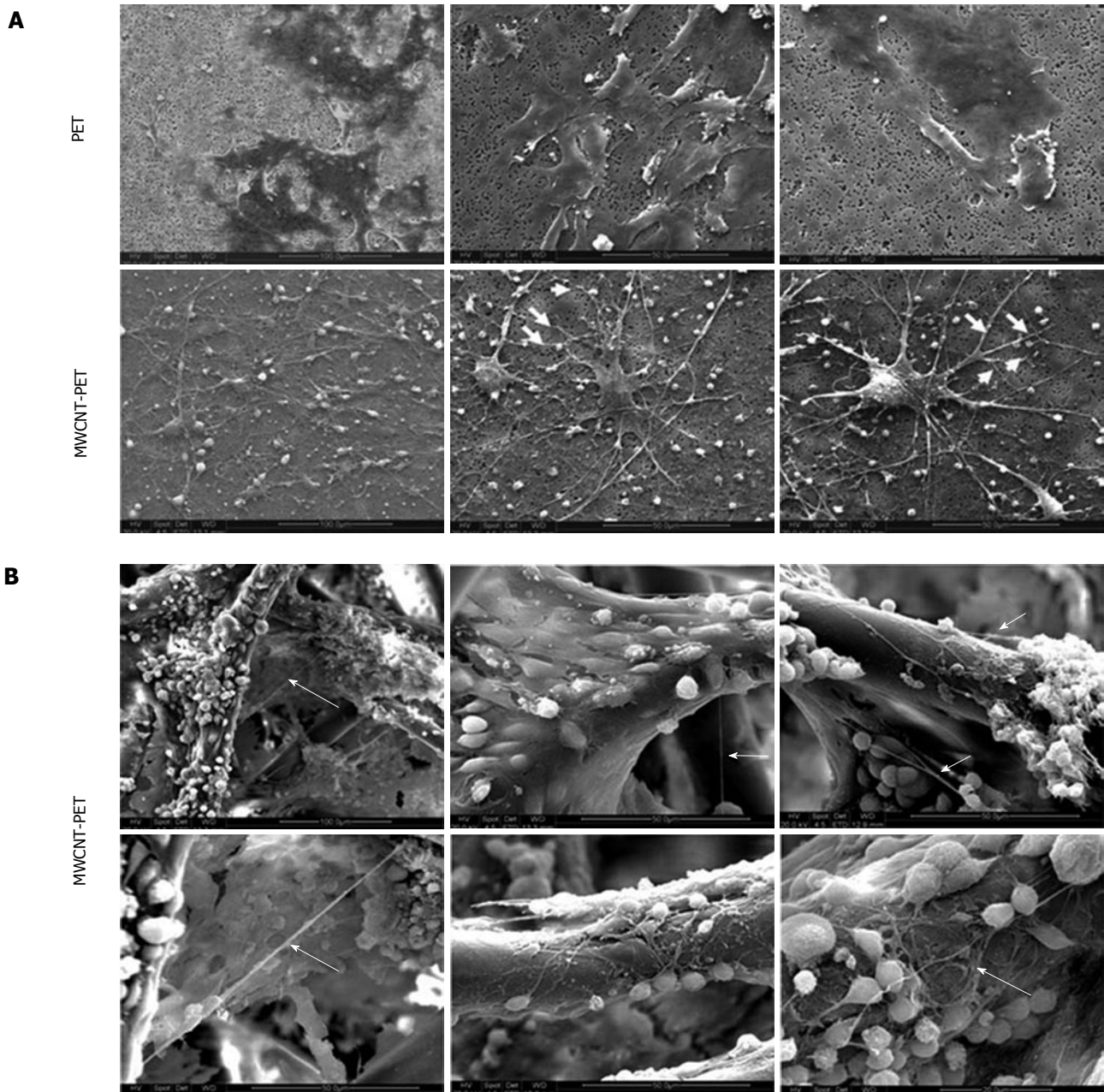


Figure 3 Neural differentiation of pluripotent stem cells. A: Neural cells derived from murine embryonic stem cells (mESCs) cultured on 2-D PET surface with or without multiwalled carbon nanotube (MWCNT) coating; B: Neural cells derived from mESCs cultured in 3-D PET scaffolds with MWCNT coating. Arrows point to neurite fibers. Images are adapted from Zang *et al.*^[83]

secreting neurons were derived from hiPSCs and used for screening anti-A β drugs for the treatment of Alzheimer's disease^[52]. β -secretase inhibitor and γ -secretase inhibitor were shown to inhibit A β 40 and A β 42 secretion from hiPSC-derived neuronal cells. Overexpressing synuclein in hESC-derived dopamine neurons led to the selective cell death; thus drugs interacting with this process or reducing the accumulation of synuclein in cells can be used to treat Parkinson's disease^[91]. Quantitative analysis of neural cells derived from hiPSCs harboring mutations associated with neurodegenerative disorders (*e.g.*, Parkinson's, ALS and schizophrenia) indicated the defects in cell growth, migration, and function compared to healthy

donors^[90]. These disease-relevant cells are more suitable for assessing the outcome of drug treatment. For examples, anti-psychotic drug loxapine has been shown to improve neuronal connectivity in Schizophrenia models established from hiPSCs^[92]. The selective loss of motor neurons derived from iPSCs of spinal muscular atrophy patients was also decreased by treating with drugs such as valproic acid and tobramycin^[91].

High-throughput analysis and high-content imaging platforms need to be developed for efficient screening. Various automated platforms, including IN Cell Analyzer (GE Healthcare), Cellomics Arrayscan (ThermoFisher), and ImageXpress (Molecular Devices), have been devel-

oped to collect information about cell physiology and function, including cell viability and apoptosis, cell number and proliferation, cell migration *etc*^[90]. 3-D culture conditions are necessary to recreate the phenotype better representing *in vivo* neural tissues. The main challenge of hPSC-based drug screening is that the cells generated from hPSCs are developmentally immature^[91]. Thus, functional maturation of hPSC-derived cells is being actively pursued in the field. Compared to 2-D platform of drug screening, 3-D ECM scaffold-based screening has been shown to be more predictive in terms of cell sensitivity to the drugs^[93]. Hence, efficient 3-D neural differentiation systems that can enhance neural cell functions are in a great demand. High-throughput electrophysiology is also a critical component in drug screening because it can provide functional readouts during the screening. Therefore, the pharmaceutical industry is developing the platform such as PatchXpress to assess the effect of ion channel modulators. Given the challenges in cell therapy and transplantation, disease modeling and drug screening have been considered as two immediate applications of hPSCs.

CONCLUSION

Neural cells (including oligodendrocyte progenitors and neural progenitors) derived from hPSCs have great potential in drug screening, disease modeling, and regenerative medicine. High-purity neural cells can be derived from hPSCs induced by various biological and biochemical cues. Natural and synthetic ECMs, including their composition, mechanical properties, and physical structures play important roles in regulating cell survival, proliferation, migration, and differentiation. Therefore, there is an urgent need to optimize ECMs for efficient neural differentiation and functional maturation, especially 3-D ECM scaffolds, which can interact with other niche factors (*e.g.*, cytokines, accessory cells and nutrients) and provide the physiologically relevant microenvironment to guide neural tissue development. Understanding the biochemical and biomechanical interactions of hPSCs and the ECMs should accelerate the applications of hPSCs, especially in the immediate applications in drug screening.

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P- Reviewers: Guo ZK, Holan V, Sauer H S- Editor: Qi Y
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