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Review

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Application of microfluidic systems for neural differentiation of cells

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Abstract

Neural differentiation of stem cells is an important issue in the development of the central nervous system. Different methods such as chemical stimulation with small molecules, scaffolds, and microRNA can be used for inducing the differentiation of neural stem cells. However, microfluidic systems with the potential to induce neuronal differentiation have established their reputation in the field of regenerative medicine. Organization of the microfluidic system represents a novel model that mimics the physiologic microenvironment of cells among other two- and three-dimensional cell culture systems. The microfluidic system has a patterned and well-organized structure that can be combined with other differentiation techniques to provide optimal conditions for neuronal differentiation of stem cells. In this review, different methods for effective differentiation of stem cells to neuronal cells are summarized. The efficacy of microfluidic systems in promoting neuronal differentiation is also addressed.

Keywords: Neural stem cells differentiation, Chemical stimulation, Scaffold, MicroRNA, Microfluidic systems

Purpose and Rationale

In this review, the concept of microfluidics and their combination with small molecules and three-dimensional (3-D) scaffolds are addressed. The neural differentiation of stem cells in molecular and cellular biology branch of microfluidics application is also highlighted.

Introduction

In the early 20th century, it was more like fiction rather than reality to carry a well-equipped laboratory in your pocket! Yet, in the mid-1990s, by the advent of micro-technology to biologic studies and using microfluidic systems, a dramatic development occurred in miniaturizing laboratories. Now it seems that fiction is becoming fact and objectivity. Micro-technology is a complex mix of procedures, instruments, and techniques used for constructing structures with features in order of micrometer, commonly within the range of 10^{-4} to 10^{-7} meters.[1] Microfluidics are typically microfabricated systems that deal with processing and conducting microliter volumes of fluids through micron size (10 to a few hundred) chambers and channels.[2,3] Nowadays, it seems that the

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number of applications of microfluidic systems even in general aspects is becoming uncountable; some instances in the field of medicine include diagnosis (pathology), disease and injury models, synthesis of drug delivery carriers, radiopharmaceutical synthesis, *in vitro* models for drug screening (safety and discovery), toxicology studies and animal microsurgery. Furthermore, cell manipulation (mechanical, magnetic, optical and electrical) studies, cell analysis, tightly controlled cell culture and tissue engineering are of the most relevant biological applications of microfluidic systems. Based on facilities provided by the microfluidic system, they have become considerable candidates for the induction of neural differentiation of stem cells. Main incorporated advantages in neural differentiation of stem cells are highlighted in Figure 1.

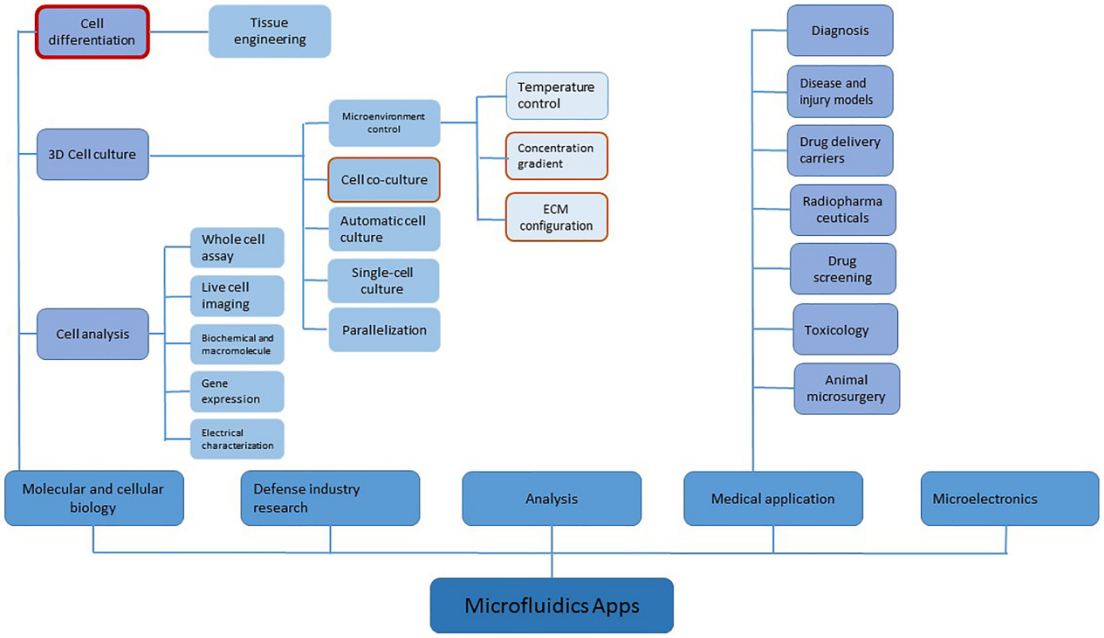

Figure 1. *Molecular and cellular biology branches of a microfluidics application. ECM = extracellular matrix.*

Based on medical evidence, nervous system injury may occur as a result of trauma or disease. Stem cells are widely considered as biopharmaceuticals and cell therapies due to their potential proliferation, genetic modification with external gene delivery systems, and differentiation to neurons, astrocytes, and oligodendrocytes. [4] Consequently, inducing the differentiation of stem cells toward neural cell lineages plays an important role in the repairing process. There are 3 main types of stem cells involved in trans-differentiation studies and cellular replacement therapy including embryonic stem cells (ESC), adult stem cells (ASC), and induced pluripotent stem cells (iPSC). However, it seems that ESCs and ASCs are the major stem cells involved in neural differentiation.[5] There have also been a large number of investigations on iPSCs in recent years in spite of their shorter history in stem cell biology research.[6] Relative neural differentiation has been shown using different types of stem cells while there is no consensus on optimum stem cells to use.

Several approaches have been developed over the years for promoting the differentiation of stem cells. The most often applied techniques in biological and clinical research include stimulation with small molecules like growth factors (GF), different types of scaffolds, gene editing, and micro-RNA (Ribonucleic acid) incorporation. Beside them, microfluidic devices have paid more attention to this area recently. Microfluidic systems are highlighted in the differentiation of stem cells due to their specific advantages in conquering the limitations of

conventional systems including a decrease in reagents consumption, reduction in expenses, and flexibility in designing various microfluidic structures based on each experiment objectives. Moreover, they have their own biological and medical related merits such as the possibility of cellular co-cultures on a unique chip, capability to mimic the natural microenvironment of cell including physical structure, appropriate medium flow and shear stress, desired oxygen and CO_2 tension, utilization of specific ECM for target organ, continuous medium flow, lower contamination risk and tight control of biochemical gradient.[7] The advent of microfluidics in rapid response to environmental changes such as fast temperature equilibrium and mass media transfer in microscale should also be noted.[8]

Summary of relevant literature and discussion

Role of Microfluidic Devices in Neural Differentiation

As previously mentioned, stem cells in microfluidic systems possess the merit of easy and precise control of the optimal microenvironment for cell culture. The combination of physicochemical and mechanical parameters can organize the cell microenvironment in terms of pH, temperature fluctuations, oxygen, osmolality, GF concentration gradient, cell-cell contact/signaling, and cell-ECM interactions and cell migration, which altogether determine cell function and behavior.[9,10] Although microfluidics can be utilized individually for stem cell differentiation, they mainly provide a suitable platform for combining differentiation techniques. For instance, the effect of shear stress as a physical intervention or GFs as small molecules can be investigated along with 3-D scaffolds simultaneously in micron size channels.

Microfluidic devices in combination with small molecules

Cellular differentiation is completed through a well-orchestrated procedure where a cell changes the expressing proteins to generate a new phenotype.[11] The incorporation of small molecules has many advantages such as providing a high degree of temporal (reversible and rapid influences) and spatial (effects narrowed to a different cell or tissue organ) control over protein function and possessing more tunable properties by changing their combinations and concentrations.[12] Small molecules may include GFs, ECM components, cytokines, and vitamin derivatives which their incorporation in different processes may accelerate the differentiation of stem cells. Some of the frequently used stimulant molecules are retinoic acid (RA),[13, 14] ascorbic acid,[15] basic fibroblast growth factor (bFGF),[16] nerve growth factor (NGF),[17] and insulin-like growth factor (IGF-I).[18]

Although these macroscopic cell culture dishes are widely utilized with standard protocols and are relatively easy to set up, they face some limitations in practice including the rigid cell culture surface, stagnant culture media, fixed device architecture, difficult to achieve perfusion, and chemical gradients. Mentioned constraints minimize the simulation of *in vivo* environment for cultured cells. Moreover, mostly requisiteness of end-point analysis and high amounts of reagent consumption followed by higher expenses are other impediments.[19] With the event of microfluidic systems, the mentioned limitations are progressively dissolving. Various types of small molecules, mainly, ECMs and GFs have been incorporated in most research on the neural differentiation of stem cells in microfluidic platforms. Among all the advantages of microfluidics, the possibility of gradient generation and gradient control sounds supreme for small molecules. In micron size channels, the flow behavior of fluids is regulated by viscous dynamics and therefore is laminar. This laminar flow favors the concentration gradient generation which is based on the fluid flow and shows acceptable temporal and spatial stability.

Yuta Nakashima and Takashi Yasuda in 2007 investigated the effect of GFs on cell differentiation and axon elongation guidance.[20] They fabricated a microfluidic device consisted of a cell culture chamber, a microchannel, a nano-hole array (containing GF) and a micro-valve which allowed precise control for releasing the chemicals from nano-hole. They used NGF for stimulating the differentiation of adrenal pheochromocytoma (PC12) cells. The results indicated that the cell growth, differentiation, and

axon elongation were depended on micro-valve switching and release gradient of NGF. Therefore, they suggested that the designed microfluidic device would be useful for further studies on nerve regeneration.[20] Continuing combinational investigations, more recently Nayeon Lee and co-workers analyzed the concomitant effect of GFs and ECM in a microfluidic device. Human embryonic stem cells (hESC) were co-cultured with PA6 stromal cells to form rosette-like structure and inducing neurosphere formation. Neurospheres were transferred to a microfluidic device which was double coated with poly-L-ornithine and fibronectin as ECM and brain-derived neurotrophic factor (BDNF) was used to achieve mature neurons. Immunocytochemical (ICC) analysis utilizing various markers including human neurofilaments (NF-H) and Tau (for axon identification), microtubule-associated protein (MAP2) (dendrite identification) confirmed the migration of neural cells and differentiation of these cells into mature neurons. However, ECM free and GF free systems were also used independently in order to evaluate their specific role.[21] Following GF studies, a GF gradient-generating microfluidic device was designed. Cells were exposed to a continuous gradient of GFs to optimize the proliferation and differentiation of human Neural Stem Cell (hNSC) into astrocytes (GF mixture contained epidermal growth factor [EGF], fibroblast growth factor 2 [FGF2] and platelet-derived growth factor [PDGF]). A similar study was designed by using neural stem cells (NSCs) and an EGF and bFGF gradient-generating microfluidic chip. Results interestingly confirmed that the proliferation and differentiation of hNSCs were directly depended on GF concentration.[4,22] Many other bio-molecules can influence the differentiation of stem cells based on their physiologic role in specific organs in vivo.

Consequently, Joong Yull Park et al. examined the effect of cytokines instead of GFs in microfluidic systems to evaluate the effect of this combinatorial system on the differentiation of neural progenitors into neurons.[23] They incorporated sonic hedgehog (Shh), FGF-8, and bone morphogenetic protein 4 (BMP-4) cytokines. The microfluidic platform was built in a way to produce a stable concentration gradient of signaling molecules in one week. Results demonstrated that neural progenitors successfully differentiated into neurons, producing complex neuronal networks. The average numbers of neuronal cell body clusters and neurite bundles also showed a direct relation to Shh concentrations in the gradient chip.[23] Utilization of small molecules in stem cell differentiation leads to higher efficiencies; meanwhile, microfluidic systems provide additional favorable outcomes.

Microfluidic devices in combination with 3-D scaffolds

Considering cells are embedded in a 3-D environment and surrounded with other tissues in the human body, it is quite clear that the solution state is not sufficient to provide an appropriate mechanical and physical support for optimal differentiation.[24] On the other hand, communication between cells, transportation of oxygen and nutrients and removal of waste products requires arranged cellular orientation and possibility of movement. In order to overcome the aforementioned problems, various scaffolds have been developed. It is easier to obtain the desired tissue by mimicking the ECM using 3-D scaffolds.

3-D scaffolds are able to organize the stem cells into a higher ordered construct to achieve the optimal neural tissue ECM. Microspheres,[25] fibers,[26,27] (self-assembling peptides[28,29] or polymeric[30]) hydrogels,[31] and conductive scaffolds[32,33] are examples of different scaffolds used for neural differentiation of stem cells. While scaffolds provide the possibility of 3-D cell culture, limitations on dynamic fluid flow and probable gradient generation still remain. In addition, since cell culture studies on scaffolds are performed in conventional cell culture dishes, high amounts of reagent expenditure are imposed.[34] A strategy to overcome the possible limitations of 3-D scaffolds in neural cell differentiation is mixing these structures with microfluidic systems.

Hence, S Han et al. investigated the role of ECM-containing hydrogels in guiding NSC differentiation into neuron and oligodendrocyte.[35] To develop the 2-D cell culture system into 3-D cell culture, they employed ECM-containing hydrogel scaffold in

micro-channels. Three different hydrogel groups were compared in this research including collagen type I (Col I), Matrigel and their 1:1 mixture. Quantitative real-time polymerase chain reaction (qRT-PCR) and microscopic results showed that neuronal and oligodendrocyte differentiation of NSCs was significantly higher in 3-D culture in comparison to 2-D culture. Moreover, Matrigel containing ECM groups revealed a better differentiation compared with the group containing bulk Col. The presence of laminin (LN) in the structure of Matrigel influence of ECM on stem cell differentiation was confirmed by Sewoon Han et al., it's supposed that a combination of other stimulation techniques may have synergistic effects on differentiation yield. Furthermore, it is possible to compare the role and efficiency of various techniques on cellular differentiation.[35] In this regards, Kisuk Yang et al. evaluated the effect of hypoxic condition along with various types of ECM on neural differentiation of human NSCs in a 3-D microfluidic device.[36] They compared 4 ECM hydrogel groups including (1) Col, (2) Col+fibronectin (FN), (3) Col+LN, and (4) Col+FN+LN, in both hypoxia and normoxia conditions. The microfluidic chip contained 8 units in a single device. Each unit consisted of one central channel for hNSC culture in ECM and 2 side channels for supplying with growth medium. Immunofluorescence and qRT-PCR results showed that ECM proteins may promote the formation of an appropriate 3-D microenvironment which induced the differentiation of hNSCs into the neuronal lineage. They also found that neuronal differentiation of hNSCs was increased in low oxygen tension using most of the 3-D ECMs (e.g., Tuj1 expression was significantly increased in Col+FN and Col+LN ECM groups in day 7). It was concluded that ECM proteins such as LN affected the neuronal differentiation of hNSCs at an early stage in culture, but the effect of hypoxia was dominant at later time points in neuronal commitment.[36] Furthermore, Thibault Honegger et al. used an unmodified collagen scaffold in order to guide, accelerate, slow down and push up neuritis in a microfluidic device, incorporating AC electrokinetic forces.[37] They established an *in vitro* brain structure motif which reveals the 3-D neuronal networks as a minimal microenvironment to study the brain circuitry.[37]

While hydrogel scaffolds have been combined with microfluidics in some aforementioned studies, to the best of our knowledge, only one investigation has incorporated nanofibrous scaffold along with microfluidic system yet. Hesari et al. developed a hybrid microfluidic system consisting of poly(lactic-co-glycolic acid) (PLGA) nanofibrous scaffold and Polydimethylsiloxane (PDMS) microchip for differentiation of hiPSCs into neurons.[38] The neural differentiation of hiPSCs was confirmed using qRT-PCR and immunostaining evaluations. Incorporation of a hybrid microfluidic system for hiPSC differentiation revealed an increased level of β-tubulin III (a neuron-specific marker) gene expression while the expression of glial fibrillary acidic protein (GFAP) gene (a classic marker for astrocytes) was decreased. Consequently, the hybrid device was turned to be optimum for neuronal differentiation. The results obtained from this study were in complete agreement with the observations of Yang et al.[40] Next, the cell loaded scaffold was implanted in a spinal cord injury (SCI) model of rats, for further *in vivo* evaluation of this hybrid microfluidic system. Results demonstrated that although animals receiving this implant showed functional improvement during 28 days of study, but the difference with the control group was statistically insignificant. (Figure 2).[38]

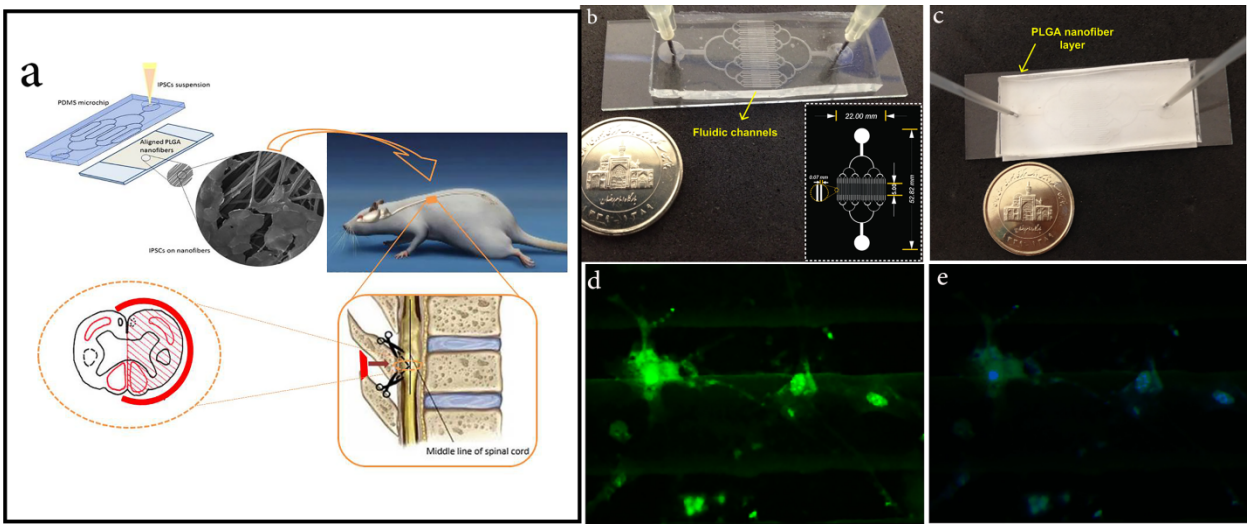

Figure 2. Hybrid microfluidic device. (a) Schematic representation of in vitro and in vivo experiments, (b) PDMS microchip with fluidic channels. Cells were cultured in 32 identical microchannels and were subjected to slow fluidic flows concurrently, (c) Hybrid device consisted of the PDMS chip on top and glass substrate coated with PLGA nanofibers at the bottom, (d) Fluorescent image of the dendritic spines in differentiated hiPSCs, inside the microchannels after 8 days. A neural marker of NSE (Neuron specific enolase) was utilized, (e) Nuclear co-staining with DAPI.

Nowadays investigations on microfluidic combination systems especially with 3D scaffolds are widely considered.[35]

Microfluidic devices for specific types of neural cell differentiation

Recently, efforts are guided toward the stem cells differentiation into a specific type of neural cells with the desired function such as differentiation of stem cells toward dopaminergic neurons for treating Parkinson's disease. In this regards, Edinson Lucumi Moreno and co-workers designed a 30 days differentiation protocol in microfluidic system in which they utilized 3-D cell culture in combination with chemical stimulation, using small molecules such as ascorbic acid (AsA), CHIR, phorbol myristate acetate (PMA), and dibutyryl cyclic AMP (dbcAMP) and specific GFs (BDNF, glial cell-derived neurotrophic factor [GDNF] and Transforming growth factor β3[TGFβ3]).[39] 3-D cell environment was achieved by the use of Matrigel. In addition, 2 types of microfluidic bioreactors (2-lane and 3-lane) was incorporated in which in the 3-lane bioreactor, gel-embedded cells are bordered by medium perfusion in 2 sides, while in a 2-lane bioreactor medium perfusion was applied in one side.

Results expressed high percent of βIII tubulin expression that confirmed the neural differentiation beside the low tyrosine hydroxylase (TH) expression, indicating efficient differentiation into dopaminergic neurons. They achieved neuronal differentiation efficiency of 91%, among the differentiated neurons; however, the dopaminergic neuronal differentiation efficiency was 19% in 2-lane and 11% in 3-lane bioreactors. They hypothesized that lower dopaminergic differentiation in 3-lane systems may be related to higher dilution of paracrine factors; hence logically proposed that regulation of the medium perfusion rate may result in higher differentiation efficiency (Figure 3).[39]

In 2015, another effort was made by Kisuk Yang et al.[40] on neuronal differentiation, especially dopaminergic differentiation of hNSCs, in which, co-culture of hNSCs with GDNF expressing human mesenchymal stem cells (hMSCs) was used as a paracrine signaling imitation. In combination with cellular co-culture, 3D hydrogel cell culture was incorporated in micro-channels.

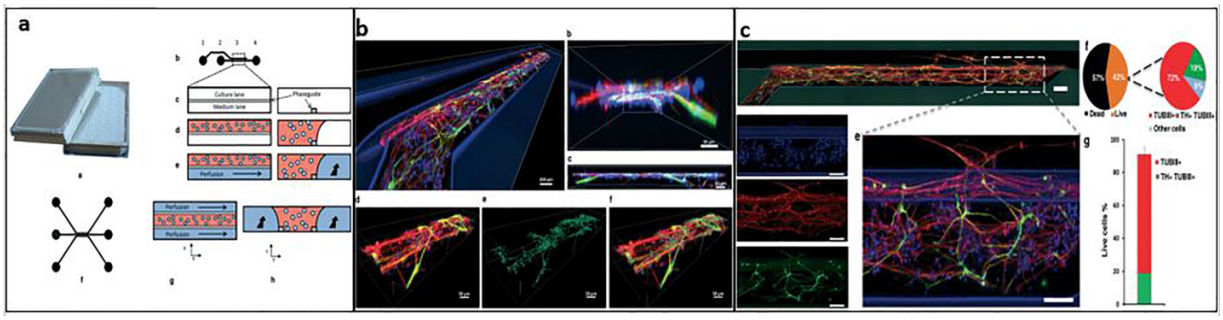

Figure 3. Device design and differentiation of stem cells to neurons. (a) Schematic representation of 2-lane bioreactor consisting of (1) gel inlet, (2) perfusion inlet, (3) optical readout window, (4) perfusion outlet, (b) Survival efficiency and differentiation of hNESCs into neurons, (c) Correlation of live–dead cells attributing to differentiation of hNESCs into neurons and differentiation efficiency.

In microfluidic platform, hNSCs were cultured in 3-D collagen hydrogel filled central channels, while GDNF-hMSCs were cultured in 2 channels on both sides of the central channel (Figure 4). Immunofluorescent staining and qRT-PCR analysis demonstrated that microfluidic co-culture of hNSCs with GDNF-hMSCs significantly increased the expression of neuronal markers (Tuj1 and MAP2) at day 5 in culture in comparison to either hNSCs cultured without hMSCs, co-cultured with no transfected hMSCs and co-cultured with enhanced green fluorescent protein (EGFP) transfected hMSCs. In contrast, glial lineage differentiation markers including GFAP, oligodendrocyte marker (O4), and olig2 in hNSCs showed a decrease after 5 days of co-culture with GDNF-hMSCs compared to culture without hMSCs and co-cultured with hMSCs. Observations confirmed the reduced glial differentiation of hNSCs significantly enhanced the differentiation rate into neuronal cells such as dopaminergic neurons. The released GDNF from genetically-modified hMSCs enhanced the differentiation of hNSCs into dopaminergic neurons in a 3-D ECM micro-environment. Immunofluorescent staining and qRT-PCR revealed that co-culture of hNSCs with GDNF-hMSCs in microfluidics upregulated the expression of TH, the dopaminergic neuronal marker in hNSCs after 7 days in culture.[40] Although dopaminergic neuron differentiation was achieved in 3-D (hydrogel filled) micro-channels in both experiments, a more similar structure to an *in vivo* system was provided for stem cells. Evidently, co-culture of GDNF-hMSCs adjacent to hNSCs accelerated the dopaminergic differentiation in 7 days in comparison to the utilization of small molecules in 30 days protocol in Moreno's study. However, precise quantitative comparison in differentiation efficiency was impossible.

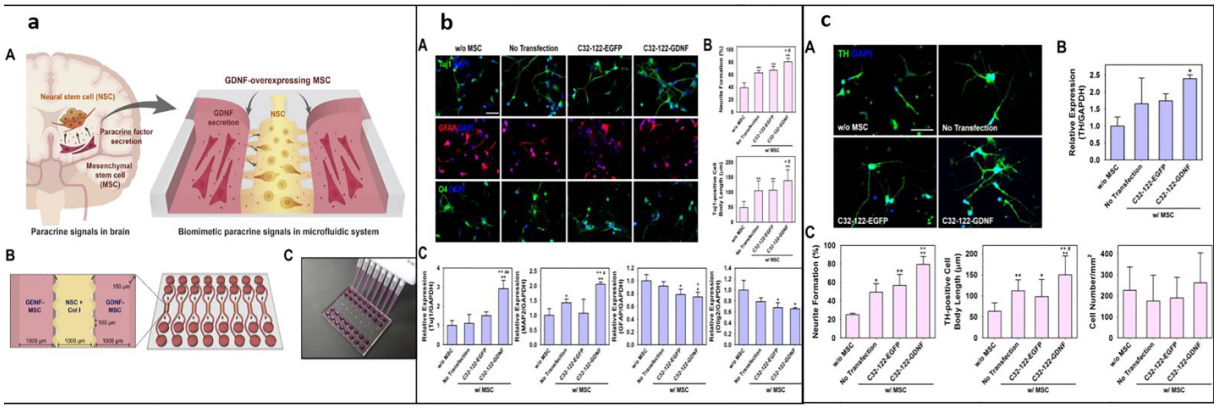

*Figure 4. Schematic representation of (a) Microfluidic array device for co-culture of NSCs and GDNF-MSCs, (b) EGFP expression quantified by flow cytometry (n ¼ 3; *p < 0.05 and **p < 0.01 versus no transfection, ##p < 0.01 versus LIPO2000) and cell viability measured by MTT assay (n ¼ 5~12; **p < 0.01 versus no transfection, #p < 0.05 versus LIPO2000) 2 days after transfection, (c) Fluorescence microscopy and flow cytometry analyses indicating the expression of EGFP in hMSCs 2 days after transfection (n ¼ 3).*

Additionally, Jeein Choi et al. designed a gel-free 3-D microfluidic cell culture device consisting reservoir and microchannel layers for inducing neural differentiation of human adipose tissue-derived stem cells (hATSCs) to gamma-aminobutyric acid (GABA) positive neurons in hypoxic condition.[41] This study showed that the growth of immobilized cellular aggregates (neurospheres) in micro-channels was higher than control cells in a culture dish. Moreover, presenting Tuj or NF160 positive long neuritis compared to control cells confirmed the significant increase in neuronal-like cell structures induction. They also revealed that microfluidic system resulted in a higher level of GABA positive neurons which includes about 60% of cells. The cells were also transplanted into the mouse SCI lesion for further *in vivo* evaluations. One month after surgery, the neuronal differentiated hATSCs were significantly transdifferentiated into NF160 positive motor neurons. Their CHIP/PCR analysis proved that low oxygen level induced the expression of HIF1a, which resulted in increased expression of Wnt5A/β-catenin and Oct4 via the direct binding of HIF1a to their regulatory regions in nucleus and the activation of the Wnt5A/β-catenin signaling pathway in hATSCs resulted in self-renewal and differentiation of hATSCs into neurons. It was logically concluded that incorporation of signaling factors such as Wnt5 protein could be safer and easier for stem cells differentiation instead of genetic manipulation.[41]

Microfluidics, topography, mechanical and electrical interventions

Topography plays an effective role in differentiation platforms, after the vast incorporation of chemical stimulants, Yu-Che Cheng et al. designed a microfluidic system capable of analyzing the effect of both mechanical and chemical stimulation on neural differentiation of stem cells.[43] Due to the possibility of better microenvironmental control in microfluidic devices in comparison to the traditional cell culture, they analyzed the effect of shear stress with various flow rates on placenta-derived multipotent stem cells (PDMCs) beside 3-isobutyl-1-methylxanthine (IBMX) as the chemical stimulant.

However, topography and stiffness of ECM sway the differentiation fate: soft environment enhances neural differentiation while rigid environment enhances myogenic and osteogenic differentiation.[42] It was found that shear stress cannot lead PDMCs differentiation into other cell types. It seems that chemical stimulation plays a critical role in PDMCs differentiation and physical stress can accelerate the neuronal differentiation of PDMCs. The highest cell differentiation ratio of 42.4 % was found in the highest flow rate and IBMX in 48-hour condition.[43] Beside numerous chemical and biological techniques to induce neural differentiation, a type of electrical intervention was incorporated by Kang et al.[44] They invented an on-chip localized electroporation device (LEPD) for gene editing and gene expression. In this study, they validated the device by electroporation of Hela and HT1080 cells with a transfection efficiency of ~95% with propidium iodide and up to 50% with GFP plasmid. In the next step, NSCs were differentiated into neurons in a day with the incorporation of LEPD. Differentiation efficiency was also confirmed by βIII tubulin staining.[44] Therefore, the role of electricity was also confirmed in the field of microfluidics in neural tissue engineering.

Organ on-chip models such as brain on-chip have been studied by microfluidics. Brain microenvironment was mimicked by concave microarrays containing neuro-spheroids and a constant fluid flow which is observed in the interstitial space.[45] These platforms can respond to the growing need for in vitro disease models for investigation of etiologies and treatment approaches.

According to these studies, microfluidic systems provide advantageous effects on neural differentiation of stem cells. Microfluidics mainly have been combined with chemical stimulants and sometimes with scaffolds (hydrogel or nanofiber), beside the incorporation of possibly effective additional factors including hypoxic conditions, various flow rates or electroporation (Table 1).

Table 1. Neural Differentiation Strategies in Microfluidic Devices

Microfluidics differentiation strategies	Stem cell	Differenti-ation inducer	ECM	Time days	Evaluation technique	Key findings	Ref.
Gradient generation	NSCs	EGF FGF2 PDGF	LN, poly-L-lysine	7	ICC Cell counting	Direct correlation between GF gradient, cell numbers and differentiation	[22]
	hESC-NPC*	Shh FGF8 BMP4	-	8	ICC FC*		[23]
Chemical stimulation	PC12	NGF		9-11	Optical imaging	Induced neural differentiation by chemical stimulation, synergistic role of physical stimulation on neural differentiation	[20]
	hESC-NCs*	BDNF	poly-L-ornithine FN	5-7	ICC Optical imaging		[21]
Physical intervention	PDMC	IBMX flow rate (shear stress)	-	3	ICC Cell density counting		[43]
Electrical inter-vention, On chip electro-poration,LEPD[4]	NSCs	Gene transfection and expression	poly-D-Lysine	6	ICC, Electroporatio n studies	NSC differentiation into neurons within 24 hours after plating on LEPD	[44]
3-D cell culture	hNESC	AsA, CHIR, PMA, dbcAMP BDNF, GDNF,TGFβ3	Matrigel	30	Calcium imaging, ICC	Production of dopaminergic neurons	[39]
3-D cell culture	NSCs	ECM hydrogel	Col I, Matrigel, (1:1)	4 days	ICC, qRT-PCR	Promoting neuronal and oligodendrocytic differentiation by 3-D micro-scale hydrogel culture in microfluidics, better differentiation by Matrigel containing groups compared with bulk collagens	[35]
3-D cell culture	hNSC	Hypoxic condition, ECM hydrogel	Col I, Col I+ FN, Col I+ LN, Col I + FN+ LN	7	qRT-PCR ICC	Promoted expression of the neuronal marker by ECM (primarily), neuronal differentiation was enhanced by low oxygen tension (in later time points)	[36]
3-D cell culture	hNSC	GDNF-hMSC	Col	7	ICC, qRT-PCR Electrophysiol ogy	Enhanced differentiation into neuronal cells including dopaminergic neurons, Reduced glial differentiation of hNSCs	[40]
3D cell culture	hATSC	BDNF	FN	1-14 days	BrdU*, SEM* incorp.,RT-PCR, ICC, CHIP-on-chip analysis,	Efficient neuronal differentiation and higher level of GABA secreting neurons, via Wnt5a-mediation	[41]
3-D cell culture	hiPSC	Nanofibrous scaffold BDNF, EGF	FN	8 days	ICC, qRT-PCR	Enhanced neural differen-tiation in hybrid microfluidic system in comparison with other control groups.	[38]

hESC-NPC= Human embryonic stem cell-derived neural precursor cell, NCs= Neural Cells, FC= Flow cytometry, BRDU= Bromodeoxyuridine, SEM= Scanning electron microscope,

Conclusion

Nowadays, neural differentiation of stem cells has been performed utilizing 4 main techniques including chemical stimulation with small molecules, polymeric scaffolds, MicroRNAs, and microfluidic devices. Chemical stimulation is the first practicable method in traditional culture plate that provides rapid and reversible differentiation in comparison to other differentiation methods. However, this approach offers minimal similarity to *in vivo* microenvironment. Polymeric scaffolds with the advent in providing ECM incorporated 3-D structures simulate more in vivo like microenvironmental systems. MicroRNAs and genetic manipulation is a biotechnology instrument which provides a continual differentiation of stem cells that requires its own facilities and costs. The microfluidic device is the most recent platform which is highly flexible in design for the intended differentiation goal and the possibility of tight environmental control over cells and consequently providing the most *in vivo* similar conditions. The microenvironmental advantages along with lower reagent consumption and costs are the reason for the welcoming of microfluidics in molecular and cellular biology research. However, we paid attention to neural differentiation of stem cells in this review, and microfluidics application's domain extends to various fields such as neurodegenerative disease models,[46] and cellular interactions.[47]. In future studies, development of novel microfluidic systems in combination with other types of biomedical scaffolds would be of potential interest. For example, there has been great attention on various types of aligned hydrogel which seems to be an ideal candidate for neural tissue engineering because of the proved influence of alignment on neural differentiation of stem cells.

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Conflict of Interests

The authors declare no conflicts of interest. For signed statements, please contact the journal office: editor@precisionnanomedicine.com

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