Micro/nanoengineered technologies for human pluripotent stem cells

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Abstract

Human pluripotent stem cells (hPSCs) are a promising cell source for cell replacement therapies and modeling human development and diseases *in vitro*. Achieving fate control of hPSCs with high yield and specificity, however, remains challenging. The fate specification of hPSCs is regulated by biochemical and biomechanical cues in their local cellular microenvironment. Recent advances in micro/nanoengineering have developed a broad range of tools for the generation and control of various extracellular biomechanical and biochemical signals that can control the behaviors of hPSCs. In this review, we summarize these micro/nanoengineering technologies for controlling hPSC fate and highlight the role of biomechanical cues, such as substrate rigidity, surface topographies and cellular confinement.

1 Introduction

Human pluripotent stem cells (hPSCs) are a unique category of cells that carry unlimited self-renewing capability and the potential to differentiate into all the cell types in the human body, including the derivatives of three germ layers [1]. They reside in a variety of niches in the human body. Signals received in the niche will prompt them to self-renew or differentiate. Because of hPSC potential for developmental studies as well as cell replacement therapy development, it is important to understand how the niche microenvironment regulates their self-renewal and differentiation. This type of study requires *in vitro* culture systems that are able to capture key aspects of the in vivo niche. Over the past few years, various micro/nanoengineering methods for hPSC fate and function control have been developed and applied for biomedical and biological research [7-12]. Using two main sources of hPSCs, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), researchers have found that cell-extracellular matrix (ECM) and cell-cell interactions as well as biochemical factors including soluble factors have an essential role in the regulation of fate and function of the cell populations [2-4].

The development of the human embryo is one of the most dynamic processes that occurs in the human body. It involves a plethora of events including cell sorting, self-organization into 3D structures, patterning, migration, and specification. The complexity of embryonic development highlights the amazing capability of hPSCs to respond to a wide range of environmental parameters in very distinct and specific ways. The vast number of responses that hPSCs can display has prompted researchers to design different biomimetic and biological systems that allow for multiparametric microenvironmental control. Studies have started to reveal the significance of biomechanical cues such as substrate rigidity, nanotopographical features [5-7], and geometrical confinement, all of which require the application of different

nano- and micro-fabrication techniques. For example, substrate rigidity and dimensionality has been controlled with both hydrogels and the use of micro-post arrays and has been shown to affect hESC differentiation and self-organization [8]. Geometrical confinement is commonly achieved though micro-contact printing and has been shown to affect both cytoskeletal traction force [9] and morphogen distribution among cells [10]. Nanoscale ridge/groove patterns fabricated using UV-assisted capillary force lithography have been used to induce hESC differentiation into a neural lineage [11].

The goal of this review is to present an overview of the state of the art of existing micro/nanoengineered technologies for controlling hPSC fate and function. First of all, we summarize diverse culture platforms and the biochemical cues used for maintaining pluripotency and self-renewal of hPSC. We then discuss the roles of biomechanical cues such as substrate rigidity, surface topographies, and cellular confinement in determining hPSC fate. Further, we discuss the application of microfluidic devices for engineering hPSCs. In the end, we present a summary of recent advances in human organoid technologies using hPSCs including brain organoids, kidney organoids, and endodermal organoids.

2 Biochemical approaches for in vitro hPSC maintenance

Research with hPSCs requires long-term cell culture without loss of pluripotency. Traditionally, hPSCs have been cultured on feeder cells, which are cells that secret multiple growth factors that support hPSC self-renewal (Fig 1a)[12, 13]. For example, mitotically inactivated mouse embryonic fibroblast cells (MEFs), which have been successfully used to maintain mouse ESC self-renewal, are commonly used in the maintenance of hPSCs. However, there is a risk of murine pathogens transferring from the MEFs to the hPSCs. These pathogens can cause zoonosis

in cell transplantation recipients [14, 15]. Additionally, feeder-based cultures suffer from cytogenic aberrations due to the repeated enzymatic treatments, which poses a challenge for achieving controllable hPSC culture systems [16]. Murine feeder cells can be replaced by human feeders such as human foreskin fibroblasts and human adult marrow cells [17-19]. However, using feeder cells increases the cost of hPSC production, limiting the scaling-up of hPSCs for clinical applications [14]. More recently, feeder-free systems have been developed with the use of conditioned medium (CM) in conjunction with human serum [26], and purified ECM proteins like Matrigel [17, 27, 28] (Fig 1b). In the case of ECM protein substrates, researchers found a twofold increase in the expansion of cells as compared to hESCs grown in MEF-CM [17]. Batch to batch variation of biological materials and the need for costly tests to ensure the absence of pathogens have led researchres to develop synthetic substrates. Thus far, defined peptide and protein surfaces have beed used as synthetic ECM for cell culture (Fig 1c). Melkoumian *et al.* [20] developed synthetic peptide- acrylate surfaces (PAS) to create an appropriate environment for pluripotency maintenance of different hPSC lines in several commercially available media including KnockOut SR-supplemented medium, and the chemically defined medium mTeSR1 for more than ten passages. Their study showed that high functional peptide density on the plating substrate and uniform peptide distribution result in hESC expansion, cell morphology, and phenotypic marker expression similar to that on Matrigel.

Similarly, Kolhar *et al.* [21], have developed a novel peptide-based surface using a high-affinity cyclic RGD peptide for the long time culture of hPSCs. This substrate provides a surface supporting integrin-mediated cell attachment, which protects hESCs against apoptosis caused by loss of attachment to an extracellular matrix substrate (anoikis). There are several other studies

using synthetic polymers to provide a desirable environment for the long-term self-renewal of hESCs. For informative discussions, readers are referred to these excellent papers [21-27].

Cells in culture respond to a plethora of biochemical and biomechanical signals. When using polymers as substrates, polymer features can be used to increase cell control and cultivation efficiency. Microarrays are a great tool for identifying appropriate polymer features [28, 29]. In this process, a large number of monomers with different ratios can be synthesized in nanoliter volumes. Brafman *et al.* [30], reported the use of array-based high-throughput screening approach to identify a synthetic polymer, poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA), which could promote attachment, proliferation, and self-renewal of several hPSC lines over five passages. In another related study, Hansen et al. [31], reported a two-step method for the rapid fabrication of 7316 polymer features on a glass slide for the discovery of the best substrate for cultivation and self-renewal of hESC. The process consists of generating a fluorous-mask in which two monomers along with a photo-initiator and cross-linker are printed. This process is done in a way that generates a large number of compositions with various chemical characteristics [41].

In addition to functionalizing the surface of the substrate as described above, physical methods can also be used to prepare new 2D surfaces for long-time self-renewal of hESCs. For example, oxygen plasma-etched tissue culture polystyrene (PE-TCP) surfaces can be generated by placing polystyrene substrates under radio frequency oxygen plasma (Fig 1d). This treatment raises the oxygen content at the surface of the substrate by 1.6 fold, enabling attachment and proliferation of hESCs. Mahlstedt *et al* [32] investigated the use of PE-TCP for long-time pluripotency maintenance of various hESC cell lines including HUES7 and NOTT1. Furthermore, oxygen plasma etching can be used to modify the surface chemistry of the standard

tissue culture polystyrene (TCPS) to support hESC growth and proliferation. Supporting this view, Saha et al [33] developed culture conditions based on UV/ozone radiation modification of cell culture plates to provide a suitable substrate for hPSC culture. This attractive cell culture platform generates more than three times the number of the cells generated by feeder containing substrates.

3 Biomechanical approaches for in vitro culture or differentiation of hPSC

3.1 Mechanical stiffness of extracellular matrix

It has been demonstrated that hPSCs have mechano-sensitive and mechano-responsive properties that affect their self-renewal and differentiation [9, 34-36]. Substrate rigidity modulates hPSC behaviors partially through intracellular cytoskeleton and actomyosin contractility [34]. The idea of using tissue-mimicking matrix stiffness to observe how mechanical properties of the ECM affect hPSC differentiation can be traced back to the research done by Engler *et al.* [37]. They showed that culturing human mesenchymal stem cells (hMSCs) in polyactrylamide (PA) hydrogel substrates with brain-mimicking stiffness led to neurogenesis, while muscle- and bone-like stiff PA substrates promoted cardiogenesis and osteogenesis, respectively. Keung et al. also used PA hydrogels to modulate substrate rigidity and found that soft substrate stiffness *in vitro* promoted hPSC neural ectoderm differentiation [34].

In addition to PA hydrogel substrates, elastomeric micropost arrays can be used to modulate substrate rigidity and study its effect on cytoskeleton contractility and differentiation of hESCs [9, 35] (Fig. 2a). Top surfaces of the micropost array are functionalized with adhesive ECM proteins to promote hPSC attachment. The substrate rigidity of the array can be easily modulated by changing post height while leaving other substrate properties such as surface chemistry and adhesive ligand density unchanged. Moreover, each post functions as a cantilever

to measure subcellular contractile force [36, 38]. The micropost array has been successfully applied to study mechanotaxis [38], single-cell mechanical homeostasis [39], and stem cell differentiation [40]. In this section, we will mainly discuss the use of polydimethylsiloxane (PDMS) microposts to modulate substrate rigidity and measure contractile forces to study the differentiation of hPSCs.

By using a PDMS micropost array, Sun *et al.* [9] demonstrated that hESCs are mechanosensitive, as they could increase their contractility with increasing substrate rigidity. They also showed that rigid substrates support the pluripotency of hESCs, while soft substrates promote the differentiation of hESCs as reflected by the down-regulation of E-cadherin. Another work from Sun *et al.* [35] showed that neural induction and caudalization of hPSCs could be accelerated with the use of a PDMS micropost array of low rigidity (Fig. 2b). Furthermore, the authors demonstrated that such mechanotransductive neuronal differentiation of hPSCs involved Smad phosphorylation and nucleocytoplasmic shuttling, which was regulated by mechanosensitive Hippo-YAP activities. The micropost array has also been used to study hPSCs in mechanically controlled 3D culture environments. For example, Shao *et al.* studied the effect of substrate rigidity on hPSC self-organized amniogenesis using a PDMS micropost array [8] (Fig. 2c). Interestingly, the authors found the development of squamous amniotic ectoderm-like cysts occurred only in hPSCs cultured on microposts with low rigidity. In addition, they found that hPSCs cultured on both soft and rigid microposts in 2D conditions without Geltrex overlay maintained pluripotency and did not form cysts. These results demonstrated that both low mechanical rigidity and 3D dimensionality of the ECM were needed to trigger the amniotic differentiation of hPSCs.

3.2 Nano topography controls hPSC fate

Within native tissues, cells interact with different nanoscale features of the surrounding extracellular matrix which varies from porous fibrous connective tissue to more tightly woven basement membranes [41]. These nanometer to micrometer topographical features possess a complicated mixture of ridges, grooves, fibers, and pores [42] which regulate cell–cell interaction, cell-soluble factor interaction, cell–ECM interaction, and cell-mechanical stimuli interactions [43-46]. Basement membrane, a common type ECM, is an example of an *in vivo* substrate that presents a mixture of different surface topographies regulating fate and function of different types of cell. The effects of surface topography on cell behavior have been under investigation for several years and research has shown that mammalian cells respond to synthetic nanotopographies [47-49]. Various nanoengineering tools and synthesis methods have been successfully developed and utilized to generate nanotopographical surfaces, nanopatterns, and scaffolds for *in vitro* stem cell research.

In brief, nanotopographical features are used for both maintenance [50, 51] and differentiation [52-55] of many cell types. In particular, recent studies have demonstrated that some types of topographical features can provide regulatory signals for adhesion, proliferation, and self-renewal of hPSCs [50, 56-59]. For example, Bae *et al.* [50] cultured cells on a nanopillar topography to investigate its effect on colony formation and the expression of pluripotency markers in hESCs. Cell-nanopillar interaction leads to cytoskeletal reorganization by the formation of focal adhesions and restricted colony spreading, which increases E-cadherin mediated cell–cell adhesions in hESC colonies. It was demonstrated that formation of a compact colony is indispensable for hESC undifferentiated state results in the expression of pluripotency markers higher than those cultured on the flat substrate. In another study, Chen *et al.* [58] used

nanorough glass coverslips with various levels of roughness and reported an optimized level of nanoroughness that promotes proper cell function and enhanced expression of pluripotency markers (Fig 3a).

Nanotopographical features can also be utilized to direct differentiation of hESCs into different cell types (Fig 3b, c) such as neural [11, 60-63], cardiac [64] , and pancreatic cells [65]. Lee *et al.* used nanoscale ridge/groove patterns to induce hESCs into a neural lineage [11]. They showed that hESCs seeded on gelatin-coated nanoscale pattern arrays in DMEM/FBS medium could rapidly and effectively differentiate into neuronal lineage without using any differentiation-inducing agents. Elongation of the cytoskeleton guided by ridge/groove patterns led to a transfer of tensional force to the nuclei which influenced signal transduction and gene expression. Similarly, in another study by Pan *et al*. [62], it was observed that hPSCs cultured on the nanografted substrates efficiently differentiate into the neuronal lineage and show aligned and elongated nuclei in the direction of nano/microstructures with increased contact guidance [62, 66]. Another study from Lu et al. [67] showed that nanofibrous scaffolds can cause differentiation of hESCs into the neural lineage when combined with treating the cells with neural induction medium containing Noggin/retinoic acid. It was further proposed that topographical features might improve the cardiomyogenic differentiation of hESCs. Interestingly, Lee *et al.* [64] reported that hPSCs cultured with no exogenous chemicals for differentiation on a nanorough graphene substrate show enhanced cardiomyogenic differentiation compared to cells cultured on glass or Matrigel. hESCs cultured on the nanorough graphene showed enhanced cell adhesion which led to the cardiomyogenic differentiation through ERK signaling pathway. More recently, Kim et al [65] demonstrated that nanopore-patterned surfaces can remarkably promote the pancreatic differentiation of hPSCs. In this study they showed that

nanopores of 200 nm diameter lead to a 3-fold increase in the percentage of pancreatic cells as compared to hESCs cultured on flat surfaces. TAZ was identified as a significant player in the nanopore-induced mechanotransduction facilitating the pancreatic differentiation of hPSCs.

3.3 Cellular confinement

In addition to the surface topography, cells *in vivo* exist in limited spaces either encapsulated in ECM or surrounded by other cells, and exposed to different gradients of soluble factors and local adhesive motifs [75]. Geometrical confinement of the cells in ECM is crucial for regulating dynamic cellular behaviors including asymmetrical cell division and cell migration. Also, it is important for wound healing, fibrosis, and embryo development *in vivo* [76, 77]. Conventional culturing systems, such as homogeneous plates and tissue culture dishes with uniform surface treatments, do not properly recapitulate the spatial cell confinement *in vivo*. In the past 15 years, a plethora of techniques has been developed to generate micro/nanoscale patterns of ECM proteins in various shapes and sizes both on 2D and 3D culture systems in order to get insights into the role of spatial confinement in tissue morphogenesis [78]. In particular, a handful of technologies such as microcontact printing (μCP) [79-81], microstencils [82], microwell culture [83, 84], and photopatterning [10] processes have been implemented to study hPSCs.

Micro-contact printing is the most common method for generating micro/nanoscale adhesive ECM patterns on glass substrates and tissue culture dishes [81, 85, 86]. In this process, an elastomeric PDMS patterned stamp is coated with adhesive proteins or solution of thiol-containing molecules that can be spontaneously absorbed by the stamp owning to hydrophobic interactions. After the stamp is dried, it is brought into conformal contact with a second substrate which effectively creates protein patterns (Fig 4a). Since its invention, μCP has been widely

adapted to create micro/nanopatterns of ECM proteins on substrates despite the drawbacks of requiring of a two-step coating process and specific humidity conditions [75]. μCP has also been used as an efficient method for generating patterning with hPSCs. It has been demonstrated that the trajectory and rate of hESC differentiation can be affected by engineering niche properties such as the organization of hESCs in μCP to induce neural lineage and mesodermal cell lines from hESCs by generating circular colonies of hESCs of various sizes. It was observed that the ratio of the neural-associated marker (Pax6) to mesodermal associated marker (Gata6) expression increased with the use of bigger colonies. Similarly, Lee *et al.* [80] reported that by treating hESCs with BMP2 and activin A and by precisely controlling colony size, the cells could differentiate into either mesoderm or definitive endoderm lineages. Activin A and BMP2 act synergistically to activate the expression of endoderm-specific genes and mesoderm-specific genes in the system. However, colony size is able to selectively guide these primitive streak-like cells to either definitive endoderm or mesoderm lineages. In another related study, Hoof et al achieved hESCs differentiation into pancreatic endoderm-like cells by seeding cells onto a patterned substrate [87].

An alternative method to create regular micropatterns of hESCs is stencil-assisted micropatterning. The stencil is a thin sheet with an array of microscale through-holes that will self-seal against the target substrate. As early as 1967, nickel stencils [88] and stainless-steel stencils [89] were used to generate cellular micropatterns on non-adherent acetate. However, the metallic stencils cannot be completely sealed against the target substrate. Researchers have reported the fabrication of a rubberlike stencil that allows for the creation of cellular micropatterns of different cell types on a substrate. In this case, the stencil is applied onto the cell-culture substrate before the seeding process and peeled off after (Fig 4b). There are several

studies describing the microstencil method as a robust and simple method for generating hPSC micropatterns that is capable of working with various ECM proteins and different culture substrates [90]. Yao *et al* [82] used stencil micropatterning to generate multilayered hPSC-derived colonies and induce them toward hepatocyte-like cells by performing a multi-staged 17-day differentiation protocol on the cells. In multilayered colonies, cell-cell interaction was enhanced leading to more mature hepatocyte-like cells with higher levels of Albumin (hepatic marker) as compared to hepatocyte-like cells obtained through more conventional methods.

Both μCP and stencil-assisted micropatterning technologies need access to microfabrication tools, limiting their usage for laboratories which don't have access to microfabrication technologies [75]. A new approach that overcomes this limitation is deep UV-activated micropatterning. This method can create dynamic and stable ECM adhesion patterns on target substrates with a sub-micron resolution [91-94]. It consists of deep UV exposure that oxidizes a polymer coating (e.g., poly(L-lysine)- g-poly(ethylene glycol), PLL-PEG) on a cell culture substrate (e.g., glass or PS) covered by a photomask. Exposed surface areas will become hydrophilic and undergo covalent binding to ECM proteins (Fig 4c). Using such micro-patterned substrates, Warmflash *et al.* [10], showed that confinement of hESCs to a disk-shaped pattern is a key factor for recapitulating germ layer patterning. It was shown that colonies with larger diameters resulted in differentiation of hESCs into spatially organized three germ layers. However, in smaller colonies, the inner layer (ectoderm layer) disappeared and the two outer layers were extended into the center of the colony.

Embryoid bodies (EBs) are three-dimensional aggregates of hPSCs which recapitulate early stages of embryonic development. Using microwell for generating EBs is a new method that can homogenously form EBs by controlling the initial number of cells, shape and the size of

the EBs (Fig 4d). hPSCs within the EBs will undergo differentiation into the three germ layers, which could potentially differentiate into all somatic cell types. It has been shown that EB size can affect the differentiation patterns of hESCs. Mohr et al [95] generated EBs with various sizes to investigate the effect of EB size on cardiomyocyte formation. They showed that microwell-engineered EB size regulates cardiogenesis by controlling the passive diffusion on the substrate, and can be considered as a reproducible and efficient method for the formation of hESC-CMs for therapeutic and research applications. Similarly, Hwang et al [96] reported that endothelial cell differentiation was enhanced in smaller EBs while cardiogenesis differentiation was increased in larger EBs. It has been shown that larger EBs generate inductive signals of early endoderm tissue which leads to differentiation into mesoderm cells in the EBs. However, in smaller EBs, the absence of cues from the endoderm tissue leads to the endothelial cell differentiation.

3.4 Local mechanical perturbation

Tissues and cells in human and animal bodies are continuously subjected to different types of mechanical stresses including shear, tensile, and compressive stresses. Mechanical stimuli play essential roles in different biological actions including proliferation, differentiation, migration, and contraction [164-167]. Many techniques and tools have been developed study the role of mechanical forces in tissue engineering, cell biology, and regenerative medicine. These technologies can be used to study force-dependent dynamics or measure local mechanical properties of some molecules in mechanotransduction. In this section, four different techniques which have been used to study the mechanical properties of hPSCs are discussed.

Optical tweezers [97, 98] and magnetic tweezers [99-101] are techniques commonly used to provide force and displacement on the surface of the cell or within a defined region of a cell

(Fig 5a). In this technique, microbeads are functionalized with an antibody or adhesive ligand to bind to the specific receptors on the surface of the cells. The tweezers apply forces to the microbeads to balance forces transferred from cells to the beads. This force can be calculated with parameters of the microbeads and optical/magnetic fields. [102]. Optical tweezers have been used to compare the mechanical properties of undifferentiated hPSCs with the mechanical properties of differentiated hPSCs [103, 104]. Tan *et al*. [103], used optical tweezers to explore how dynamic and static micromechanical properties of hESCs vary by differentiating toward cardiac cells. It was shown that differentiated hESC-CM have a higher stiffness than undifferentiated hESC due to an increase in organized myofibrillar assembly. Similarly, atomic force microscopy (AFM) can probe cell components by applying force in the resolution of 10^{-12} N and displacement with a resolution of 1nm. In this technique, an electronic controller is used to move an elastic cantilever beam over the cell which causes mechanical perturbations (Fig 5b) [105, 106]. There is a nano-microscopic tip at the end of the cantilever beam functionalized with an adhesive ligand that binds to cell receptors. Cantilever movement caused by the electronic controller generates a local stretch or indentation to the cell that can be calculated by measuring the deflection of the cantilever beam. It was found that AFM can quantify beat force of either a cluster or a single cardiomyocyte cell. Liu *et al.* [107] used AFM to measure the mechanobiological properties of pluripotent stem cell-derived cardiomyocytes (hPSC-CM) including cellular elasticity, contraction rate, beat force, and duration.

Acoustic tweezer cytometry (ATC) is another technique used to apply a local mechanical load to the cells [108, 109]. In this method, lipid microbubbles functionalized with specific ligands can covalently attach to the surfaces of the cells via adhesive ligand-receptor binding (Fig 5c). An acoustic wave is utilized to vibrate the lipid microbubbles and apply force to the

cells. The parameters of the exerted force include frequency, magnitude, period, and duration and are determined by the ultrasound parameters. To improve survival rate and cloning efficiency of hESCs, Chen *et al*. [109] used ATC to provide mechanical stimulation to the disassociated single hESCs. In this way, integrin-mediated adhesion formation and strengthening by ATC stimulations led to facilitating disassociated hESC spread which rescues the cells from hyperactivated actomyosin activities that prompt downstream apoptotic signaling pathways.

Cell stretching devices are utilized to carry out stretching of single cells, colonies, and tissue samples in a way that captures the patterns of deformation experienced by different cell types in the body including vascular cells, cardiomyocytes, fibroblast, and skeleton muscles. Several studies have reported that mechanical strain can direct differentiation of hPSCs. Li *et al*.[110, 111] investigated how uniaxial mechanical strain in parallel to the signaling pathways regulated by TGF-β can modulate the differentiation of neural crest stem cells (NCSCs) into smooth muscle cells (SMCs). In another recent study, Teramura *et al*. [111] showed that cyclic strain alters the alignment of actin fibers in hiPSC and the expression of pluripotency markers. In another study, Xue *et al*. [112] reported a micropatterned hPSC-based neuroectoderm developmental model, in which pre-patterned geometrical confinement induces emergent patterning of neuroepithelial and neural plate border cells. To see the effect of mechanical force on cell differentiation, a custom designed cell stretching device (Fig 5d) was developed and implemented for stretching central regions of micropatterned cell colonies leads to the activation of BMP signaling pathway and differentiation into the neuro plate border cells in the central region of the pattern.

4 3D biodegradable scaffolds

3D tissue scaffolds are often used to provide support for biological applications such as tissue engineering [113]. Porous biodegradable scaffolds can provide a desirable environment to host cell adhesion and proliferation. Furthermore, they can provide a complex 3D matrix for cell maintenance and differentiation. The application of scaffold biomaterials to mimic ECM requires that the biomaterial have a high biocompatibility, proper chemistry to induce cell adhesion and proliferation, and the mechanical properties and degradation rate of the ECM of interest. The level of porosity, pore distribution, and exposed surface area also play a major role in the architecture of the ECM and penetration of cells into the scaffold volume [114]. Various natural and synthetic biomaterials have been successfully utilized to generate scaffolds for *in vitro* stem cell research. Scaffolds which have been implemented for hPSC culture are classified into three groups: bioactive hydrogel scaffolds, synthetic biodegradable polymers, and micro/nano fibrous scaffolds.

4.1 Natural scaffold

The bioactive hydrogel is a scaffold with bioactive molecules that provides good spatial control for hESCs maintenance and differentiation [115]. Naturally derived hydrogels include collagen, alginates, and chitosan extracted from animals, plants, and human tissues. They exhibit promising biocompatibility and low toxicity for cell culture but suffer from batch-to-batch variability [116, 117]. Collagen is a widely used natural material for making scaffolds that is composed of fibrous proteins with a stiff helical structure that provides a suitable structure for cell distribution and capillary formation [116, 118-120]. Chen *et al.* [121] incorporated hESC-MSCs within a silk-collagen sponge scaffold that provided mechanical strength in conjunction

with neo-ligament tissue regeneration to induce tendons like cells. hESC-MSCs positively expressed tendon-related gene markers including Epha4, Scleraxis, and Collagen type I & III. They also exhibited tenocyte-like morphology when exposed to mechanical stimuli.

Alginate, present in the cell walls of brown alginate, is another naturally derived polysaccharide which is a proper candidate for making 3D scaffolds. Gerecht-Nir et al [122] used alginate as a scaffold to direct differentiation of hESCs. They reported the generation of human embryoids (hEBs) and induced vasculogenesis in the forming hEBs within three-dimensional porous alginate scaffolds. They showed that the environment provided by the alginate scaffold pores enables the formation of round, small-sized hEBs and subsequent vasculogenesis. It was concluded that in addition to chemical cues, physical constraints can also induce and direct differentiation of hESCs.

4.2 Synthetic biodegradable polymers

Synthetic biodegradable polymers are recognized as a good scaffold biomaterial due to their good workability, reproducibility, and their ability to be processed easier than natural polymers [113, 123]. Researchers have used synthetic biodegradable polymers for making scaffolds used for tissue engineering and other biomedical applications (Fig 6a). Recent efforts have been directed toward using different types of polymeric biomaterials including poly(lactic-co-glycolic acid) [124-126], poly(- glycerol sebacate) [127, 128], poly(methyl methacrylate) [129], and poly(caprolactone) [130-132] as a supportive structure for hESC viability, attachment, and differentiation. To this end, Levenberg *et al.* [133], explored the neuronal differentiation of hESCs on 3D polymeric scaffolds made from poly(lactic-co-glycolic acid) and poly(L-lactic acid). In this study, neural rosette-like structures developed throughout the scaffolds in the

presence of differentiation factors in the medium including neurotrophin 3 [NT-3], retinoic acid [RA], and nerve growth factor [NGF]. A notable study by Zoldan *et al.* [134] showed that using 3D scaffolds made from synthetic polymers with varying concentration ratios can induce differentiation of hESCs into the three germ layers by providing specific mechanical properties such as substrate stiffness. In another related study, Subrizi et al [135] reported the in vitro generation of functional retinal pigment epithelium (RPE) on a supporting scaffold consisting of a transplantable, biopolymer-coated polyimide membrane which is clinically approved and has been shown suitable for subretinal transplantation. After co-culturing of hESCs with rat retinal explants, the hESCs showed a distinctive hexagonal, cobblestone morphology and expression of RPE specific proteins and genes.

A noteworthy achievement was accomplished by developing polymer grafted carbon nanotubes (CNTs) scaffolds for directing differentiation of hPSCs toward neuron cells. CNTs are of high strength, but flexible. Furthermore, they are conductive and their conductivity remains unchanged during harsh situations [136]. These characteristics make polymer grafted CNTs a promising scaffold material for inducing neuronal lineage from hESCs. Supporting this view, Chao *et al.* [137] generated a thin film scaffold comprising of biocompatible polymer Poly(acrylic acid) (PAA) grafted CNTs which can promote differentiation of hESCs into the neuron cells. According to the observations, PAA is a weak acid by nature has a negative effect on neuron differentiation. However, the nanoscale fiber morphology of CNTs can enhance both protein adsorption and cell adhesion, making PAA grafted onto CNTs a proper substrate for neuron differentiation and neuron cell attachment. In addition to the neural differentiation of hESCs, studies have used CNTs to study the effect of matrix properties on hESC differentiation

into other cell types. Sridharan et al [138] reported the differentiation of hESCs into the ectodermal lineage on the collagen-carbon nanotube (collagen/CNT) composite material.

4.3 Micro/Nano fibrous scaffolds

Nanomaterials have emerged as a great candidate for making scaffolds due to their resemblance to natural ECM, which provides an appropriate environment for cell adhesion, proliferation, and differentiation [139, 140]. Furthermore, they are biodegradable and have suitable surface chemistry, appropriate mechanical properties, and the capability to be formed into various sizes and shapes. It has been demonstrated that nanofibrous scaffolds can support self-renewal of hESCs. Gauthaman et al. [141] cultured hESCs on a scaffold made from Polycaprolactone /gelatin (PCL/gelatin) nanofibrous and PCL/collagen. It was observed that hESCs could proliferate on both scaffolds, showing the capability of nano-fibrous scaffolds for long-term maintenance of stemness characteristics of hESCs (Fig 6b). One possible reason is that the porous nature of the scaffold and large surface to volume ratio offer proper cell and matrix interaction for MEFs attachment and prevent the direct contact of hESCs and MEFs due to the fibroblast-like cell growth of MEFs and colony formation of hESCs in vitro. Supporting this view, Lu et al [142] reported using an engineered 3D microfiber system supporting long-term hPSCs self-renewal under defined conditions. The unique ability to form microscale fibrous matrices allowed cells to be encapsulated in the scaffold with excellent viability. One advantage of the micro-fibrous system is its ability to support both cell culture and differentiation within the same 3D system by manipulation of specific medium components. Another study from the same group [67] indicated that nanofibrous scaffolds can also be used for differentiation of hESCs into

the neural lineage by treating the cells with neural induction medium containing Noggin/retinoic acid.

5 Controlling hPSC fate by microfluidic devices

Microenvironment, including soluble factors, extracellular matrix, and mechanical cues, is very important for control of hPSC behavior. Microfluidic systems allow researchers to precisely modulate the microenvironment to control hPSCs maintenance and differentiation through microscale biochemical [143-147] and mechanical stimulation [148-150]. Microfluidic platforms have also been widely used in cell sorting [151-154] and high-throughput single cell analysis [155-160].

Several recent studies have used microfluidic devices to precisely control the hPSC microenvironment and study its effect on hPSC maintenance and differentiation [143, 161-164]. For example, a cell culture platform named inverting microwell array chip was developed to generate hiPSC aggregates with controlled size and geometry [161] (Fig. 7a). The cell aggregates were first formed on the bottom of the PEG-based microwells. After the cellular aggregates formed, the chip was inverted to plate the aggregates onto the polystyrene surface. This platform has the potential to study autocrine and paracrine signaling by modulating aggregate size and spacing. Additionally, Sikorski *et al*. developed a microfluidic device to support the robust generation of colonies derived clonally from single ESCs to study heterogeneity of hESCs [162]. The single ESCs cultured in individually addressable chambers to track cell proliferation, morphology, and OCT4 expression. They revealed that low OCT4 expression was correlated with low growth rate and a less compact morphology. Microfluidic devices were also used to identify the optimal culture conditions of hESCs and hiPSCs [163, 164]. Matsumura *et al.* [163]

found that laminin promoted hiPSC proliferation better than Matrigel. In another study, Yoshimitsu *et al.* [164] found laminin and fibronectin to be better than collagen and gelatin in terms of attachment and growth rate in hiPSC maintenance.

Microfluidic devices can generate chemical gradients to precisely assess the phenotype of hPSCs or to model early development [145-147]. Park *et al*. [144] cultured hESC-derived neural progenitor cells in microfluidic chambers for eight days under gradients different growth factors including Shh, FGF8, and BMP4. They observed the opposing effect of Shh and BMP4 on proliferation and differentiation of hESC-derived neurons; BMP4 inhibited the SHH mediated proliferation of neural projector cells. A microfluidic device was also used to provide a temporal and spatial gradient of multiple morphogens (Wnt3a, Activin A, BMP4, and their inhibitors) on embryoid bodies (EB) to study the effect of these molecular factors on the fate specification and mesoderm differentiation of hESCs [145] (Fig. 7b). This study showed that a linear concentration of morphogen gradients resulted in non-linear EB differentiation responses. More recently, Kamei *et al*. developed PDMS devices using soft lithography and 3D printing in which they exposed hESCs in a micro-channel to 3D gradients of chemicals created by differences in molecular weight [146]. They showed that the concentration of growth factors in the culturing medium is critical for the sphere formation of hESCs.

Label-free, microfluidic cell sorting platforms have been widely investigated because of the minimal sample preparation required, the ability to apply precise forces, and their greater compatibility with downstream analysis as compared to conventional cell sorting methods such as fluorescence-activated cell sorting (FACS) [151-154]. For example, Wang *et al.* integrated optical tweezers with microfluidic technologies to handle small cell population sorting [151]. They isolated OCT4-GFP^{+} hESCs from OCT4-GFP^{-} differentiated cells with a 90% recovery

rate and 90% purity. Choudhury *et al.* developed a microfluidic platform to separate hESCs from differentiated cells based on the difference in their cytoskeletal elasticity [152]. The elastic cells were more likely to flow along narrow separation channels than the inelastic ones. In another study, undifferentiated hESCs were isolated from a heterogeneous population based on the hESC surface marker SSEA-4 using an antibody-functionalized PDMS channel. Singh *et al.* [153] utilized the differential adhesive strength between hPSCs and somatic cells to rapidly isolate fully reprogramed hiPSCs from heterogeneous reprogramming culture with 95%-99% purity and >80% survival [154] (Fig. 7c). In the future, microfluidic sorting platforms could be integrated with imaging technologies and downstream biochemical and genomic analysis.

One significant advantage of microfluidics is the integration of lab-based testing in a single chip to perform high-throughput single cell analysis such as on-chip immunoassays [155, 156], and single cell real-time PCR [157-160]. Recently, such technologies have been utilized in hPSC research to study the heterogeneity of hPSCs. Kamei *et al.* [155] demonstrated the culture and analysis of hESC colonies in an integrated microfluidic platform termed hESC-μChip. hESC-μChip is capable of culturing hESCs in addressable chambers and running phenotypical and functional analyses including live cell imaging and immunocytochemistry. In another study, Kamei *et al*. performed single-cell profiling of protein expression (OCT4 and SSEA-1) with a similar device [156]. In this device, every single chamber could run immunocytochemistry under different hPSC culture conditions. They found that culture in different conditions resulted in the generation of hPSC lines of different phenotypes in which growth rate, morphology, and pluripotency and differentiation markers all varied. High-throughput single cell analysis methods are essential to study how heterogeneity in hPSC populations can lead to different fate determinations. Microfluidic devices are a powerful tool for single-cell gene expression

measurements with low sample population, reduced cost, and high sensitivity [157-160]. White *et al*. [159] developed a fully integrated microfluidic device able to perform RT-PCR from hundreds of single cells per run. All steps including cell capture, cell lysis, reverse transcription, and quantitative PCR were processed in the chip. They observed coregulation of miR-145 and OCT4 in the single cells, which is not apparent from population measurements. Another study used microfluidic-based single cell gene expression analysis and showed that hiPSCs were more heterogeneous in gene expression than hESCs [160].

6 Model Organoid Systems: Applying Bioengineering Approaches

Organoids have been generated from both PSCs and adult stem cells (ASCs) by mimicking the biochemical and physical cues of tissue development and homeostasis [165]. The homeostasis of many tissues in vivo is maintained through self-renewal and differentiation. Both of these processes can be recapitulated in vitro using specific culture conditions that lead to self-organized tissue organoids. The generation of organoids is influenced by biochemical and biophysical signals, cell-cell interactions, and cell-extracellular matrix (ECM) interaction [166]. By providing the proper biophysical and biochemical factors, differentiated cells from PSCs will self-organize to form tissue-specific organoids including the optic cup[167], brain [168, 169], intestine [169], liver [170], and kidney [171].

6.1 Brain Organoids

Human brain development involves a high degree of coordination between the neural stem cells (NSCs) and the dynamic niche in which they exist. PSCs can differentiate into different neural subtypes including spinal cord motor neurons [15, 172, 173], cortical pyramidal neurons [174],

and midbrain dopaminergic neurons [175-177] by subjecting them to different levels of morphogens (i.e., BMP, Wnt, Shh, RA, and FGF). Also, more complex architectures such as sub-brain regions like the cerebral cortex [178-181] and the pituitary [182] have been generated using serum-free floating culture of embryoid body-like aggregates with quick re-aggregation (SFEBq) protocol. Alternatively, Lancaster et al. [168] developed a culture system to generate heterogeneous neural organoids that contained multiple brain regions within individual organoids. In the presented study, the generated neuroectodermal tissues were maintained in 3D Matrigel for further expansion, growing as large as 4 mm in diameter in 2 months. They generated distinct brain regions such as the dorsal cortex, ventral telencephalon, choroid plexus, hippocampus, and retina. Although cerebral organoid systems have been used to model human brain development, several limitations still exist. Due to the absence of body axis and surrounding tissue, the current models are not able to form the brain structure as they exist in vivo. Patterning factors can be used to increase control over tissue organization. Bioengineering approaches such as cell patterning signals using customized scaffolds with immobilized signals or signal gradients created with microfluidic devices will have the potential to guide the differentiation and patterning of brain regions in the organoids. Another challenge is that, as the organoid grows, there is inadequate supply of nutrients and oxygen to some regions, limiting the size and sometimes leading to undesired differentiation [183]. A potential solution could be the implementation of microfluidic perfusion networks or co-culture systems that can vascularize the brain organoids.

6.2 Kidney Organoid

Kidney regeneration is one of the target goals of the study of hPSCs. Its complex structure and blood filtration functionality present many challenges. Several different approaches have been successfully used to generate kidney organoids from hPSCs. In one approach, hPSCs are induced to differentiate into Nephron Progenitor cells (NPCs), which will subsequently generate kidney organoids [184, 185]. Taguchi et al. [185] reported the first protocol to differentiate hPSCs into kidney organoids with nephron-like structures. In this work, mouse embryonic spinal cords were used to stimulate epithelialization of NPCs on a polycarbonate filter. They saw the formation of early-stage nephron structures resembling S-shaped bodies. These cells expressed markers for tubules (cadherins CDH1 and CD) and podocytes [WT1 and nephrin (NPHS1)] while still expressing NPC markers including spalt-like transcription factor 1 (SALL1) and PAX2. This resulting expression profile indicated the successful use of this approach for the generation of immature nephrons.

Drug discovery and disease modeling require the generation of organoids in culture conditions suitable for high-throughput screening. Protocols for kidney organoid generation that require coculture with mouse embryonic spinal cords may limit disease modeling due to the presence of undefined components as well as limited access to mouse embryonic spinal cords [185]. To address these issues, Morizane et al. [184] developed differentiation protocols for kidney organoid formation suitable for high throughput screening with the use of two approaches. The first approach uses a 2D culture system to prompt differentiation of hPSCs from the NPC stage to kidney generation. The second approach involves placing NPCs into ultra-low attachment, 96-well round-bottom plates. This leads to the generation of a large number of kidney organoids in small well 3D culture. These kidney organoids contained segmented

nephrons with regions bearing characteristics of proximal tubules, podocytes, loops of Henle, and distal convoluted tubules in an organized arrangement.

Some studies have achieved the generation of kidney organoids containing nephron-like cells without the use of NPCs as a preliminary stage. Freedman *et al*. [186] generated cavitated spheroids made up of pluripotent cells by 'sandwiching' cells between two layers of Matrigel. The cavitated spheroids were then treated with CHIR for 1.5 days, and they were differentiated for up to 16 days. The results showed WT1+SYNPO+ podocyte-like cells and LTL+ tubular structures resembling proximal tubules. However, the approach resulted in less-specific differentiation into nephron-like cells, with possibly less-mature nephron phenotypes. In another study, Takasato *et al.* [187] reported the generation of kidney organoids containing multiple lineages. In this study, hiPSCs were differentiated through CHIR stimulation for four days. The cells were subsequently treated with 200 ng/ml FGF9 for three days and formed into pellets. The pellets were then cultured on a transwell dish with 200 ng/ml FGF9 for five days following CHIR pulse treatment. After an additional 13 days of treatment, the resulting organoid had segmented nephron-like structures along with mature proximal tubules, podocytes, loops of Henle, and endothelial-like cells.

6.3 Endodermal organs

HPSCs can be used to generate endodermal tissues and organoids [188]. So far, researchers have generated a variety of endodermal organs from hPSCs including small intestine, lung, liver, pancreas, and stomach. Studies have shown the generation of functional liver tissue from iPSC-derived liver buds containing human mesenchymal and endothelial cells. In the study by Takebe *et al*. [170], a vascularized and functional human liver was generated from human iPSCs by

transplantation of liver buds created *in vitro* (iPSC-LBs). Immature endodermal hepatic cells self-organized into iPSC-LBs by recapitulating organogenetic interactions between endothelial and mesenchymal cells. Some additional experiments including gene-expression analyses and immunostaining showed that *in vitro* grown iPSC-LBs resemble the *in vivo* liver buds. By connecting iPSC-LBs to the host vessels, vascularized and functional human livers were generated within 48 hours. In another related study, Camp *et al*. [189] derived three-dimensional liver bud organoids from hPSCs by reconstituting stromal, hepatic, and endothelial interactions during liver bud development. They found a striking correspondence between the three-dimensional liver bud and fetal liver cells by evaluating three-dimensional liver buds against adult and human fetal liver single-cell RNA sequencing data.

Dye *et al.* [190] recently developed a strategy to generate a lung organoid from hPSCs. In this study, hPSCs were exposed to developmental signaling pathways that prompted differentiation toward ventral-anterior foregut spheroids. The ventral-anterior foregut spheroids expanded into human lung organoids (HLOs) with structural features similar to the native lung. A notable study by McCracken *et al.* [191] reported that the manipulation f WNT, FGF, BMP, and retinoic acid signaling pathways, in conjunction with a three-dimensional culturing system achieved generation of gastric organoids from hPSCs. The generated gastric organ contained cell types from the pit, gland, and neck regions of the antral stomach but it did not contain corpus cell lineages.

To generate intestinal tissue, Spence *et al*. [169] developed an efficient method in which temporal series of growth factor manipulations were used to direct the differentiation of hPSCs into intestinal tissue. The generated three-dimensional intestinal ‘organoids’ contained a polarized epithelium and crypt-like proliferative zones expressing intestinal stem cell markers.

6.4 Organoids: new models to study old diseases

One of the most critical applications of hPSCs is *in vitro* modeling of human diseases in order to study the mechanisms of disease and develop new therapeutic approaches. This can be done in a high-throughput manner with the use of organoids. Gastrointestinal organoids have successfully used to investigate specific diseases that are difficult to study in animal models. Intestinal organoids infected with Clostridium difficile or Helicobacter pylori have been used to understand some of the earliest processes in the epithelial response to pathogens [191-193]. Kidney organoids have been used to model inherited kidney diseases and explore possible treatments [194, 195]. Also, cerebral organoids provide a unique opportunity to study specific neurological disease processes, such as the microcephaly that is secondary to infection with Zika virus [196-199].

Additionally, with the advent of gene editing processes such as CRISPR/Cas9, mutation correction and personalized medicine are now possible in patient-specific iPSC-derived organoids [200]. CRISPR/ Cas9 gene editing has enabled the study of polycystic kidney disease within kidney organoids [186]. In the future, patient-specific iPSC-derived organoids could be used to predict individualized drug efficacy and epithelial response, as has recently been shown for patients with cystic fibrosis using adult tissue-derived organoids [201].

7 Conclusion and future perspective

hPSCs constitute a promising cell source for human tissue and organ regeneration, *in vitro* modeling of human diseases, and screening for patient-specific therapeutic and drug responses. Over the years, progression in the study of hPSC has revealed the importance of the

stem cell niche in stem cell development. In the last 15 years, the fields of micro/nanoengineering have remarkably advanced in the generation of novel micro/nanoengineered culturing systems in terms of efficiency, robustness, and control of hPSC function. This paper presents a comprehensive review on developed micro/nanoengineered approaches for accurate regulation of various aspects of the cell microenvironment for control of hPSC fate and function. These approaches incorporate a variety of engineering technologies including biomaterials, microfabricated systems, and microfluidics. We anticipate that in the near future, researchers will be able to better address issues in fundamental hPSC studies and biomedical applications including toxicity and drug screening, and regenerative medicine.

As in vitro niches become more able to capture the in vivo environment, the limits of what we are able to study in a laboratory setting will continue to be pushed, leading to the better understanding of a plethora of human organoids. By providing the proper biophysical and biochemical factors, differentiated cells from PSCs will self-organize to form tissue-specific organoids including the optic cup, brain, intestine, liver, and kidney. However, scalability and improving the maturity of organoids in vitro is a great challenge facing the field of organogenesis. There has been recent success in growing some organoids like brain organoids which has the potential for large-scale organoid generation and high-throughput drug screening [198]; however, it still needs to be pushed forward.

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Figure 1

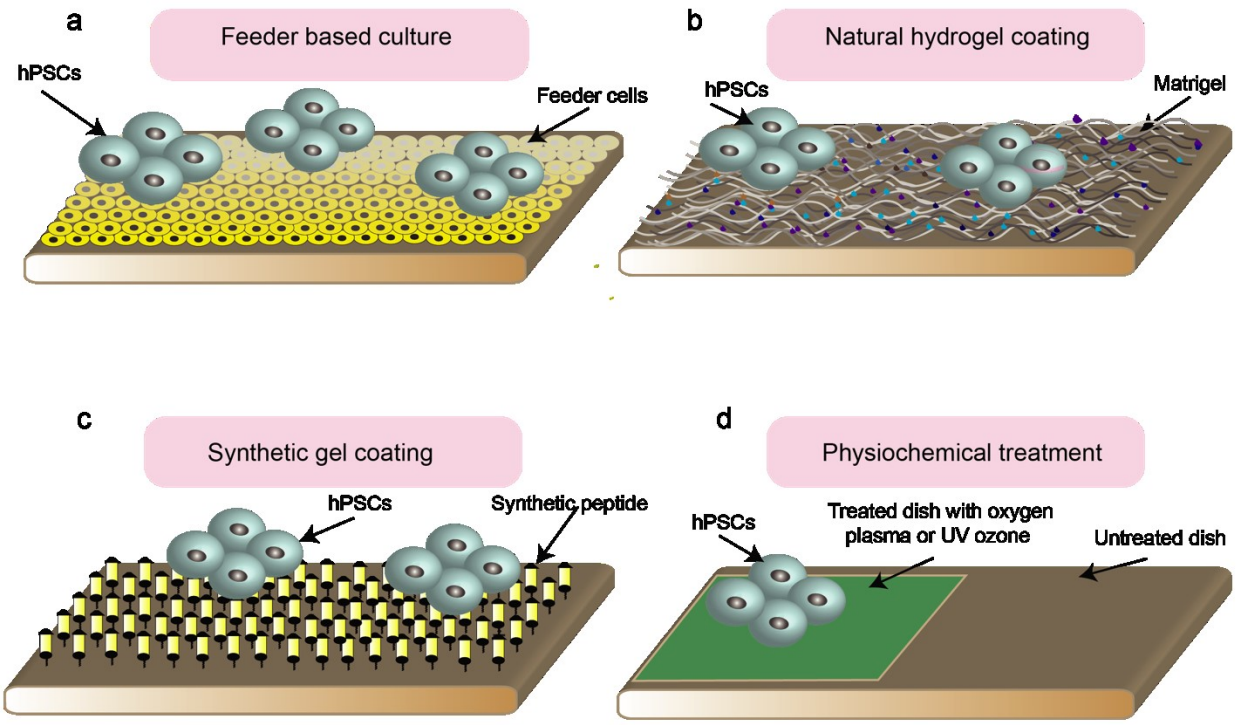

Figure 1. Culture platforms for hPSC maintenance and expansion. (a) Culturing hPSCs on the feeder culture. (b) Feeder free culture of hPSCs on the substrates coated with natural hydrogel. (c) Feeder free culture of hPSCs on the substrates coated with synthetic gel. (d) Feeder free culture of hPSCs on the dishes treated with UV ozone or oxygen plasma.

Figure 2

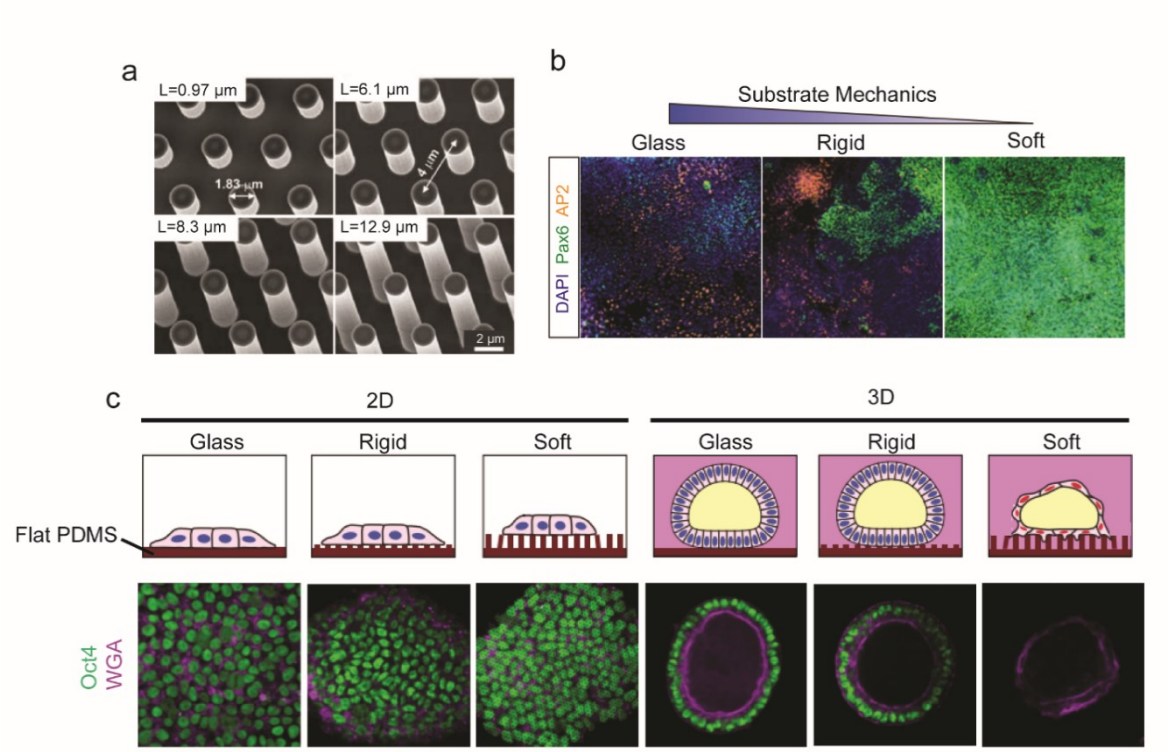

Figure 2. Microposts arrays are used to study the mechanoresponsive behaviors of hPSCs**. (**a) Scanning electron microscopy images of microfabricated silicon micropost array masters with different post height. Adaped from [8]. (b) Immunofluorescence and quantitative results showing $Pax6^+$ Neural epithelial cells and $AP2^+$ neural crest cells cultured on vitronectin-coated coverslips and rigid and soft PDMS micropost array. Adapted from [3]. (c) Confocal micrographs showing staining of Oct4 and WGA for hPSCs cultured in the indicated conditions. Adapted from [8]. (You need to make sure to obtain the re-print license for ALL the figures that you adapted from other papers)

Figure 3

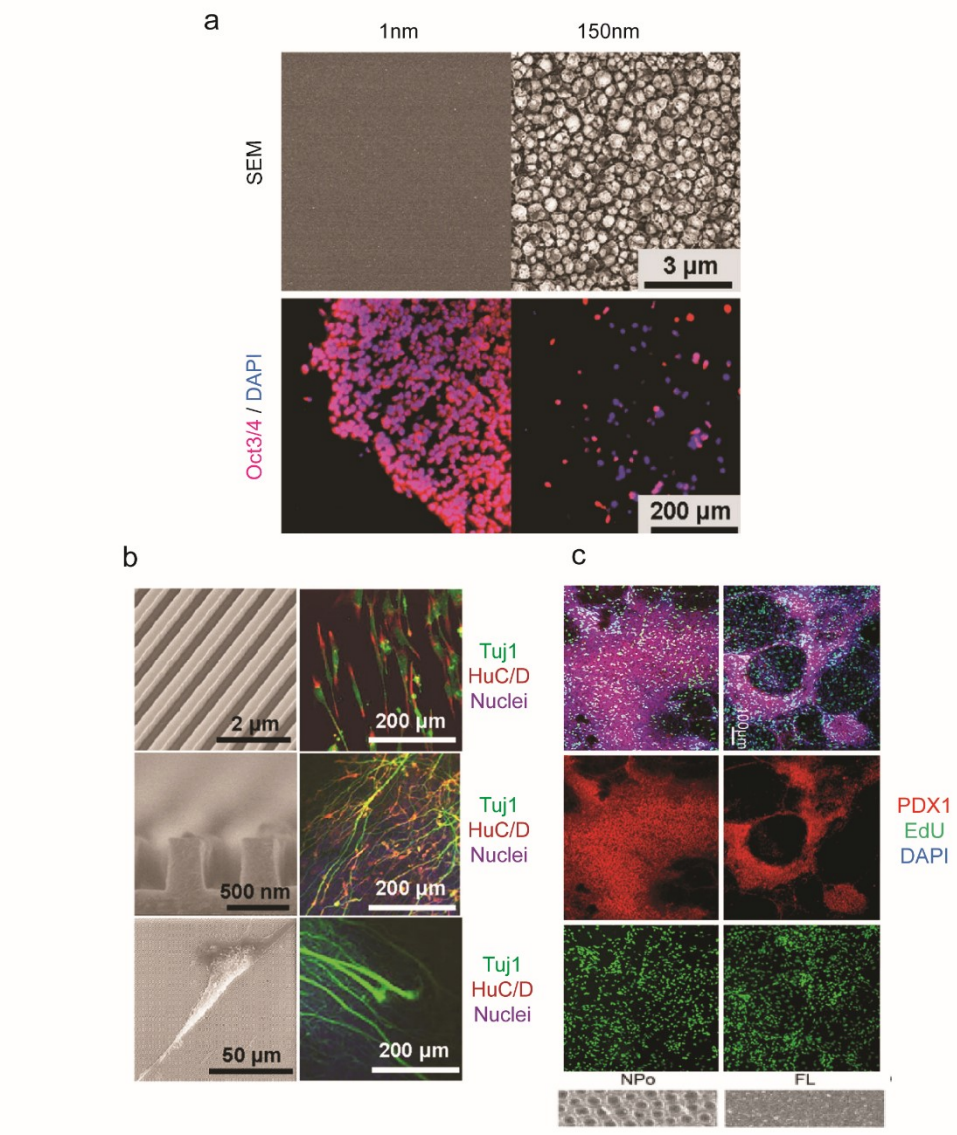

Figure 3. Nanotopography regulates hPSCs self-renewal and differentiation. (a) Culturing hESCs on platforms with surface topographies. SEM images of glass surfaces with surface topographies (top) and immunofluorescence images of hESCs (bottom) cultured on a glass surface with their indicated root-mean-square (RMS) nanoroughness *Rq*. The cells were stained for nuclei (DAPI; blue), and pluripotency marker (Oct3/4; red) [58]. (b) Differentiation of hESCs into selective neurons on ridge/groove patterns. SEM images of a bird's eyes view of 350-nm ridge/groove pattern arrays (height of 500 nm, the spacing of 350 nm) (left top), a cross-section (left middle), and a SEM image which shows hESCs on the ridge/groove pattern arrays (left

bottom). Immunofluorescence images of hESCs stained with nuclei, neural and glial marker (HuC/D, Tuj1) [11]. (c) Differentiation of hESC-derived endoderm to pancreatic progenitors on nanopores with 200 nm diameter. Immunofluorescence images of hESC-derived endoderm with nuclei (DAPI), and critical transcription factor for pancreatic development (PDX1) (If available from the original paper, add SEM images or cartoons in c, to show what the topography looks like) [65].

Figure 4

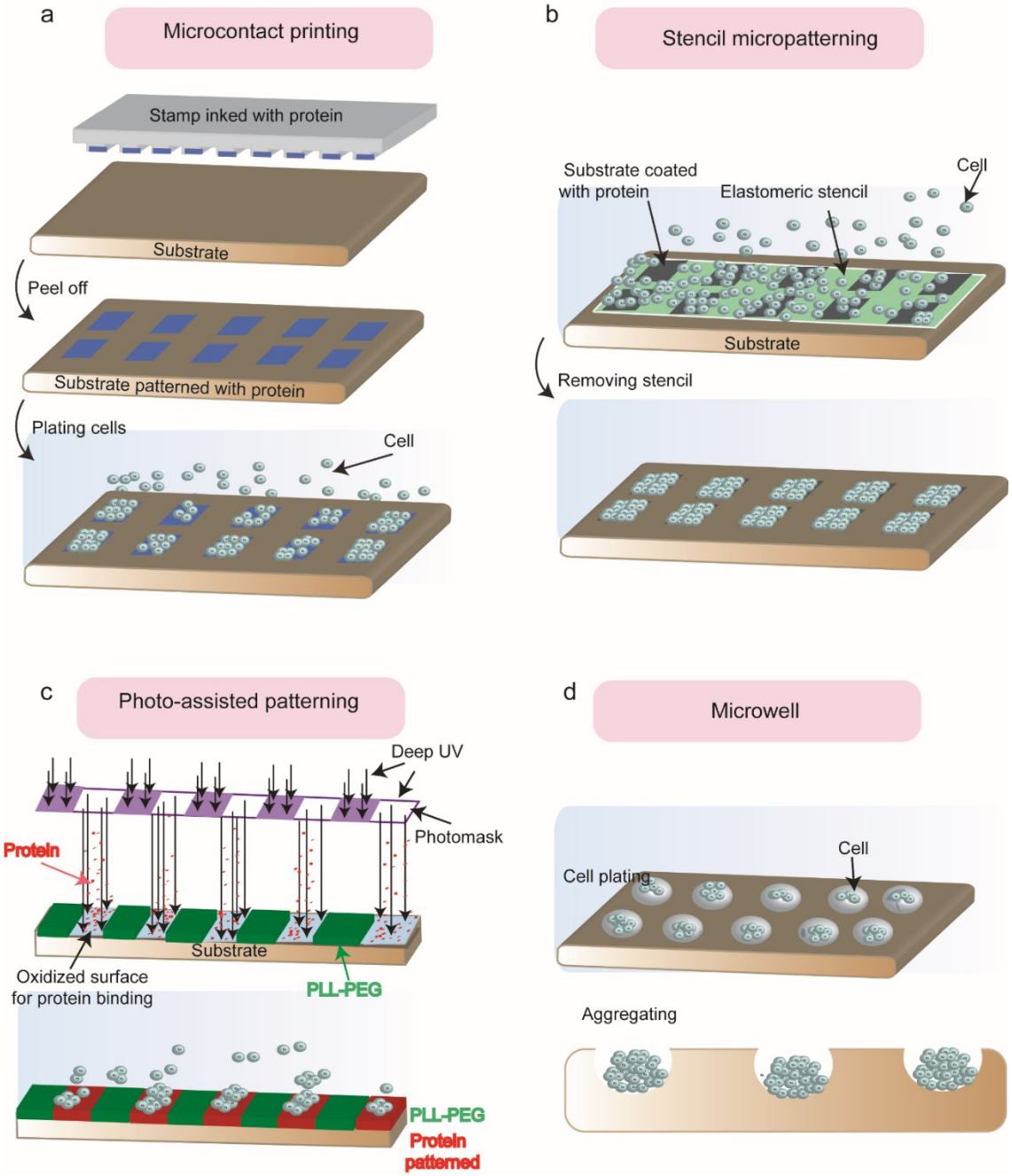

Figure 4. Generating micro/nano-patterns of cell adhesive cues on the substrate. (a) Schematic of microcontact printing (μCP). The PDMS stamp inked with proteins bring into contact with the activated substrate. After peeling the stamp off, the patterned protein is transferred into the substrate. (b) Schematic of elastomeric stencil micropatterning. After coating the substrate with ECM protein, the stencil is applied onto the cell-culture substrate during seeding process and

peeled off after cell plating. (c) Schematic of deep UV-activated micropatterning. Deep UV removes the cell-repellent PLL-g-PEG coating and oxidized the surface underneath for proper binding to the soluble ECM protein (fibronectin) molecules. Therefore, micro-patterns will be transferred from the photomask to the substrate in the presence of cells. (d) Schematic of the microwell for patterning hPSCs. A specific number of cells are seeded into each microwell depending on the size of the well. Cells will aggregate and form the shape of the well.

Figure 5

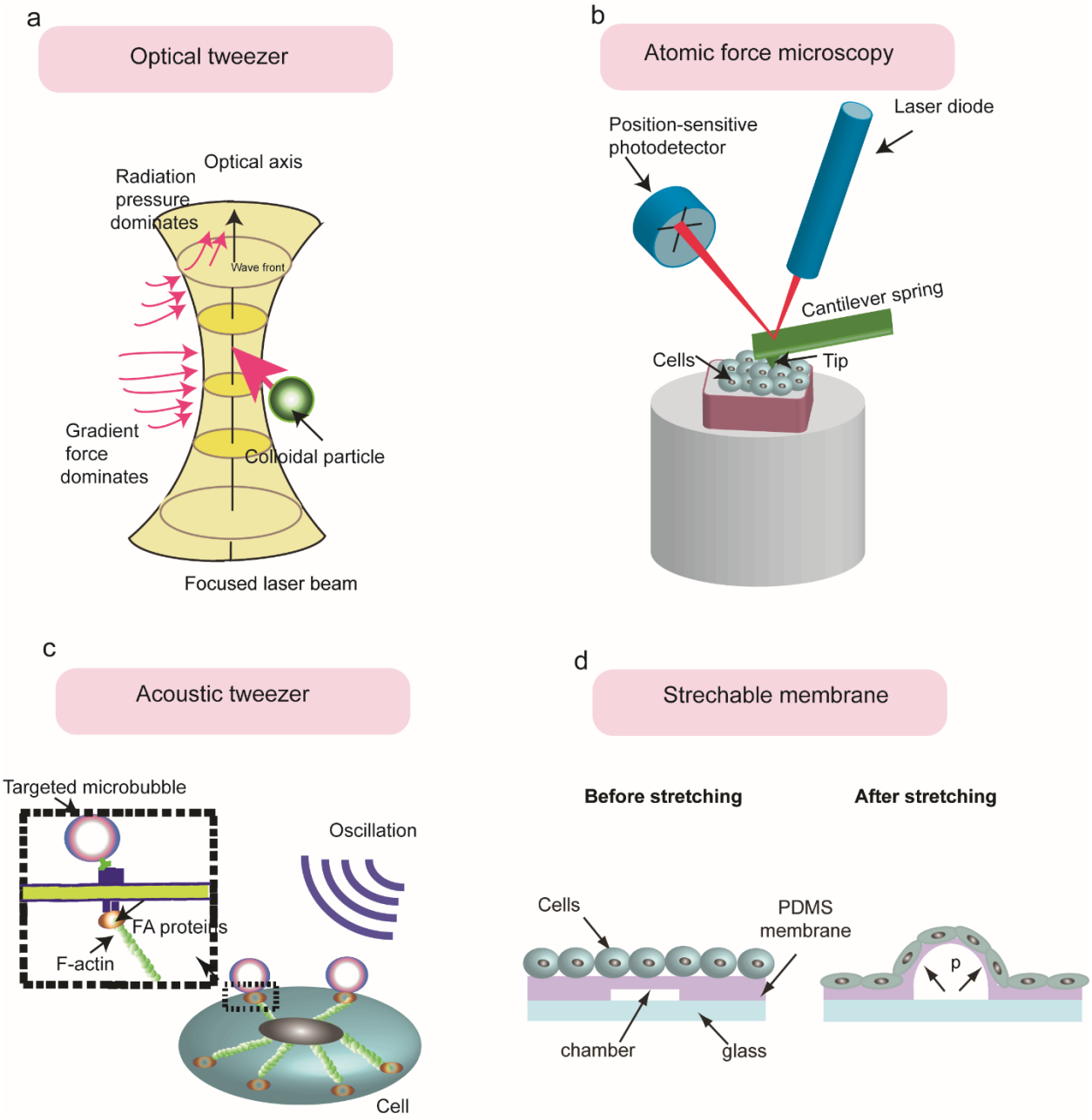

Figure 5. Techniques to apply mechanical perturbations to hPSCs. (a) Optical tweezer provides force and displacement on the surface of the cell or within a defined region of a cell by controlling the displacement of microbeads. (b) Atomic Force Microscopy to apply mechanical perturbations to the cells. An electronic controller moves the cantilever beam which is functionalized with the adhesive ligand to bind the cell surface via adhesive ligand-receptor binding and provide mechanical perturbations to the cells. (c) In the acoustic tweezers cytometry

(ATC) method, Acoustic wave vibrating the lipid microbubbles covalently attached the surface of the cell and applied force to the cell. (d) Stretchable substrate technique to apply mechanical strain to the cells. The cells are plated on the PDMS membranes containing some chambers. By connecting chambers to the vacuum, the PDMS membranes and cells that are attached to the membrane will be stretched.

Figure 6

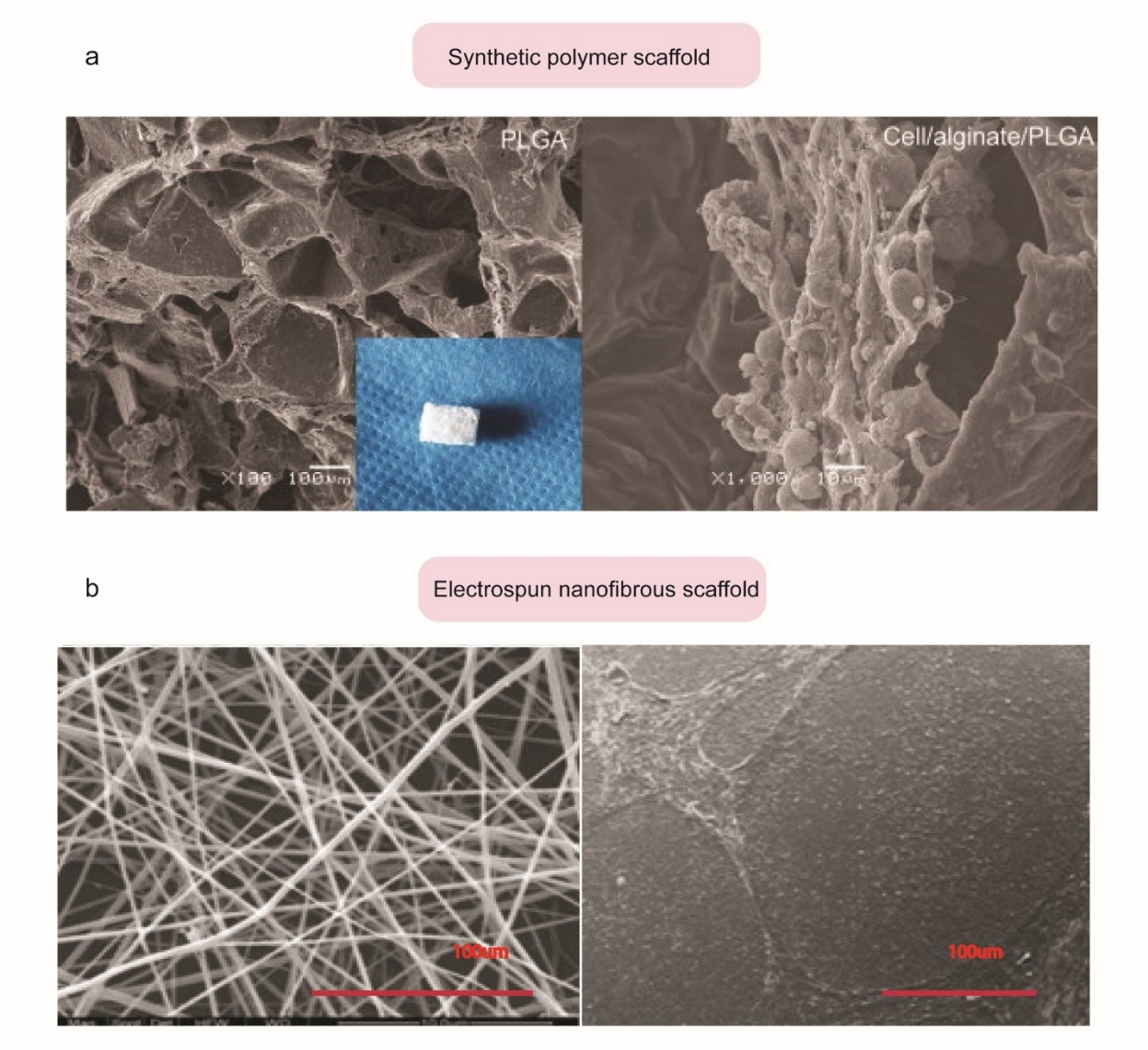

Figure 6. Scaffold regulates hPSCs self-renewal and differentiation. (a) Derivation of cartilage-like tissue from hESCs on alginate/PLGA (Synthetic polymer) scaffolds, SEM examinations of the PLGA scaffold (left) and the cells/alginate/PLGA complex. (b) Nanofibrous scaffold supports colony formation and maintains stemness of hESCs. SEM examination of the electrospun nanofibrous scaffold (PCL/gelatin (1:9%w/v)) (left). hESCs cultured on PCL/gelatin nanofibrous scaffolds and MEFs.

Figure 7

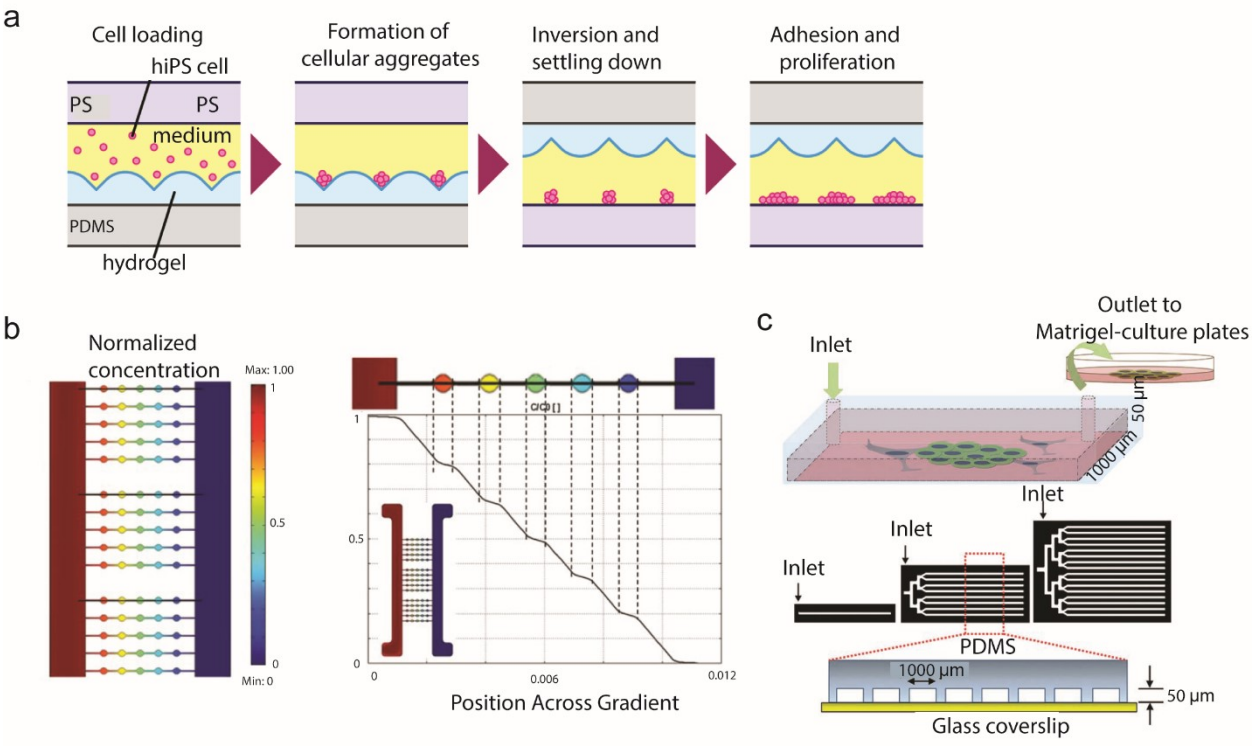

Figure 7. Different applications of microfluidics in hPSC culture. (a) Schematics showing the culture process of hiPSCs in the inverting microwell chip. Adapted from [161]. (b) Computational modeling of mass transport whithin the microbioreactor. Adapated from [145]. (c) Schematics of adhesion strength-based isolation of pluripotent stem cells in microfluidic devices. Adapted from [154].

Figure 8

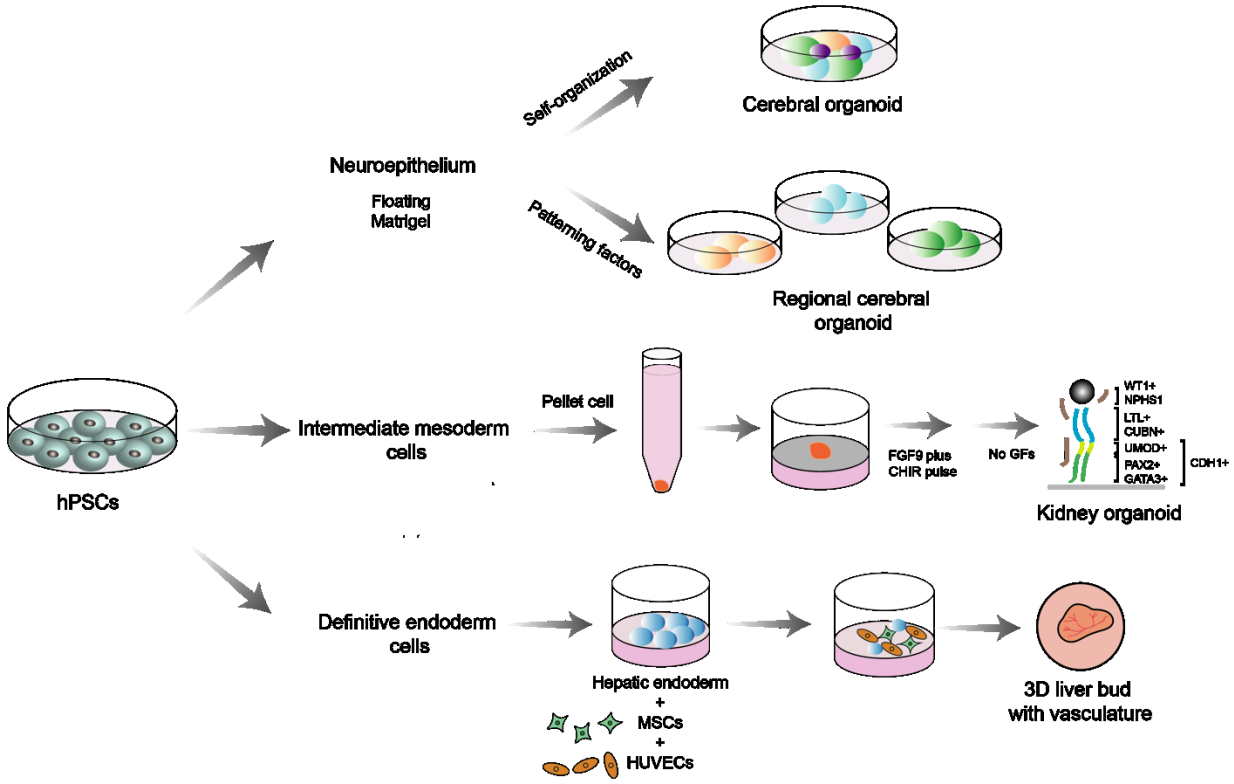

Figure 8. hPSC-derived organoids. hPSCs were differentiated into brain organoid, kidney organoid, and liver organoid.

Table1. Summary of various nanotopographic methods for stem cell studies.

APPLICATION	FEATURE SIZE	FABRICATION TECHNIQUE	TOPOGRAPHY	MATERIAL	CONCLUSION
HPSC MAINTENANCE	1-150nm	Lithography and replica-molding	Nano roughness	Silica-based Glass wafer	Alter cell morphology, adhesion, and proliferation [58]
HPSC MAINTENANCE	1-150nm	Photolithography and reactive ion etching (RIE)	Nano roughness	Silica-based Glass wafer	Mediate hESCs function including attachment, morphology, proliferation and differentiation [68]
HPSC MAINTENANCE	30nm	Chemical vapor deposition (CVD)	Multi-walled carbon nanotube–graphene hybrid	CNT-graphene	Maintain attachment, proliferation, and stemness of hESCs [56]
NEURAL DIFFERENTIATION	360 nm	Laser inference lithography (NIL) and replica-molding	Ridge/groove-patterned surface	PDMS	Ridge/groove nanotopography direct differentiation of hiPSCs towards the neuronal lineage [62]
NEURAL DIFFERENTIATION	-	Nano-imprinting	Ridge/groove-patterned surface	Glass coverslip	Direct differentiation of human embryonic stem cells into selective neurons on nanoscale ridge/groove pattern arrays [11]
NEURAL DIFFERENTIATION	80-250 nm	Nanoimprinting	Grating-pillar	Thermoplastic polycarbonate	Substrate topography, with optimal dimension and geometry modulates the neural fate of hESCs [60]
NEURAL DIFFERENTIATION	250 nm	Soft lithography	Nano-grating	PDMS	Nano-grating substrates directs neural differentiation of hPSCs through actomyosin contractility [66]
NEURAL DIFFERENTIATION	1-200 nm	Reactive-ion etching (RIE)	Random nanoscale	Glass coverslip	Nanotopographic

					substrates promote hPSC motor neuron progenitor cell differentiation[63]
CARDIOMYOGENIC DIFFERENTIATION	-	Chemical vapor deposition (CVD)	Nanorough graphene	Graphene	Improving cardiomyogenic differentiation of hESCs on the nanorough graphene [64]
ENDOTHELIAL DIFFERENTIATION	-	Salt leaching process	Porous sponges	Poly-(l-lactic acid) (PLLA) and polylactic-glycolic acid (PLGA)	Endothelial cells derived from human embryonic stem cells [69]
PANCREATIC DIFFERENTIATION	-	Electrospinning	Nanofibrious	Poly-L-lactic acid and polyvinyl alcohol (PLLA/PVA)	Synthetic scaffolds lead to the differentiation of hiPSC to pancreatic cells [70]
PANCREATIC DIFFERENTIATION	100-400 nm	Electrochemical method	Nanopillar/nanopore	Oxalic AAO (O-AAO) and phosphoric AAO (P- AAO	Nanotopographical surface improve 3-dimensional differentiation of pancreatic cells from hPSCs [71]
CHONDROGENIC DIFFERENTIATION	-	Electrospinning	Nanofibruous	Polyethersulfone (PES)	Nanofiber-based polyethersulfone scaffold directs differentiation of hiPSCs to chondrogenic [72]
RETINAL DIFFERENTIATION	150-190um	-	Porous structure	Gelatin, chondroitin sulfate, and hyaluronic acid (GCH)	Biodegradeable scaffold improve differentiation of hPSC into the retinal cells [73]
HEPATOGENIC DIFFERENTIATION	-	Electrospining	Nanofibrous	Polyethersulfone/ collagen	Enhancing hepatogenic differentiation of hPSC on the aligned polyethersulfone [74]

Table 2. Comparison of methods for generating cellular confinement

Methods for patterning hPSCs	Cost	Throughput	Controllability of feature shape	Controllability of feature size	Easy to use	Appropriate for small sizes	Need access to microfabrication methods
Microcontact printing	***		***	***	*	***	***
Stencil	***		**	**	**	*	***
Photo-assisted	**						