Biomaterials 52 (2015) 26–43

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Review

On human pluripotent stem cell control: The rise of 3D bioengineering and mechanobiology

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ARTICLE INFO

Article history:
Received 4 December 2014
Accepted 28 January 2015
Available online 21 February 2015

Keywords:
Human pluripotent stem cells
Mechanobiology
Bioengineering
Regenerative medicine
Development
Human disease modeling

ABSTRACT

Human pluripotent stem cells (hPSCs) provide promising resources for regenerating tissues and organs and modeling development and diseases *in vitro*. To fulfill their promise, the fate, function, and organization of hPSCs need to be precisely regulated in a three-dimensional (3D) environment to mimic cellular structures and functions of native tissues and organs. In the past decade, innovations in 3D culture systems with functional biomaterials have enabled efficient and versatile control of hPSC fate at the cellular level. However, we are just at the beginning of bringing hPSC-based regeneration and development and disease modeling to the tissue and organ levels. In this review, we summarize existing bioengineered culture platforms for controlling hPSC fate and function by regulating inductive mechanical and biochemical cues coexisting in the synthetic cell microenvironment. We highlight recent excitements in developing 3D hPSC-based *in vitro* tissue and organ models with *in vivo*-like cellular structures, interactions, and functions. We further discuss an emerging multifaceted mechanotransductive signaling network – with transcriptional coactivators YAP and TAZ at the center stage – that regulate fates and behaviors of mammalian cells, including hPSCs. Future development of 3D biomaterial systems should incorporate dynamically modulated mechanical and chemical properties targeting specific intracellular signaling events leading to desirable hPSC fate patterning and functional tissue formation in 3D.

1. Introduction

Starting from fertilization, embryonic development comprises a complex process that unfolds the complete form of human life. During early embryonic development, each single cell in the morula holds equal totipotency – the capability to divide and differentiate into all differentiated cells in a living organism [1]. Derived from the inner cell mass of human blastocyst, which comes right after the morula stage, human embryonic stem cells (hESCs) inherit most of the totipotency and maintain the potential for unlimited self-renewal and differentiation into all types of somatic cells *in vitro* [2]. Interestingly, although the pluripotency of cells decreases as the embryo develops and the organism ages, it does not completely disappear. Instead, many kinds of adult stem cells exist in the human body throughout the life span, serving as resources for replenishing dying cells and regenerating damaged tissues [3]. Although considered as an irreversible process – as an analogy to a rock spontaneously rolling downhill – the transition from pluripotent cells to terminally differentiated cells has recently been found to be reversible through a "reprogramming" process under certain "driving forces", such as nuclear transfer [4], transcription-level interference [5], and treatments with small molecules [6]. Such human induced pluripotent stem cells (hiPSCs), together with hESCs, are termed human pluripotent stem cells (hPSCs), holding great promise for studying human development and disease, regeneration of tissues and organs, and constructing patient-specific disease models for drug and toxicology screening [7,8].

The fate and organization of cells in the human body are tightly regulated in the three-dimensional (3D) cell microenvironment through intricate interactions with neighboring cells, the surrounding extracellular matrix (ECM), and soluble biochemical cues [9,10]. Thus, to recapitulate *in vivo*-like cellular structures, properties, and organizations using hPSCs, it is critical to control and modulate the 3D cell microenvironment mimicking tissue- and

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http://dx.doi.org/10.1016/j.biomaterials.2015.01.078
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organ-specific niches. Since the end of last century, biologists, biomaterials scientists, and bioengineers have been endeavoring to develop efficient strategies for maintaining hPSC culture and directing hPSC differentiation and their spatiotemporal organization by modulating the physiochemical culture environment [11–15]. So far, a majority of achievements for hPSC culture have been witnessed through development of various two-dimensional (2D) systems, using either tissue culture plastics (TCPs) or synthetic biomaterial surfaces. These 2D culture platforms have enabled drastic advancements in controlling long-term clonal growth and multilineage differentiations of hPSCs [11], and they have been shown effective in inducing early developmental structures such as neural rosettes [16].

However, 2D hPSC culture platforms are intrinsically limited for delivering full promise of hPSCs, given the 3D nature of human tissues and organs. Compared to 2D platforms imposing unnatural geometric constraints on cells, 3D culture systems using functional biomaterials create a more physiologically-relevant cell microenvironment. Supporting this notion, it has been shown that 3D biomaterial systems can improve survival and regenerative potential of hESC-derived multipotent stem cells and human adult stem cells after *in vivo* implantation [17–19]. 3D hPSC cultures are also needed for modeling human diseases related to abnormal ECM remodeling during development and aging [20], a process difficult if not impossible to recapitulate in a 2D environment. Furthermore, 3D spatiotemporal patterning and organization of cytosystems is one of the most prominent features of embryonic development, tissue morphogenesis, and organogenesis and is also key to proper functionalities of human tissues and organs. Such dynamic cellular patterning and organization can only be simulated in a 3D environment using functional biomaterials of appropriate properties [21].

Fundamental understanding of cell–biomaterial interactions in a 3D environment is critical for guiding rational designs of biomaterials for bioengineered control of cell fate. Interestingly, recent studies of human stem and adult cells have revealed potent roles of mechanical aspects of cell–biomaterial interactions in regulating cell fate, through mechanotransductive signaling mechanisms intricately connected to classical cellular pathways important for cell fate control [22]. In particular, a signaling network centering around two transcriptional coactivators YAP and TAZ has emerged recently for its important role in growth control and fate regulation of human stem cells, including hPSCs [23–25].

The goal of this review, therefore, is to present an overview of existing biomaterial systems for fate control of hPSCs in both 2D and 3D environments, in accompany with a summary of the current understanding of cell signaling pathways, which are potentially mechanosensitive, in hPSC fate and function control. We first summarize existing 2D and 3D culture platforms for regulating hPSC behaviors, laying a foundation of hPSC fate and function regulation by inductive microenvironmental cues. We then discuss recent excitement on using 3D biomaterial systems with hPSCs for generating microtissues and organoids with *in vivo*-like tissue structures and functions. We further discuss current understanding of YAP/TAZ-mediated intracellular signaling in controlling stem cell fates and highlight mechanotransductive mechanisms acting upstream of YAP/TAZ through Rho GTPases, cytoskeleton tension, and F-actin mechanics. We offer some speculations on future opportunities to develop large-scale, multifunctional hPSC culture systems that integrate multiparametric control of 3D microenvironmental cues and high-throughput, automated cellular processing and analysis techniques.

2. Engineering hPSC fate and function: from 2D to 3D

2.1. 2D hPSC culture platforms

2.1.1. 2D culture platforms for hPSC self-renewal

Maintaining long-term hPSC culture without losing their pluripotency is a prerequisite for hPSC-based applications. Conventionally, mitotically inactive mouse fibroblasts (MEFs) are used as feeder cells to support hPSC self-renewal (Fig. 1A). However, mouse feeder cells may raise potential issues owing to immunogenicity and microbial or viral transmission [26]. In addition, feeder-based cultures suffer from batch-to-batch variations and cytogenetic aberrations due to repeated enzymatic treatments, posing a challenge for controllable and repeatable hPSC culture. As an attempt to eliminate feeder cells from hPSC culture, Kim et al. recently developed a strategy using porous polymeric membranes to physically separate hPSCs from feeder cells (Fig. 1B) [27]. In their culture system, MEFs were seeded to the bottom surface of the porous membrane before hPSCs were cultured on its top surface. This setup allowed continual interactions between hPSCs and MEFs as well as an efficient separation mechanism without enzymatic treatments, resulting in reduced contamination from MEFs, as

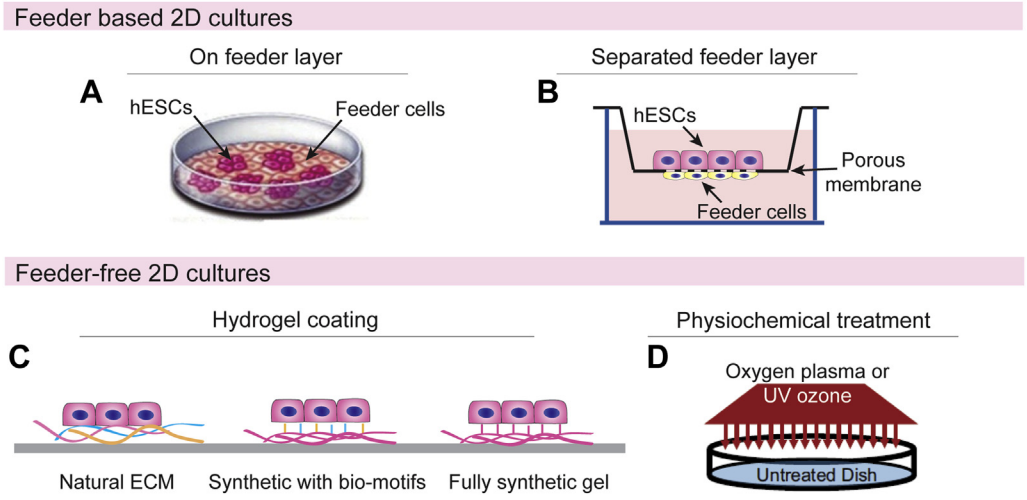

Fig. 1. 2D culture platforms for hPSC self-renewal and expansion. (A) Culturing hESCs directly on feeder cell layer. Adapted with permission from Ref. [169]. Copyright 2011, InTech. (B) Culturing hESCs on feeder cell layer separated by a porous membrane. Adapted with permission from Ref. [27]. Copyright 2007, Wiley-VCH. (C) Feeder-free 2D culture of hPSCs using substrates coated with natural ECM (*left*), synthetic hydrogel with bioactive motifs from natural ECM (*middle*), fully synthetic hydrogel (*right*). (D) Feeder-free culture of hPSCs using 2D culture dishes treated with oxygen plasma or UV zone. Adapted with permission from Ref. [34]. Copyright 2011, United States National Academy of Sciences.

evidenced by significantly decreased mouse vimentin gene expression in hPSCs.

To fully address issues associated with feeder cells, most recent efforts have been directed toward developing feeder-free, chemically fully-defined 2D culture platforms (Fig. 1C) [28]. A noteworthy achievement was accomplished by functionalizing 2D culture surfaces with either naturally derived proteins or synthetic polymers. For example, the first feeder-free culture system, as reported by Xu et al. [29], applied Matrigel (secreted by Engelbreth-Holm-Swarm (EHS) sarcoma cells and composed of ECM proteins such as laminin, collagen IV, and heparin sulfate proteoglycan) to coat 2D culture surfaces to support hPSC self-renewal in conjunction with MEF conditioned medium (MEF-CM). hPSCs on Matrigel in MEF-CM can maintain a normal karyotype and an undifferentiated and pluripotent state for >130 population doublings (>180 days).

Alternatively, researchers have taken resort to synthetic polymeric materials for feeder-free hPSC culture (Fig. 1C). The first successful strategy is to incorporate active components of natural ECM proteins into synthetic polymers, to mimic native ECM functions and thus support adhesion and self-renewal of hPSCs. For example, bioactive peptide sequences including RGD, DGEA, P15, IKVAV, KRSR, and GROGER are commonly used to construct ECM-mimicking biomaterials [13], among which RGD is the most popular one. Another powerful strategy is to develop completely synthetic polymers without using any animal-derived component, rendering a fully-defined surface biochemistry for hPSC culture. This method was demonstrated recently for supporting long-term self-renewal of hPSCs using synthetic polymers such as aminopropylmethacrylamide (APMAAm) [30], poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA) [31], and poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] (PMEDSAH) [28]. There were some other studies using high-throughput screening techniques to identify optimal combinations of different synthetic polymeric materials and natural ECM proteins to promote hPSC self-renewal [31,32].

In addition to surface functionalization using ECM proteins or synthetic polymers, physical methods have also been adopted for developing novel 2D substrates for hPSC culture (Fig. 1D). Using oxygen plasma-etched tissue culture polystyrene (PE-TCP) surface, Mahlstedt et al. successfully cultured hPSCs in MEF-CM for >10 passages with the cells maintaining a stable karyotype and pluripotency markers (Oct4, SSEA-4, and TRA1-60) [33]. Recently, Saha et al. applied UV ozone radiation to modify TCP surfaces, and such modified TCPs were shown to out-perform feeder-based hPSC culture in terms of supporting hPSC self-renewal [34]. Long-term culture (>10 passages for 2 months) of hPSCs on UV ozone-modified TCPs provided undifferentiated hPSCs with a normal karyotype and robust expression of pluripotency markers (SSEA-4, Sox2 and Nanog) as well as the ability to form teratomas *in vivo*.

2.1.2. 2D platforms for directed differentiation of hPSCs

During embryonic development, cell growth, differentiation, and morphogenesis are spatiotemporally regulated by an intricate and dynamic gene regulatory network as well as local environmental signals. Knowledge of such dynamic gene regulatory network and environmental signals, especially those related to growth factors and cytokines, have been well documented through decades of studies in developmental biology. In the past decade, soluble factors such as retinoic acid (RA), basic fibroblast growth factor (bFGF), bone morphogenetic factor 4 (BMP4), activin A, wnt3a, and vascular endothelial growth factors (VEGFs), have been extensively applied in 2D cultures for directed differentiation of hPSCs into different lineages such as epidermal, neural, cardiac, and vascular cells. For informative discussions about different soluble factors and their effects on *in vitro* hPSC differentiation, the readers are referred to an excellent recent review by Hazeltine et al. [11].

In contrast to soluble factors, ECM proteins constitute the first known family of insoluble factors in the cell microenvironment that can directly regulate the fate and function of hPSCs. Different ECM molecules such as vitronectin, fibronectin, laminin, and collagen have been demonstrated to support hPSC adhesion, proliferation, and differentiation in conventional 2D cultures. Detailed discussions of biological activities of ECM proteins and their roles in controlling hPSC fate and function can be found in excellent reviews published elsewhere [12,35]. Herein, we focus on discussing the emerging theme of controlling extracellular insoluble biophysical signals for regulation of hPSC differentiation (Fig. 2) [13,14,36].

As a measurement of the resistance to deformation, mechanical stiffness of the embryo varies in space and over time during development, generating a distinct pattern of tissue stiffness throughout the human body [37,38]. The interesting idea of using tissue-mimicking matrix stiffness to regulate hPSC differentiation

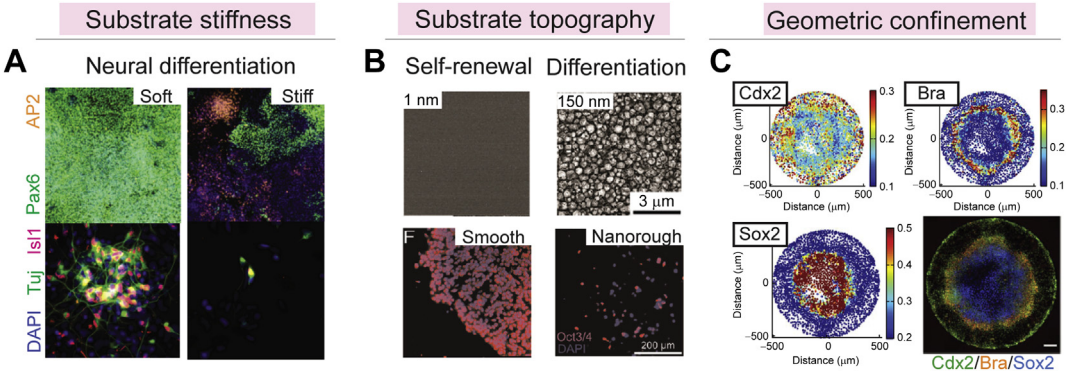

Fig. 2. 2D culture platforms for directing hPSC differentiation *via* modulating substrate physical properties. (A) Promoting neural differentiation of hPSCs using soft elastomeric substrates. Upon neural induction, hPSCs cultured on soft substrates committed highly efficient specification (~90%) towards neuroepithelial lineage (Pax6+, *top left*), while those cultured on stiff substrates exhibited a mixture of neuroepithelial and neural crest (AP2+) differentiations (*top right*). Upon further induction of caudalization and ventralization, soft substrates yielded a much larger amount of motor neurons (β-III-tubulin, Tuj+) than stiff substrates did. Adapted with permission from Ref. [46]. Copyright 2014, Nature Publishing Group. (B) Regulating hESC self-renewal and differentiation *via* substrate nanotopography. While hESCs cultured on smooth glass substrates exhibited strong self-renewal (Oct4+, *red*) (*left panel*), those cultured on nanorough glass surfaces demonstrated enhanced differentiation (*right panel*). Adapted with permission from Ref. [53]. Copyright 2012, American Society of Chemistry. (C) Using geometric confinements to recapitulate cell fate patterning during early embryonic development. When culturing hESC colonies on circular islands and applying external BMP4 stimulation, cells on the outmost ring committed trophectodermal lineage (Cdx2+), while the inner most cells exhibited ectodermal differentiation (Sox2+), with a mesendodermal ring (Bra+) between them. Adapted with permissions respectively from Ref. [58]. Copyright 2014, Nature Publishing Group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

can be originated back to the seminal work by Engler et al., which demonstrated that brain-like soft polyacrylamide (PA) hydrogel substrates enhanced neurogenesis of human mesenchymal stem cells (hMSCs), while PA substrates with muscle- and bone-mimicking stiffness promoted cardiogenesis and osteogenesis, respectively [37]. Using microfabricated elastomeric polydimethylsiloxane (PDMS) micropost arrays with different post spring constants – a physical analogy of varying substrate stiffness, Fu et al. also demonstrated that substrate stiffness could serve as an extracellular switch to direct hMSC differentiation between osteogenesis and adipogenesis [39]. The idea of controlling stem cell differentiation by modulating matrix mechanics has also been recently extended to other types of adult stem cells. Holst et al., for example, showed that only tropoelastin substrates with intermediate elasticity enhanced expansion of human hematopoietic stem cells (hHSCs) [40]. Gilbert et al. reported that instead of rigid substrates such as TCPs, applying softer hydrogels with stiffness close to native muscle elasticity promoted self-renewal of skeletal muscle stem cells (MuSCs) *in vitro* [41]. A very recent study from the same group reported that subjecting the MuSC population from aged mice to transient inhibition of p38-mediated mitogen-activated protein kinase (MAPK) pathway in conjunction with culturing the cells on soft hydrogel substrates rapidly expanded MuSC population and more interestingly, rejuvenated its potential for regeneration and strengthening damaged muscles after serial transplantation in aged mice [42].

Effect of matrix rigidity on hPSC behaviors has not been examined explicitly until recently. Different groups including our own have lately investigated whether the survival, self-renewal, and differentiation of hPSCs are regulated by substrate rigidity. For example, we utilized the PDMS micropost array system to show that substrate rigidity plays a significant role in regulating pluripotency of hPSCs, as a significantly higher percentage of hPSCs after culture on rigid PDMS microposts coated with vitronectin for 24 h remained as undifferentiated Oct4+ cells compared to cells on soft PDMS microposts [43]. Similarly, the study by Musah et al. using a modified PA gel system functionalized with adhesive peptide GKKQRFRHRNRKG derived from vitronectin also showed that only rigid PA gels but not soft ones maintained hPSC proliferation and pluripotency [44]. Interestingly, another study by Keung et al. using Matrigel coated PA gels showed that substrate rigidity had no significant effect on proliferation or expression of pluripotency markers for hPSCs after culture for 3 d under a self-renewal medium condition. By using a neurogenic culture protocol, Keung et al. further demonstrated that soft PA substrates promoted neuroepithelial differentiation of hPSCs [45]. A more convincing recent study from our group further utilized soft PDMS micropost arrays to accelerate neural induction and caudalization of hPSCs (Fig. 2A). Excitingly, the purity and yield of functional motor neurons derived from caudalized, hPSC-derived neural progenitor cells obtained from soft PDMS micropost arrays were improved four- and twelve-fold, respectively, compared to coverslips or rigid PDMS micropost arrays [46]. Mechanistic studies revealed a multi-targeted mechanotransductive process involving Smad phosphorylation and nucleocytoplasmic shuttling, regulated by rigidity-dependent Hippo-YAP activities and actomyosin cytoskeleton (CSK) integrity and contractility [46]. Together, these studies reveal a functional synergy between biophysical and biochemical cues in the cell microenvironment providing a localized autonomous control of hPSC fate and function. Different observations between our group and Musah et al. and Keung et al. on the rigidity effect on hPSC pluripotency maintenance suggest that rigidity sensing by hPSCs may critically depend on the specific cell adhesion molecules employed by hPSCs to bind surrounding ECM proteins. Different observations from these studies also support the importance in recognizing differences in molecular-scale material properties (such as porosity, surface chemistry, molecular backbone flexibility, and binding properties of immobilized adhesive ligands) when comparing different synthetic substrates and hydrogel systems. Recent studies using synthetic hydrogels have supported that certain molecular-scale variations in material properties can have profound effects on hPSC behaviors [32].

Another exciting new trend is to develop novel hydrogel systems with temporally modulated mechanical stiffness to modulate stem cell behaviors. In recent studies, dynamic substrate stiffening was shown to either switch hMSCs from adipogenesis to osteogenesis [47] or promote their cardiogenic differentiation [48].

In addition to biomimicry of tissue mechanical stiffness, researchers have also developed different bioengineering strategies to recapitulate *in vivo* ECM micro/nanoscale topography and architecture onto 2D culture surfaces. A notable study by Dalby et al. showed that synthetic substrates with disordered nanoscale topographical features enhanced osteogenesis of hMSCs [49]. Substrates with highly aligned textures (*e.g.*, microengineered grooves and electrospun fiber arrays) were utilized to enhance neuronal differentiation of murine ESCs as well as neural progenitor cells [50,51]. Substrates composed of nanotubes were also applied for stem cell culture, with results demonstrating that hMSC osteogenesis was promoted on nanotubes with greater diameters [52]. By using reactive ion etching-generated nanorough coverslips, our group demonstrated nanoroughness-dependent adhesion, spreading, and self-renewal of hPSCs (Fig. 2B) [53].

Cell shape is another important cell property dynamically regulated during embryonic development. Indeed, numerous studies have supported cell shape as a potent biophysical regulator of stem cell fate and function. In one of the earliest studies, McBeath et al. applied microcontact printing to coat flat PDMS surfaces with distinct patterns of adhesive ECM islands and reported that single hMSCs confined to small ECM islands selectively underwent adipogenesis, whereas single hMSCs on large ECM islands were biased toward osteogenesis [54]. More recent studies further revealed that in addition to cell shape, cell geometry could also influence hMSC differentiation. hMSCs of elongated morphology preferred osteogenesis, whereas those with a low aspect ratio morphology were biased toward adipogenesis [55]. Similarly, Lee et al. observed on soft PA substrates that a confined cell shape would promote hMSC adipogenesis, whereas a spread, branching cell morphology promoted hMSC neurogenic differentiation [56].

Direct investigation on the effect of cell shape on hPSC fate and function is not as straightforward as those reported for adult stem cells, given the rapid proliferative nature of hPSCs. Peerani et al. patterned hPSC colonies onto adhesive ECM islands with defined colony diameters, with results showing that larger hPSC colonies with a high cell density promoted pluripotency maintenance through a niche size-dependent spatial gradient of BMP-mediated Smad1 signaling, which was generated through antagonistic interactions between hPSCs and hPSC-derived extraembryonic endoderm [57]. Another study by Chambers et al. showed that cell seeding density had a direct effect on neuroepithelial (high seeding density) *vs.* neural crest (low seeding density) differentiation of hPSCs [16]. Very recently, using patterned adhesive ECM islands, Warmflash et al. recapitulated spatial cell fate patterning during early embryonic development by leveraging the self-organization of differentiating hPSCs (Fig. 2C) [58]. In their study, when cultured on circular adhesive islands, hPSCs exhibited patterned pluripotency marker expression, with greater expression in cells closer to colony border. Upon further induction by extracellular BMP4, hPSCs spontaneously self-organized into “germ-layer-like” structures, with a trophectoderm-like ring (Cdx2+) on the outmost edge, a mesendodermal layer (*i.e.*, endodermal/mesodermal

mixture; Sox17+/Brachyury, Bra+) next to it, and an inner circular ectodermal layer (Sox2+). The authors further reported that spatial patterning of hPSC fate and corresponding Smad signaling were defined relative to the outer boundary of cell colony with a fixed length scale.

2.2. 3D hPSC culture platforms

2D hPSC culture platforms have been proven effective in supporting long-term self-renewal and large-scale expansion of hPSCs as well as directed hPSC differentiation toward specific lineages. However, it is arguable that a 3D culture system mimicking *in vivo* architecture and biological roles of the ECM may allow recapitulation of embryonic development *in vitro* to a degree of complexity not achievable in a 2D culture system. Recently, there are significant efforts in developing innovative 3D biomaterial systems for hPSC culture and modeling human development and disease *in vitro* [15]. Here we limit our discussions to methods using embryoid body (EB) and 3D biomaterial scaffolds for 3D hPSC culture. For detailed discussions about biomaterial properties and their specific, independent effects on expansion of undifferentiated hPSCs, directed hPSC differentiation, and *in vivo* transplantation of differentiated hPSCs, the readers are referred to an excellent recent review by Kraehenbuehl et al. [59].

2.2.1. Embryoid body-based 3D culture

Embryoid bodies (EBs) are 3D hPSC aggregates (Fig. 3A). As formation and differentiation of EBs recapitulate many hallmarks of early embryonic development, EB-based hPSC culture has been used extensively for studying hPSC fate regulation [15]. For example, in EBs, it is common to observe regional expression of embryonic markers specific to the three germ lineages - mesoderm, ectoderm, and endoderm. Compared to 2D adherent culture, EB-based hPSC culture is inherently more scalable for biofabrication of hPSCs. Additionally, 3D structure of EBs with accompanying intricate cell–cell and cell-ECM interactions enables patterned and structured cell differentiation and morphogenesis, yielding microtissues similar to native tissue structures. Such microtissues are promising for regenerative medicine, development and disease studies, and *in vitro* drug and toxicology screening.

In the past years, there are significant progresses using micro/nanoengineering tools to control EB size or to incorporate into EBs polymeric microparticles (MPs) loaded with growth factors. Such functional MPs can be dynamically and remotely controlled for growth factor release. Bauwens et al., for example, used microcontact printing to pattern Matrigel islands onto TCPs to generate EBs with a uniform size [60]. Using such a strategy, Bauwens et al. showed that neural *vs.* cardiac differentiation of hPSCs in EBs depended on EB size. Such micropatterning technique was also recently applied for controlling EB size for large-scale hPSC production using bioreactors [61].

Instead of using micropatterning, Mohr et al. developed 3D microwell structures of different sizes (with a lateral dimension of 100–500 μm and a height of 120 μm) to control EB size [62]. The highest percentage of contracting EBs undergoing cardiogenesis was observed in microwells of an intermediate size (300 μm). More recently, Hwang et al. applied a similar strategy to generate EBs of defined sizes and observed a functional role of the non-canonical Wnt pathway in regulating EB size-dependent hPSC differentiation. Specifically, EBs of larger sizes (450 μm in diameter) preferentially underwent cardiogenic differentiation, whereas EBs of smaller sizes (150 μm in diameter) exhibited enhanced endothelial differentiation (Fig. 3B) [63].

Alternatively, researchers have attempted to control hPSC differentiation in EBs *via* modulating local extracellular biochemical signals. To this end, Bratt-Leal et al. incorporated polymeric MPs composed of biomaterials such as agarose, poly(lactic acid-co-glycolic acid) (PLGA), and gelatin and loaded with soluble factors such as RA and BMP4 into EBs (Fig. 3C) [64]. In response to these functional MPs, hPSCs maintained their viability and exhibited distinct gene and protein expression patterns depending on specific soluble factors and their dosages loaded in MPs.

2.2.2. Hydrogel-based 3D encapsulation culture

Various hydrogels based on native ECM molecules including Matrigel, collagen, chitosan, hyaluronic acid (HA), and alginate and synthetic hydrogels derived from polymers such as polyethylene glycol (PEG), polyester (PLA), PLGA, and poly(glycerol sebacate) (PGS) have been used for 3D hPSC culture [65,66]. While natural hydrogels can retain biological activities of native ECM molecules, they suffer from batch-to-batch viability and poor ability for biochemical modification. In addition, clinical application may be limited for natural hydrogels by the risk of immunogenicity and disease transfer. In contrast, synthetic hydrogels are fully defined in chemistry and more amenable for biochemical functionalization (for example, with growth factors, ECM adhesive motifs, and

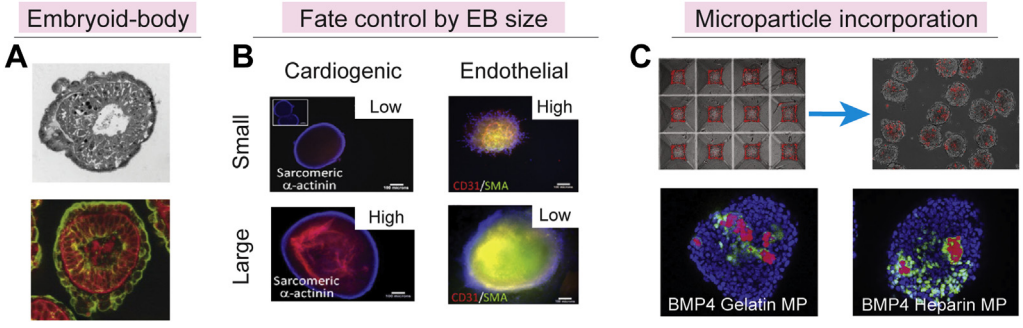

Fig. 3. 3D embryoid body (EB) culture platforms for regulating hPSC fates. (A) Microtome (*top*) and immunofluorescence (*bottom*) micrographs of an embryoid body (EB), which was formed by ESC aggregates. Adapted with permission from Ref. [170]. Copyright 2001, The Rockefeller University Press. (B) Controlling EB fate in 3D by controlling its size. It has been demonstrated that smaller EBs (*top panel*) exhibited lower cardiogenic differentiation but stronger endothelial differentiation, while larger EBs (*bottom panel*) preferred cardiogenesis over endothelial differentiation. Cardiogenic and endothelial differentiations were visualized by sarcomeric α-actitin staining (*left panel*, *red*) and CD31 (*right panel*, *red*), respectively. Adapted with permission from Ref. [63]. Copyright 2009, United States National Academy of Sciences. (C) Regulating hPSC differentiation within EBs by incorporating soluble factor-loaded polymeric microparticles (MPs). (top panel) Incorporating MPs during EB formation within microwell arrays. MPs were fluorescently labeled (*red*) for visualization. (bottom panel) BMP4-loaded gelatin (*left*) and heparin (*right*) MPs (*red*) resulted in different patterns of early mesodermal induction (visualized by GFP Brachyury-T reporter, *green*) within EBs. Adapted with permission from Refs. [64] and [85]. Copyright 2011 and 2013, Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

specific molecules agonistic or antagonistic to cell surface receptors), biophysical modulations (*e.g.*, mechanical stiffness, pore size, and 3D architecture), and mimicking key degradation characteristics [65,66]. Synthetic hydrogels also have a lower risk for immunogenic reactions.

So far, attempts of using 3D hydrogels for maintaining and expansion of hPSCs have utilized both naturally derived and synthetic hydrogels. Gerechet et al., for example, developed a methacrylated HA hydrogel system to promote self-renewal of hPSCs [67]. hPSCs encapsulated in 3D HA scaffold maintained an undifferentiated state till soluble factors inductive for hPSC differentiation were added to culture. Li et al. reported another 3D scaffold composed of chitosan and alginate to support hPSC self-renewal for >21 days [68]. Similarly, Stenberg et al. developed a 3D agarose system for culturing spherical hPSC colonies [69]. This 3D agarose culture platform promoted EB formation and significantly enhanced hPSC growth. In another more recent study, Siti-Ismail et al. successfully cultured hPSCs encapsulated in alginate hydrogels for >260 days in a feeder-free environment without any enzymatic treatment, mechanical expansion, or manipulation [70].

In addition to natural hydrogels, synthetic polymers with multifunctionalities have also been developed for promoting hPSC self-renewal in 3D culture. A notable example was demonstrated by Lei et al. using a fully-defined hydrogel system composed of poly(N-isopropylacrylamide)-co-poly(ethylene glycol) (PNIPAAm-PEG), which is liquid at a low temperature (<4 °C) but can solidify into an elastic hydrogel at >37 °C [71]. This thermoreversible hydrogel system provided a simple yet efficient system for hPSC culture, passaging, harvesting, and differentiation. Excitingly, hPSCs cultured in this system showed a significantly greater growth rate compared to those reported for other hydrogel systems. After culturing for 60 passages (~280 days), an overall hPSC expansion about 1072-fold was achieved, with about 95% of cells remaining Oct4+ and maintaining differentiation potential toward specific adult lineages under inductive conditions.

Studies of directed differentiation of human stem cells including hPSCs in 3D culture have only emerged in recent years (Fig. 4) [72]. By embedding murine MSCs (mMSCs) in alginate hydrogels of different mechanical stiffness, Huebsch et al. observed that consistent with 2D culture studies, soft matrix promoted adipogenesis. Intermediate stiffness (11–30 KPa) instead of very high stiffness (100 KPa), however, was observed for maximal osteogenesis (Fig. 4A) [73]. Integrin-mediated cell adhesion and cellular traction force were suggested to play an important role in rigidity-dependent mMSC differentiation in 3D. Another important property of hydrogels, biodegradability, has also been harnessed to regulate stem cell fate in 3D. The Anseth group reported a metalloproteinase (MMP) degradable PEG-hydrogel for 3D culture of hMSCs [74]. Interestingly, hMSCs encapsulated in MMP-degradable PEG-hydrogels and cultured in osteogenic, chondrogenic, or adipogenic differentiation medium expressed increased levels of specific differentiation markers as compared to cells in hydrogels that were not cell-degradable. Khetan et al. developed another HA-based hydrogel that supported MMP-mediated degradation but lost such biodegradability after UV-initiated covalent crosslinking [75]. Using this system, Khetan et al. observed that matrix degradation, instead of ECM mechanics or cell morphology, directed osteogenic *vs.* adipogenic fates of hMSCs in 3D (Fig. 4B). In another recent work, Kloxin et al. reported a strategy to generate 3D photodegradable hydrogels with dynamically tunable physical and chemical properties [76]. Using this photodegradable hydrogel to cleave RGD ligands during the early stage of hMSC differentiation, Kloxin et al. determined that binding of integrin receptors with RGD ligands was required for integrin αvβ3 expression, and downregulation of αvβ3 promoted chondrogenic differentiation of hMSCs in 3D (Fig. 4C). More recent studies from Kloxin, Anseth and colleagues further demonstrated simultaneous orthogonal modulation of hydrogel degradation and biochemical modifications in 3D hydrogels [65,77]. In another study by Lim et al., an interesting approach for dynamic modulation of both mechanical and electrical cues was devised to expose hMSCs with a continuous electric field to enhance chondrogenesis [78].

Levenberg et al. reported the first study to use biodegradable PLLA/PLGA scaffolds for promoting hPSC growth and differentiation and formation of 3D structures [72]. Differentiation and organization of hPSCs were influenced by the scaffold and directed by growth factors known to regulate hPSC differentiation into the three germ layers. Importantly, differentiating hPSCs under the influence of different growth factors formed 3D structures with

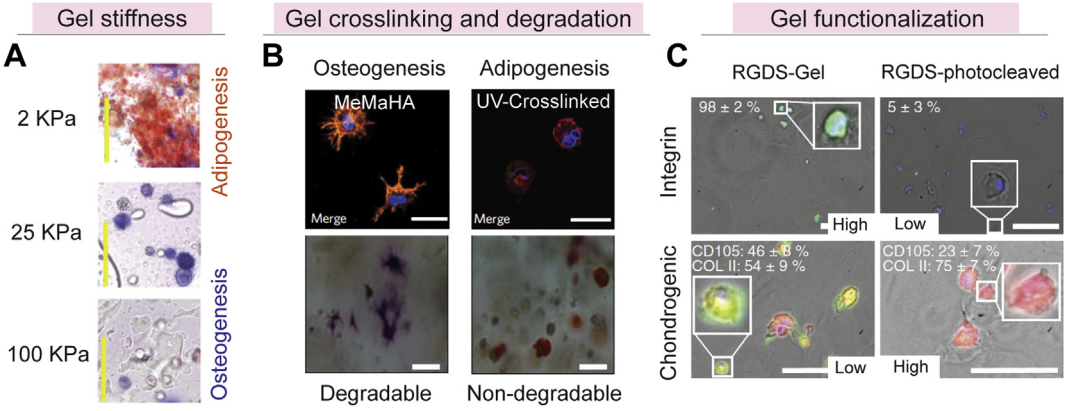

Fig. 4. Modulating 3D hydrogel biomaterial properties for controlling MSC fates in 3D: A lesson for 3D hPSC engineering. (A) Matrix stiffness-dependent differentiation of mMSCs within 3D hydrogels. Soft hydrogel enhanced adipogenesis (*red*, lipid droplet staining), while hydrogel of intermediate stiffness ~25 KPa induced optimal osteogenesis (*blue*, ALP staining). Reproduced with permission from Ref. [73]. Copyright 2010, Nature Publishing Group. (B) Controlling hydrogel-encapsulated hMSC fates by gel crosslinking and degradation. It has been demonstrated that while MMP-degradable MeMaHA hydrogel promoted branching morphology (*top left*) and osteogenesis (*bottom left*, ALP staining, *blue*) of hMSCs embedded within a 3D microenvironment, UV-crosslinking that rendered it non-degradable could apply spatial confinement to cells (*top right*) and switch the inductive potential to enhance adipogenesis (*bottom right*, lipid droplet staining, *red*). Adapted with permission from Ref. [75]. Copyright 2013, Nature Publishing Group. (C) Regulating the adhesion and chondrogenesis of hMSCs within 3D hydrogel by photo-mediated biochemical modifications. By cleaving cell adhesive peptides RGDS from the PEG-based hydrogel at early stage of hMSC differentiation, it was found that the downregulated surface integrin (*top panel*, *green*) could promote the chondrogenesis (*red*) over the self-renewal (*green*) of hMSCs (*bottom panel*). Adapted with permission from Ref. [76]. Copyright 2009, American Association for the Advancement of Science. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

characteristics of developing neural tissues, cartilage, liver, and blood vessel, underscoring the importance of 3D culture for development and disease studies using hPSCs. More recent studies further suggested that 3D culture provided a more favorable environment for directed hPSC differentiation compared to EB-based methods. For example, by embedding EBs in 3D collagen gels instead of culturing on 2D surfaces, Baharvand et al. demonstrated enhanced induction of hepatocytes with proper morphological, gene expression, and metabolic characteristics [79]. In a separate study, Ferreira et al. achieved significant improvement over EB-based cultures by developing a bioactive dextran-based hydrogel system containing RGD peptides and microencapsulated $VEGF_{165}$ to enhance hPSC differentiation toward vascular cells [80].

The concept of "switchable hydrogel" was recently applied to introduce dynamic regulation of 3D hPSC culture, as the conditions required for self-renewal *vs.* differentiation of hPSCs are very different, and a single system that efficiently achieves both outcomes will be desirable for basic biological studies of hPSCs [81]. To this end, Dixon et al. developed a combined alginate-collagen hydrogel, which could switch from alginate-dominated to collagen-dominated chemistry after a chelation treatment and removal of alginate from the hydrogel. Using this switchable hydrogel, Dixon et al. demonstrated a transition from self-renewal permissive-environment to differentiation-permissive environment for hPSCs, mimicking dynamic environmental change during early embryonic development. Excitingly, adjusting timing of the switch could preferentially steer hPSC differentiation to mimic lineage commitment during gastrulation to ectoderm (early switch) or mesoderm/endoderm (late switch).

It should be noted that even though 3D hydrogel encapsulation culture may mimic more closely native 3D tissue environment, there are some limitations that require carefully consideration. Generating perfusable vasculature networks in 3D hydrogels is still nontrivial, without which 3D tissue constructs are limited to a millimeter size range due to diffusion limitation [59]. Currently, there is also a lack of fundamental understanding of cell–biomaterial interactions in a 3D environment, limiting rationale designs of 3D hydrogel systems for hPSC culture. In addition, retrieval of hPSCs and their derivatives from 3D culture where hPSCs are encapsulated in non-degradable hydrogels can be a practical concern for hPSC-based applications.

3. Engineering 3D microtissues and organoids *in vitro*: leveraging imposed or spontaneous cellular organization

Under appropriate conditions, the intrinsic developmental potential of hPSCs allow the cells to grow, differentiate, and self-organize into complex structures, with emergent functionalities and architectures similar to those *in vivo* tissues and organs. Indeed, there is an exciting new trend of developing 3D hPSC culture systems for generating functional microtissues and organoids, for studying embryonic development and organogenesis and applications in drug and toxicology screening [82,83]. This emerging trend offers promising opportunities for cell biologists, bioengineers, and materials scientists to collaborate closely to develop novel 3D biomaterial systems to control hPSC fate, function, and organization mimicking *in vivo* tissues and organs.

3.1. Manipulating hPSC differentiation in EBs using controlled biochemical stimulation

As a common 3D culture method for hPSCs, EB usually results in heterogeneous and disorganized differentiation of hPSCs, owing to diffusion limitation and resulting inhomogeneous distribution of soluble factors in the EB. To address this, the McDevitt group synthesized MPs with degradable biomaterials (*e.g.*, PLGA, agarose, and gelatin) and loaded them with potent soluble factors, before incorporating the MPs uniformly into EBs [15,64,84,85]. Soluble factors could be released gradually to the extracellular space within the EB and thus create a uniform biochemical environment not limited by diffusion. By using PLGA MPs loaded with RA, homogeneous and organized cell differentiation appeared in the EB, wherein a cystic morphology was formed with a FoxA2+ exterior envelop surrounding a Oct4+ epiblast-like layer (Fig. 5A) [84].

In addition to using functionalized degradable MPs to control biochemical stimulation in EBs, Fung et al. employed an asymmetric microfluidic channel to achieve spatial delivery of soluble signals to EBs and thus regulate hPSC differentiation in 3D (Fig. 5B) [86]. In a microfluidic device with an asymmetric Y-shaped channel, two separate streams of medium containing different chemical factors were simultaneously delivered to the two halves of EBs immobilized in the channel. Using this method, asymmetric hPSC states in EBs was successfully demonstrated, with one half undergoing neural differentiation while the other maintaining an undifferentiated state.

3.2. Microtissue fusion for building hybrid constructs

Two juxtaposed cell aggregates can fuse into one owing to actively regulated cell–cell adhesive interactions and cell migration. As a bottom-up approach, this phenomenon has been explored to create different structured microtissues as well as heterotypic spheroid co-cultures. Bratt-Leal et al., for example, applied microtissue fusion to integrate one murine ESC EB incorporated with morphogen-loaded MPs with another blank EB using a gravitation-based mechanism (Fig. 5C) [85]. By releasing BMP4 locally from herapin-gelatin MPs in one half of fused EBs, the authors demonstrated local mesoderm induction within fused EB.

Microtissue fusion can also be achieved by magnetic force-driven tissue assembly [87]. By incorporating magnetic MPs into murine ESC aggregates, Bratt-Leal et al. demonstrated facile manipulations of murine ESC aggregates, leading to fabrication of hybrid structures in an additive manner (Fig. 5D) [88]. Using a patterned magnetic field, Bratt-Leal et al. further demonstrated versatile, patterned organizations of murine ESC aggregates at a macroscopic scale. However, applications of these methods for building functional microtissues and organoids from hPSCs still need to be explored in the future.

3.3. Physically structuring hPSC-derived tissues for functional maturation

There is exciting recent progress in controlling organizations of hPSCs or hPSC-derived cells in 3D for human microtissue fabrication and functional maturation *via* imposed external physical constraints. Here we focus our discussion on some illustrative examples of building human cardiac tissues *in vitro* using hPSC-derived cardiomyocytes (hPSC-CMs) in conjunction with externally assisted physical structuring.

In the method developed by Schaaf et al., two silicone posts were inserted into each well of a 24-well plate filled with hPSC-CMs and ECM proteins [89]. hPSC-CMs actively interacted with ECM proteins to self-assemble into microtissues with the two ends of the microtissues anchored to silicone posts. Spontaneous contraction of hPSC-CM tissues was restricted in a uniaxial fashion by the two silicone posts and thus resulted in uniaxially elongated microtissue morphology (Fig. 6A). Interestingly, such hPSC-CM microtissue displayed highly ordered cellular alignment and intracellular structural features as well as electrophysiological activities comparable to mature human myocardium. More recently, Cvetkovic

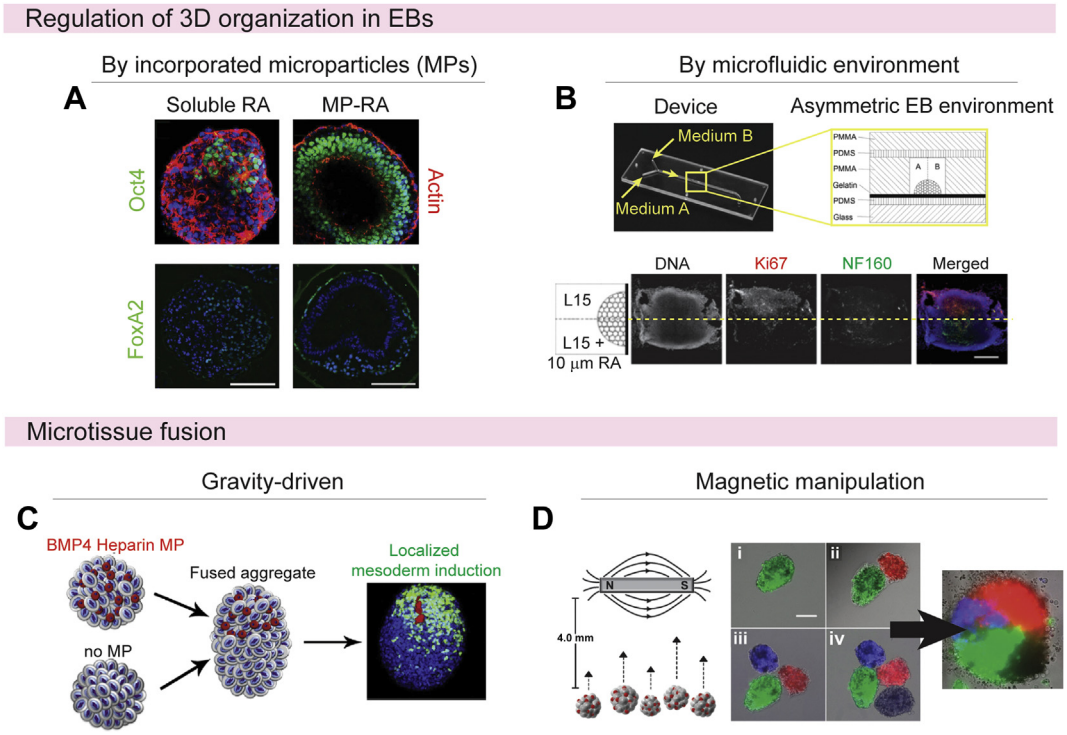

Fig. 5. Engineering the 3D cell organization within hPSC aggregates. (A) Inducing cystic morphology and visceral endoderm envelop-epiblast bilayer (FoxA2/Oct4) structures within EBs by incorporating PLGA MPs loaded with RA (*right panel*), which could not be recapitulated by simply culturing EBs within RA-supplemented medium (*left panel*). Adapted with permission from Ref. [84]. Copyright 2009, Elsevier. (B) Controlled heterogeneous differentiation within EBs by delivering asymmetric extracellular soluble cues using asymmetric microfluidic channels (*top panel*). When delivering L15 medium to one half of the EB and L15 supplemented with RA to the other half, a heterogeneous pattern of cell differentiation appeared within the EB, as visualized by the differential expression of cell proliferation marker (Ki67, *red*) and neurofilament marker (NF160, *green*) within different halves of the EB (*bottom panel*). Adapted with permission from Ref. [86]. Copyright 2010, Royal Society of Chemistry. (C) By fusing an ESC aggregate incorporated with BMP4-loaded heparin MPs (*red*) with a blank ESC aggregate, it created a hybrid microtissue expressing highly localized early mesoderm induction (*green*) co-localized with the MPs. Adapted with permission from Ref. [85]. Copyright 2013, Elsevier. (D) By incorporating magnetic microparticles into the ESC aggregates, it could achieve direct manipulation of those aggregates using specifically designed magnetic field (*left*). Through magnetic manipulation and fusion at single aggregate resolution, it could assemble a hybrid microtissue containing multiple parts of different properties (*right*, schematized using different colors). Adapted with permission from Ref. [88]. Copyright 2011, Royal Society of Chemistry. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al. further demonstrated applications of such bioengineered cardiac tissues as soft robotic devices [90].

Several other hPSC-derived cardiac microtissues were developed using a similar method. For example, in the "cardiac-patch" developed by Zhang et al., a 3D fibrin-based network structure was fabricated to locally confine hPSC-CMs within each segment of the network, resulting in locally aligned CMs in a biomaterial network of macroscopic scale (Fig. 6B) [91]. hPSC-CMs in the 3D cardiac-patch exhibited significantly higher conduction velocities, longer sarcomeres, and enhanced expression of genes associated with cardiac contractile function, including cTnT, αMHC, CASQ2, and SERCA2, as compared to hPSC-CMs cultured as 2D monolayers. Moreover, 3D cardiac-patches exhibited significant positive inotropy with isoproterenol treatment, demonstrating highly advanced levels of hPSC-CM maturation.

In a more recent study by Nunes et al., a miniaturized "biowire" cardiac tissue model was developed through self-assembly of hPSC-CM microtissues around a single surgical suture template [92]. Mixed with stromal cells in type I collagen gel, hPSC-CMs spontaneously assembled and wrapped around the linear suture template, resulting in robust myofibrillar alignment as well as enhanced contractile phenotype, mimicking features seen in human heart (Fig. 6C). The small thickness of the "biowire" cardiac tissue also allowed efficient nutrient diffusion into the tissue. More importantly, the "biowire" cardiac tissue could be conveniently stimulated electrically. Such electrical stimulation led to markedly increased myofibril ultrastructural organization, elevated conduction velocity, and improved electrophysiological and Ca^{2+} handling properties of the "biowire" cardiac tissue compared to nonstimulated controls.

In another study, Thavandiran et al. developed both dog-bone and circular shaped templates to form highly aligned hPSC-CM microtissues (Fig. 6D) [93]. Self-assembly of aligned 3D hPSC-CM tissues in the templates, accompanied with paced electrical stimulations, promoted gene expression associated with cardiac maturation and *in vivo*-like electrical signal propagation. Thavandiran et al. further applied this hPSC-CM microtissue platform to establish a tachycardiac model of arrhythmogenesis, underscoring translational potential of such 3D hPSC-CM microtissues for *in vitro* patient-specific disease modeling. In a more recent study, using a template with similar geometries and resident muscle stem cells, Juhas et al. demonstrated that engineered muscle tissue exhibited improved vascular integration and functional maturation *in vivo* after transplantation into mice [94].

Recently, Ma et al. fabricated a 3D parallel filamentous array using two-photon initiated polymerization (TPIP) for a UV curable organic-inorganic hybrid polymer, as linear geometric templates for guiding assembly and alignment of hPSC-CMs [95]. Using iPSCs derived from patients with long QT syndrome type 3, Ma et al. generated disease-specific 3D cardiac tissues and studied their contractility malfunction associated with electrophysiological consequences of LQT3 syndrome and susceptibility to drug-specific cardiotoxicity.

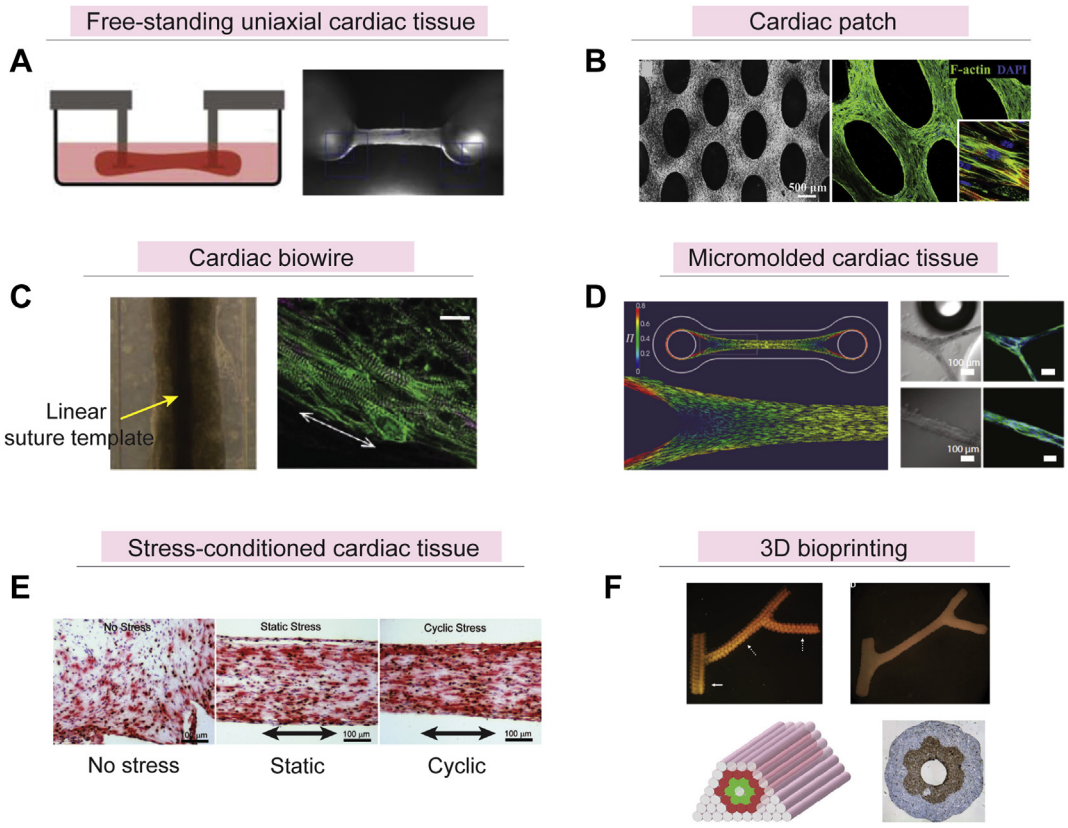

Fig. 6. Engineering 3D cell organization and functinal maturation within hPSC-derived microtissues. (A) Fabrication of a free-standing uniaxial cardiac tissue by using geometrical restraints applied by two elastomeric posts. During the gelation and spontaneous contraction of cardiomyocyte-laden hydrogel, it embedded the elastomeric posts and formed a strip of microtissue in between. As templated by the anisotropic hydrogel formation and remodeling process, cardiomyocytes embedded within the microtissue preferentially aligned with the longitudinal axis. Adapted with permission from Ref. [89]. Copyright 2011, Public Library of Science. (B) Fabrication of a 3D cardiac-patch using 3D molded hydrogel networks. Due to the geometric confinement, embedded hPSC-derived cardiomyocytes preferentially aligned with each ligand of the network. Adapted with permission from Ref. [91]. Copyright 2013, Elsevier. (C) A cardiac biowire model, wherein a linear suture template was used to geometrically template the formation of miniaturized cardiac tissue around it. Due to the geometric guidance of the linear template, embedded cardiomyocytes preferentially aligned with the axis of the wire. Adapted with permission from Ref. [92]. Copyright 2013, Nature Publishing Group. (D) A microscale cardiac tissue model fabricated by specifically designed micromolds. By culturing and forming the microtissue within a dog-bone shaped micromold, a thin wire of tissue was generated and anchored at both ends, creating an anisotropic tension and ECM remodeling. Simulations (*left*) and experimental results (*right panel*) both indicated strong alignments of cardiomyocytes and sarcomeric filaments within the microtissue. Adapted with permission from Ref. [93]. Copyright 2013, United States National Academy of Sciences. (E) External directional stresses, either static or cyclic, could also be used to condition the alignment and maturation of hPSC-derived cardiomyocytes within a microtissue. Adapted with permission from Ref. [96]. Copyright 2011, American Heart Associations. (F) Using 3D bioprinting based on micromolded build blocks, *e.g.*, spherical (*top left*) and filamentous (*bottom left*) microtissues, it could fabricate *in vitro* tissue models at a larger length scale with more complex internal and external structures, such as the branching morphology (*top right*) and multi-layer architecture (*bottom right*) mimicking vascular vessels. Adapted with permission from Ref. [98]. Copyright 2009, Elsevier.

It should be noted that in addition to templating microtissue structures using pre-defined physical constraints, mechanical conditioning has also been demonstrated useful for developing mature 3D cardiac tissues from hPSC-CMs [96,97]. For example, both static and cyclic stretches were shown to promote CM and matrix fiber alignment and enhance myofibrillogenesis, sarcomeric banding, and CM hypertrophy and proliferation in 3D cardiac tissues (Fig. 6E) [96]. Co-culture with endothelial cells in mechanically conditioned 3D cardiac tissues further promoted formation of vessel-like structures [96]. Importantly, when transplanted onto hearts of athymic rats, such hPSC-CM microtissues survived and formed grafts closely apposed to host myocardium containing human microvessels perfused by host coronary circulation [96].

3D bioprinting has shown great promise for biofabrication of artificial tissues and organs. Using special printing ink (or "modules") (Fig. 6F) [98], 3D bioprinting was successfully applied for template-free fabrication of 3D tissues with complex structures mimicking native tissues such as aortic vascular vessels. It is foreseeable that 3D bioprinting will become a powerful approach in the near future for rapid prototyping and generation of hPSC-derived artificial tissues.

3.4. Spontaneous cell patterning and organization in 3D organoids

Given their developmental potential, human stem cells including hPSCs can spontaneously develop into organized cytosystems in 3D culture mimicking human tissues and organs *via* autonomous spatiotemporal cell patterning and organization. In recent years, researchers have achieved significant progress exploring intrinsic developmental potential of hPSCs for generating novel 3D organoids with cell structures and functions comparable to native tissues and organs.

Sasai and colleagues made a seminal contribution to this field by generating the first organized neural cytosystems *in vitro*. Using serum-free culture of EB-like aggregates (SFEB), Sasai and colleagues recapitulated 3D optic cup morphogenesis during eye development using hESCs (Fig. 7A) [99]. In another work from Sasai and colleagues, hESCs were inducted toward neuroepithelial lineages using the SFEB method, and cortical tissues exhibiting distinct zones along the apical-basal direction (*e.g.*, ventricular, early and late cortical plate, and Cajal-Retzius cell zones) were generated (Fig. 7B) [100]. Interestingly, layer-specific differentiation of neural cells in such cortical tissue organoids followed a temporally

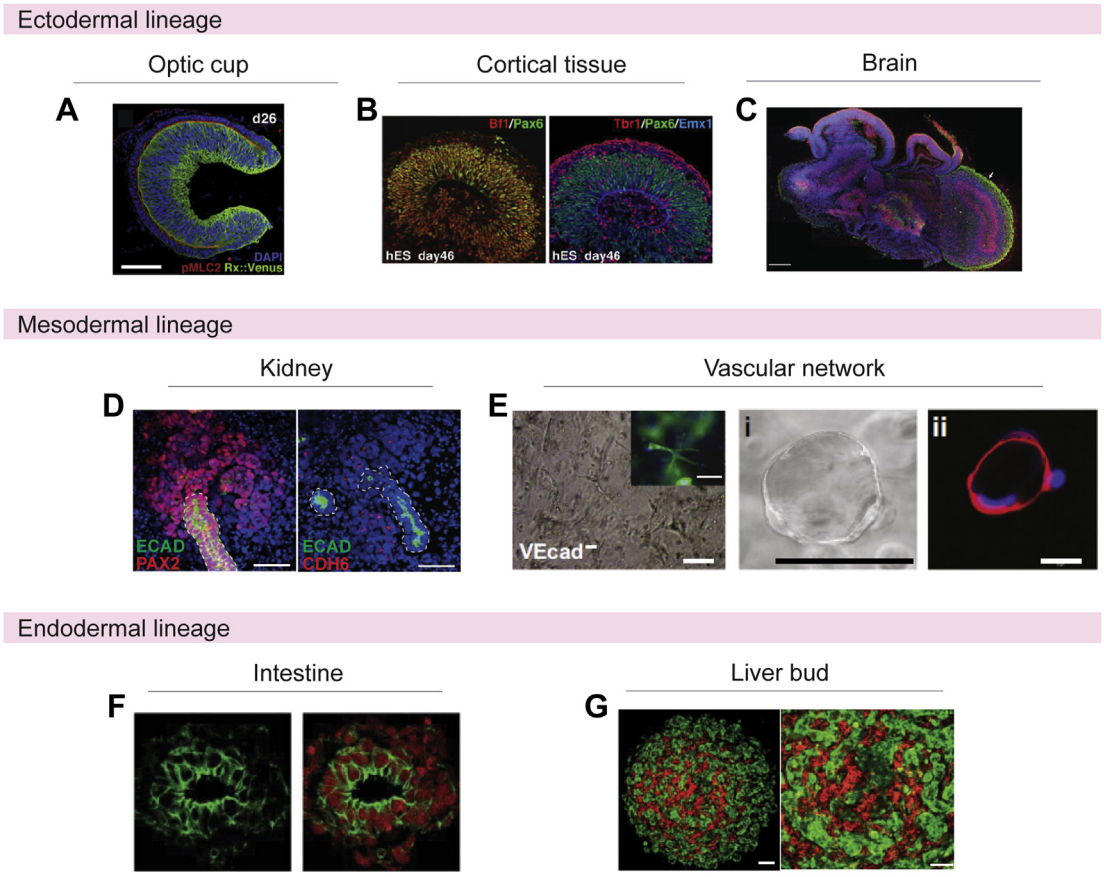

Fig. 7. Generation of 3D organoids from hPSCs. (A) A human optic cup model generated from hESCs. Adapted with permission from Ref. [99]. Copyright 2012, Cell Press. (B) A human cortical tissue model derived from hESCs, showing self-organized multi-layer cortical tissue structures. Adapted with permission from Ref. [100]. Copyright 2008, Cell Press. (C) Generation of human cerebral organoids from hESCs and iPSCs. Adapted with permission from Ref. [102]. Copyright 2013, Nature Publishing Group. (D) Creation of human kidney organoids using hESCs. It shows ureteric epithelial (UE) structures (Pax2+) surrounded by early nephrons as illustrated by CDH6+ cells. Adapted with permission from Ref. [104]. Copyright 2014, Nature Publishing Group. (E) Induction of early vascular cells and formation of 3D vascular network from hPSCs in a synthetic matrix. The phase image of the vascular network and a close-up image of the vascular vessel cross-section were shown here. Adapted with permission from Ref. [105]. Copyright 2013, United States National Academy of Sciences. (F) A human intestine organoid model established from hPSCs showing the formation of hindgut-like spheroid with lumen. Adapted with permission from Ref. [107]. Copyright 2011, Nature Publishing Group. (G) 3D human liver buds generated by co-culturing hiPSC-derived hepatic endoderm cells (iPSC-HEs, *green*), hMSCs (*black*) and human umbilical endothelial cells (HUVECs, *red*). Adapted with permission from Ref. [109]. Copyright 2013, Nature Publishing Group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

controlled pattern mimicking corticogenesis *in vivo*. Recently, Mariani et al. extended the SFEB method to hiPSCs and generated human cortical organoids mimicking developing human telencephalon [101].

In another recent exciting study, Knoblich and colleagues successfully applied 3D hPSC culture to generate cerebral organoids with distinct brain regions (Fig. 7C) [102]. By embedding neuroectoderm obtained from EB culture into Matrigel droplets and improving nutrient exchange, early corticogenesis and subsequent patterning of forebrain, midbrain, and hindbrain regions were observed in cerebral organoids. Such regional specification of neural tissues was accompanied by generation and organization of mature cortical neuron subtypes. Importantly, using hiPSCs derived from patients of microcephaly, Knoblich and colleagues successfully modeled critical pathological phenotypes of microcephaly in cerebral organoid. In a separate work, using genetically engineered human neural progenitor cells (hNPCs), Choi et al. applied a 3D Matrigel-based culture system to establish an *in vitro* Alzheimer's disease model [20], which successfully recapitulated excessive accumulation of amyloid-β peptides observed often in the Alzheimer's disease that can lead to neurofibrillary tangles with hyperphosphorylated tau. This *in vitro* Alzheimer's disease further revealed a role of glycogen synthase kinase 3 (GSK3) in mediating tau phosphorylation. In a related study, another Alzheimer's disease model established using 3D culture was reported by Zhang et al. using neuroepithelial cells derived from hiPSCs [103].

3D organotypic culture methods have also been successfully applied to generate organoids with mesodermal and endodermal origins. By inducing hESCs toward primitive streak-intermediate mesodermal lineage, Little and colleagues generated self-organized human kidney organoids that exhibited ureteric buds, metanephric mesenchyme, and nephron formation (Fig. 7D) [104]. In another work from Gerecht and colleagues, hPSC-derived microvascular network was demonstrated through co-differentiation with vascular endothelial cells and pericytes and subsequent encapsulation in HA-based hydrogel (Fig. 7E) [105]. A relevant work to engineer functional human blood vessels in 3D culture using hiPSC-derived endothelial cells and mesenchymal precursor cells was recently reported by Samuel et al. [106]. Following endodermal commitment, Spence et al. recently generated human intestinal organoids (HIOs) from hPSCs using a Matrigel-based culture system (Fig. 7F) [107]. Through temporally controlling growth factors to dictate sequential definitive endoderm, posterior endoderm, and hindgut specification and morphogenesis, Spence et al. created *in vitro* intestines containing villus-like structures and crypt-like proliferative zones. Such

human intestininal organoids was further utilized for studying the roles of Wnt3a and FGF4 in intestine development and NEUROG3 (a pro-endocrine transcription factor) in human enteroendocrine cell development and disease. More recently, Watson et al. successfully implanted human intestinal organoids generated from hPSCs *in vitro* into mice, and observed significant maturation of HIO to form human adult-like small intestinal tissue with vasculature integration with the mouse host. This work supports the feasibility of using human intestinal organoids to study human small intestine maturation and relevant adult intestinal diseases [108]. In a separate study, Takebe et al. recreated human liver buds *in vitro* by leveraging 3D self-organization of hiPSC-derive immature hepatic cells and human endothelial and mesenchymal stem cells under coculture (Fig. 7G) [109]. Interestingly, such *in vitro* engineered human liver buds matured and functioned just like adult human liver after transplantation and could even rescue drug-induce lethal liver failure mouse models.

Together, recent developments of different human organoids using 3D biomaterial-based culture methods highlight the great potential of hPSCs in conjunction with conductive 3D culture environment for fundamental organ development studies, critical for identifying therapeutic targets for treating complex developmental or degenerative diseases. For more detailed discussion on 3D organotypic cultures using both murine and human stem cells as well as cancer cells, the readers are referred to excellent recent reviews by Shamir and Ewald [82] and Lancaster and Knoblich [83].

4. Harnessing mechanobiology for bioengineered 3D hPSC culture

4.1. Mechanobiology joins forces for 3D hPSC engineering

Recent progress on engineering hPSC fate, function, and organization in 3D biomaterial systems is exciting and promising. However, future development of 3D hPSC culture is critically hinged on a few important unaddressed issues. First, the extent to which human organoids derived from 3D hPSC culture resemble *in vivo* tissue development and function remains to be fully characterized. Second, cell fate patterning and morphogenic process in existing 3D hPSC culture models lack engineering control *in situ*, significantly limiting human organoid models for development studies and other translational applications.

Retrospectively speaking, these limitations may have come from the philosophy of biomimicry that guides development of current 3D hPSC culture. Although it is sufficient to dictate early hPSC lineage commitment by recapitulating vital *in vivo* developmental regulatory mechanisms, *e.g.*, temporal patterning of soluble factors in a 3D space, pure biomimicry based on current knowledge of development may risk not incorporating critical yet unidentified factors that fine tune cell fate patterning and tissue function maturation. Although critical for developmental patterning, local gradients of multiple growth factors are also technically challenging to model and precisely control *in vitro* in 3D. Additional regulatory mechanisms other than soluble factors are needed to improve current 3D hPSC culture.

Interestingly, intricate mechanosensitive behaviors of human stem and adult cells have been well documented. Although it remains unclear to what extent such mechanosensitivity of human cells has direct impact on human development, mounting evidence has supported the functional role of mechanical signals in the cell microenvironment in controlling cell behaviors through a mechanosensitive signaling network converging on regulatory pathways downstream of soluble growth factors vital for development [22]. Given that mechanical properties of biomaterials are amenable for engineering control [110], mechanobiology of human stem cells, especially hPSCs, should provide an important leverage for future development and engineering of 3D hPSC culture.

Although mechanobiology of hPSCs is still in its infancy, cellular mechanisms underlying hPSC mechano-sensing and -transduction are beginning to be understood [111]. Here, we provide an overview of a multifaceted mechanotransductive signaling network in human stem cells, including hPSCs, with transcriptional coactivators YAP and TAZ taking the center stage (Fig. 8). Specifically, we discuss current understanding of YAP/TAZ-mediated intracellular signaling in controlling stem cell fate and highlight mechanotransductive Hippo-dependent and -independent mechanisms acting upstream of YAP/TAZ. We discuss recent studies unraveling intricate connections between YAP/TAZ activity and Smad, Wnt, GPCR, and MAPK/JNK signaling and their implications in hPSC mechanobiology. We further examine functional links between two major intracellular mechanotransductive components, Rho GTPase and the actomyosin cytoskeleton (CSK), and the Hippo/YAP pathway.

It should be noted that existing knowledge of hPSC biology and mechanobiology has been mainly established using 2D cell culture. Signaling events in 3D hPSC culture have not yet been explicitly examined, which will likely be influenced by dimensionality and different from 2D cell culture. However, we reckon that a comprehensive picture of mechanosensitive signaling in human stem cells should provide important insights into hPSC mechanobiology and hPSC-biomaterial interactions that are critical for future engineering control of hPSC fate, function, and organization in 3D.

4.2. YAP and TAZ integrate a universal signaling network to control stem cell fate

YAP (Yes-associated protein) is an evolutionarily conserved transcriptional coactivator found in both *Drosophila* (Yorkie, Yki) and mammals (YAP) [112]. TAZ (transcriptional coactivator with PDZ-binding motif) is a paralog of YAP [113]. In human, YAP and TAZ share 45% amino acid identity [113], and together, they serve as vital transcriptional coactivators shuttling between the cytoplasm and the nucleus to control transcriptional activity through binding transcription factors such as TEAD, RUNX, and p73 [23]. Recently, YAP/TAZ have been identified as universal regulators of organ size during development [114], by controlling a multitude of cell behaviors such as proliferation, apoptosis, and differentiation [23]. There is also a significant interest in the role of YAP/TAZ in human cancer, development, and tissue/organ regeneration. Comprehensive reviews on YAP/TAZ in cancer and stem cells have been published recently [23,24]. Here, we focus on YAP/TAZ-mediated signaling network that has been shown or implicated in regulation of human stem cells, especially hPSCs, and highlight mechanosensitive properties of this signaling network.

4.2.1. YAP/TAZ regulation by canonical Hippo signaling

Hippo signaling is the first pathway identified upstream of YAP/TAZ activity regulation [23,114]. In the Hippo pathway, YAP (mainly on Ser127) and TAZ (mainly on Ser89) are phosphorylated by large-tumor suppressor proteins (Lats1/2) [114]. Subsequent binding of phosphorylated YAP/TAZ to scaffold protein 14-3-3 in the cytoplasm prevent YAP/TAZ from transcriptional activities [113]. Further phosphorylation on YAP Ser381 by Lats1/2 and CK1 primes YAP degradation through E3 ubiquitin ligase β-TRCP [115]. Upstream of kinase activity of Lats1/2 is the sterile 20-like proteins MST1/2 [116], whose kinase activity are controlled by NF2 [117], an ERM family protein normally associated with the cortical cytoskeleton and plasma membrane and deactivated by phosphorylation on Ser581. Other than MST1/2, Lats1/2 can also be activated through binding scaffold proteins MOBKL1A/B

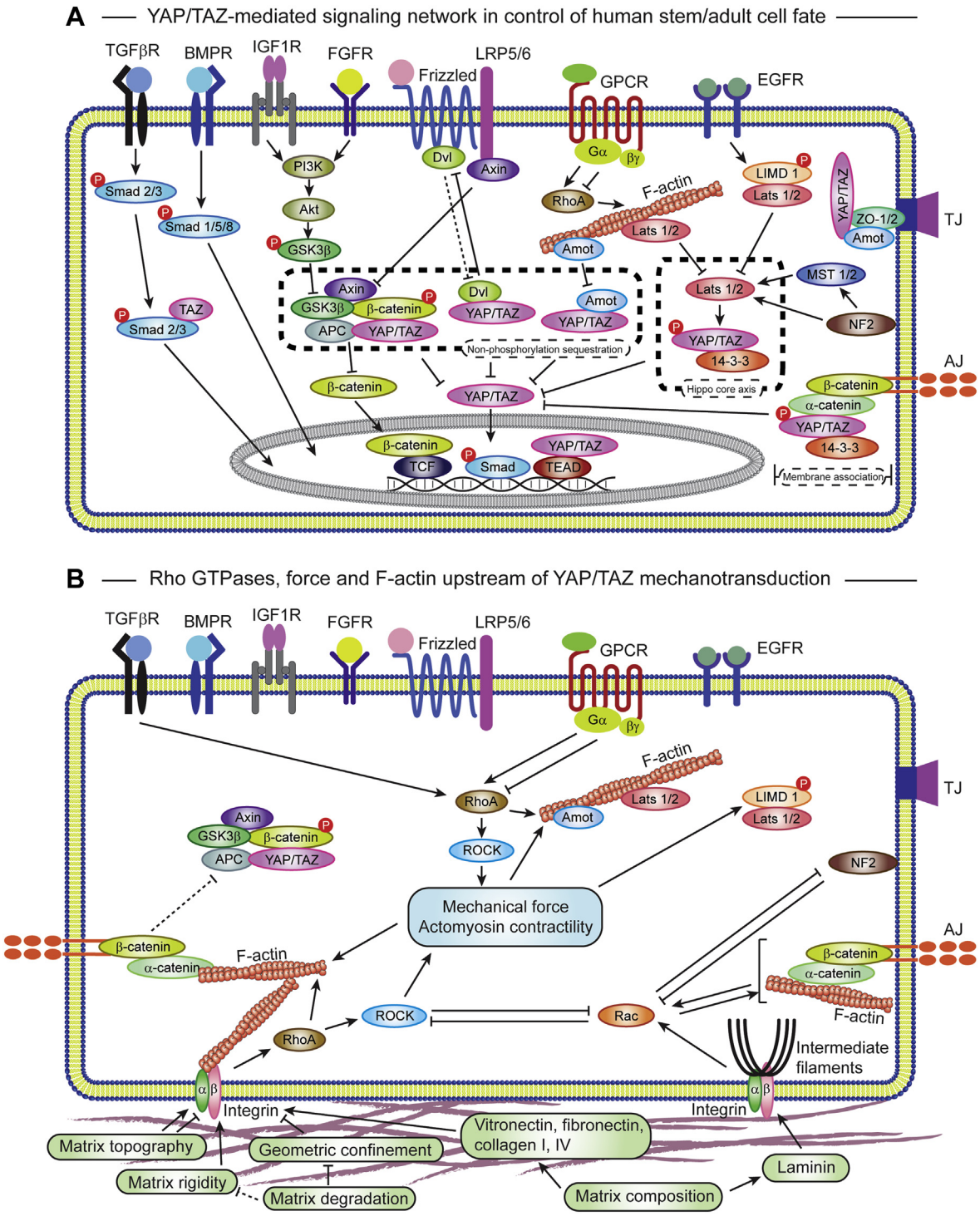

Fig. 8. (A) Schematic of a multifaceted signaling network mediated by YAP and TAZ in control of the fate and function of human stem cells as well as adult cells. In brief, the inactivation of YAP/TAZ could take place through three different principles: (1) phosphorylation inactivation of YAP/TAZ by Hippo signaling proteins and the resultant cytoplasmic retention of phosphorylated YAP/TAZ through binding to 14-3-3 proteins; (2) phosphorylation-independent cytoplasmic retention of YAP/TAZ through binding to adaptor proteins (or protein complexes) in the cytosol; such non-phosphorylation sequestration could further be coupled to phosphorylation mechanisms and then subsequent proteosomal degradation; (3) association of YAP/TAZ to plasma membrane-residing proteins. The regulation on YAP/TAZ could further crosstalk with and affect the Smad signaling, Wnt signaling, GPCR signaling, IGF, FGF, and EGF signaling. (B) Schematic of the mechanotransduction pathways acting upstream of regulatory mechanisms for YAP/TAZ. The extracellular signals, such as soluble growth factors as well as insoluble biophysical and biochemical cues, are mainly sensed and transduced through Rho GTPases, actomyosin contractility, and F-actin mechanics, which together form a tripartite mechanotransduction core and converge onto the regulatory mechanisms upstream of YAP/TAZ.

(Mob1) [118], making the upstream kinase MST1/2 functionally redundant in some cases. The highly conserved Hippo pathway has been demonstrated in control of YAP/TAZ activity both *in vitro* and *in vivo* among different species.

Recently, studies from our group [46] and the Kiessling group [119] revealed that YAP/TAZ regulated through Hippo signaling played a significant role in promoting neuroepithelial induction of hPSCs on soft substrates. YAP phosphorylation on Ser127 was

significantly increased in hPSCs on soft substrates, leading to predominant cytoplasmic retention and deactivation of YAP. Silencing of Lats1 expression in hPSCs using small interfering RNA (siRNA) suppressed YAP phosphorylation on ser127. More importantly, soft substrates did not promote neural induction of Lats1 knockdown hPSCs, further supporting that Lats-mediated phosphorylation of YAP might relay mechanical signals exerted by substrate rigidity.

Hippo-independent regulation of YAP/TAZ activity has also been reported very recently. For example, by silencing Lats1/2 in hMSCs and adult epithelial cells, Dupont et al. observed that transcriptional activity and subcellular localization of YAP/TAZ remained sensitive to changes of substrate rigidity [120]. Recent studies of YAP/TAZ regulation have further revealed a multifaceted signaling network that controls YAP/TAZ activity independent of Hippo signaling in many contexts, as we discuss below.

4.2.2. YAP/TAZ regulation by Hippo-independent cytoskeletal and membrane association

Identified firstly as YAP-associated proteins, angiomotin (Amot) and angiomotin-like proteins (Amot1/2) can interact directly with YAP/TAZ in the cytoplasm to promote their cytoplasmic retention [121,122]. Interestingly, angiomotin can also negatively regulate Hippo refractory, non-phosphorylatable TAZ mutant (TAZ S89A), suggesting regulation of TAZ by angiomotin a parallel process independent of Hippo signaling [121]. Recently, the 130-kDa isoform of Amot (Amot130) has been shown to bind F-actin and YAP competitively [123], suggesting integrity of F-actin as an upstream regulator inhibiting angiomotin-mediated cytoplasmic YAP retention. Interestingly, stability of angiomotin binding to F-actin can be regulated by Lats1/2 through phosphorylation of angiomotin, leading to its cytoplasmic accumulation and reduced association with the actin CSK, converging on YAP/TAZ inactivation [123,124]. In addition, Lats1 has recently been identified as an actin binding protein, suggesting its involvement in non-canonical Hippo signaling through sequestration to F-actin [125]. Regulation of YAP/TAZ through angiomotin family proteins and Lats1 in the non-canonical Hippo and Hippo-independent mechanisms has been so far mostly examined in human adult epithelial cells and embryonic kidney cells. Their specific effects in hPSCs remain to be elucidated in the future.

Our recent study showed that disrupted F-actin for hPSCs on soft substrates promoted YAP/TAZ cytoplasmic retention [46], which is consistent with sequestration of YAP/TAZ-inactivating proteins on the actin cytoskeleton. This observation implies that Hippo-independent mechanisms may function in parallel with Hippo/YAP signaling to regulate YAP/TAZ activity in hPSCs.

Although it has been found involved in Hippo signaling through the activation of NF2, cell–cell contacts have also been identified as a Hippo-independent mediator of YAP/TAZ activity in human epidermal stem cells [126]. Specifically, phosphorylated YAP can be associated with α-catenin residing in adherence junctions (AJs), preventing them from interacting with protein phosphatases such as PP2A phosphatase that could reactivate YAP. This cell–cell contact mediated YAP/TAZ activity plays an important role in cell density-sensing in the skin. Interestingly, ZO-1/2 proteins localized in apical tight junctions (TJs) could also directly interact with YAP/TAZ to inhibit their transcriptional activities [127,128]. Together, cell–cell contacts and their associated proteins provide another novel regulation mechanism to control YAP/TAZ activity. However, this control mechanism still needs to be examined in hPSCs in the future.

4.2.3. Crosstalk of YAP/TAZ and Smad signaling

Smad signaling mediated by TGF-β receptors (Smad 2/3 signaling) and BMP receptors (Smad 1/5/8 signaling) has been well documented for its importance in hPSC biology [129]. Binding of TGF-β ligands such as activin and nodal and BMP ligands such as BMP4 result in phosphorylation of intracellular Smad 2/3 and Smad 1/5/8, respectively. Phosphorylated Smads in turn bind Smad 4, and Smad complexes then translocate to the nucleus to serve as transcription factors for transcriptional control of hPSC fate. Smad signaling is a multifunctional pathway and has been extensively reviewed elsewhere [130]. Here, we focus on the functional crosstalk recently discovered between Smad signaling and YAP/TAZ regulation.

In their seminal work, Varelas et al. discovered that TAZ was required for nuclear shuttling of Smad 2/3 that is needed for hPSC self-renewal [131]. Silencing of TAZ expression or inhibition of TAZ function blocked nuclear accumulation of Smad 2/3 and abrogated transcriptional activity mediated by TGF-β signaling. Detailed examination further revealed that TAZ could directly bind Smads, with specifically high affinity to Smad 2/3. The role of TAZ (or YAP) in mediating Smad 1/5/8 nuclear translocation is less clear and requires further study, as TAZ knockdown by siRNA in this work did not necessarily have specific relation to BMP signaling.

Our own recent work confirmed such crosstalk of YAP/TAZ and Smad signaling in hPSCs, wherein concomitant cytoplasmic accumulations of YAP/TAZ and Smads were observed in hPSCs cultured on soft substrates [46]. Smad 1/5/8 phosphorylation was further downregulated upon YAP/TAZ deactivation on soft substrates, suggesting other mechanotransductive mechanisms (*e.g.*, rigidity-dependent endocytosis and downregulation of BMP receptors on cell surface [132]) acting in parallel to control Smads phosphorylation in the cytoplasm.

4.2.4. Crosstalk of YAP/TAZ and Wnt/GSK3β/β-catenin signaling

GSK3β/β-catenin signaling is known for its role in hPSC fate regulation *via* β-catenin nuclear translocation and association with transcription factor TCF for activation of relevant gene activities [133–136]. GSK3β controls the activity and stability of β-catenin through a destruction complex GSK3β forms together with Axin and APC. This destruction complex binds cytoplasmic β-catenin and mediates its phosphorylation and inactivation and subsequent ubiquitination and degradation [137]. GSK3β/β-catenin functions downstream of multiple signaling events, such as IGF and FGF signaling [136,138,139] and Wnt signaling, which act through PI3K/Akt-mediated phosphorylation and inactivation of GSK3β and membrane sequestration of protein Axin, respectively.

Recent studies have revealed crosstalk of GSK3β/β-catenin and YAP/TAZ in human adult cells [140–142]. Piccolo and colleagues, for example, demonstrated that the β-catenin destruction complex could directly bind YAP/TAZ and mediate their cytoplasmic retention [140,141]. Upon Wnt ligation or silencing of destruction complex proteins APC and Axin, TAZ was released from the β-catenin destruction complex and translocated to the nucleus. Importantly, cytoplasmic retention of TAZ required binding of β-catenin to the destruction complex *in priori*, supporting dependence of YAP/TAZ activity on cytoplasmic β-catenin level and environmental Wnt signal. In hMSCs, Piccolo and colleagues demonstrated that cytoplasmic β-catenin and binding of TAZ to the destruction complex were both required for adipogenesis [141]. In addition, cytoplasmic YAP/TAZ, *via* binding to the β-catenin destruction box, could facilitate β-catenin degradation and thus exert negative regulation on Wnt activation [140].

Another recent work from Camargo and colleagues demonstrated another functional link between YAP and Wnt/GSK3β/β-catenin signaling through binding of YAP to Dishevelled (Dvl) downstream of Wnt ligation and upstream of GSK3β-mediated destruction complex [143]. Binding of cytoplasmic YAP to Dvl did not require phosphorylation on YAP and was found to mainly

attenuate Wnt signaling by inhibiting recruitment of Dvl to Frizzled after Wnt ligation and therefore compromising β-catenin-dependent regeneration by intestinal stem cells. Interestingly, another recent study by Varelas et al. demonstrated that TAZ could also bind to and thus inhibit CK1-mediated phosphorylation of Dvl and therefore prevent Dvl from being involved in Wnt/β-catenin signaling [144]. Although in theory YAP/TAZ and Frizzled competitively bind Dvl, it is unclear whether and under which condition Frizzled might out-compete YAP/TAZ and thus activate Wnt by releasing YAP/TAZ from Dvl. So far, the interaction between YAP/TAZ and Wnt/GSK3β signaling in hPSCs has not yet been explored.

4.2.5. Crosstalk of YAP/TAZ and GPCR signaling

Serum-borne factors such as lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are known for their effects on cell behaviors through GPCR (G-protein coupled receptor) signaling [145]. Recently, a few important works have revealed functional connection between GPCR signaling and YAP/TAZ activity in human adult cells and hPSCs. Guan and colleagues, for example, demonstrated that GPCR signaling through $G\alpha_{12/13}$, which is stimulated by LPA and S1P, could promote YAP/TAZ activity by inhibiting Lats-mediated YAP/TAZ phosphorylation. They also showed that GPCR signaling through $G\alpha_s$, as stimulated by epinephrine, could negatively regulate YAP/TAZ activity by inducing their phosphorylation [145,146]. Activation of YAP/TAZ by $G\alpha_{12/13}$ further involved Rho GTPases, as treatment with C3 toxin, which antagonizes Rho GTPases, attenuated this effect. Inactivation of YAP/TAZ by $G\alpha_s$ involved PKA, which could restrict RhoA activity through increased RhoA-RhoGDI (Rho-GDP dissociation inhibitor) binding [147]. Another recent work by Kim et al. suggested that PKA signaling downstream of GPCR could directly promote YAP inactivation through phosphorylation on Ser381, rendering it independent of Rho [148]. Together, these studies suggest that GPCR signaling plays an important role in YAP/TAZ regulation, in which Rho GTPases may serve as a critical mediator.

Our own recent study supported that YAP/TAZ in hPSCs were indeed responsive to LPA stimulation and GPCR signaling [46]. LPA stimulation attenuated YAP/TAZ cytoplasmic retention for hPSCs on soft substrates. It remains unclear, however, which mechanism mediates crosstalk of YAP/TAZ and GPCR signaling in hPSCs.

4.2.6. Crosstalk of YAP/TAZ and EGF/MAPK/JNK signaling

MAPK/JNK signaling, which can be activated by either soluble factors such as EGF ligation or mechanical forces, is another pathway important for regulating hPSC fate [149]. Recently, Irvine and colleagues demonstrated a novel crosstalk mechanism between YAP/TAZ and EGF/JNK signaling involving Ajuba family proteins containing LIM domains, such as LIMD1. Specifically, activated MAPK/JNK could promote phosphorylation of Ajuba family proteins, *e.g.*, LIMD1 and WTIP, which could in turn bind Lats1/2 and thus sequester Lats kinases from the canonical Hippo signaling for YAP/TAZ inactivation [150,151]. MAPK/JNK signaling has been shown recently mechanosensitive in human adult cells [149] and *Drosophila* [152]. Together, it rises as an interesting question whether MAPK/JNK signaling and its crosstalk with YAP/TAZ has any direct role in hPSC mechanobiology.

4.2.7. Mechanosensitive regulation of YAP/TAZ: Roles of Rho GTPase, cytoskeleton integrity and tension

Mechanobiology studies in adult mammalian cells have identified the roles of two major intracellular mechanotransductive components, Rho GTPase and the actin CSK, in regulating mechanosensitive cell behaviors. Importantly, recent studies (including our own) have started to unravel mechanotransductive mechanisms in hPSCs acting upstream of YAP/TAZ through Rho GTPases and CSK integrity and tension (Fig. 8B).

Rho GTPases, especially RhoA, Rac and cdc42, undergo activation through binding GTP, and are inactivated after associated GTP is hydrolyzed to GDP. The functional roles of Rho GTPases in mechanotransduction have been well documented in adult mammalian cells [153]. For example, increased Rho-GTP level was observed in adult mammalian cells on rigid substrates [154], possibly through FAK/p190RhoGEF signaling. Ligation of ECM proteins such as vitronectin, fibronectin and collagen also activates RhoA. Importantly, RhoA directly mediates ROCK/MLCK signaling to control CSK tension, which positively feeds back onto and further enhances RhoA activity in adult mammalian cells [36,111,155]. There is no direct functional link reported between RhoA and YAP/TAZ activity. However, RhoA-dependent ROCK/MLCK-mediated CSK tension is indeed functionally connected to YAP/TAZ activity in mammalian cells, including hPSCs, as we will discuss below.

In addition to RhoA, Rac and cdc42 activities are also mechanoresponsive. Importantly, previous studies have implicated direct roles of Rac and cdc42 in regulating YAP/TAZ activity. Specifically, Rac and cdc42 could activate PAK through phosphorylation on Ser518, which in turn inactivated NF2 to inhibit canonical Hippo signaling [156]. Importantly, mutual inhibitory activities between RhoA/ROCK/MLCK and Rac signaling provide another level of complexity to Rho GTPase-mediated mechanotransduction [157]. For example, hyper-activation of RhoA with reduced Rac activity was identified responsible for dissociation-induced apoptosis of single hPSCs [158]. Formation of E-cadherin-mediated cell–cell contacts could rescue survival of dissociated single hPSCs by suppressing RhoA/ROCK activity [159], possibly through Rac activation. Given the role of Rac in stabilizing β-catenin in adherence junctions (AJs) (through inhibiting binding of β-catenin to IQGAP) [160], it is possible that Rac activation, in hPSCs, may inhibit formation of the GSK3β/Axin/APC/β-catenin destruction complex that prevents nuclear localization and transcriptional activation of YAP/TAZ.

The functional roles of CSK integrity and tension in regulating YAP/TAZ activity were first observed in adult mammalian cells and *Drosophila* [24,120]. Prominent F-actin stress fibers are commonly associated with nuclear localization and activation of YAP/TAZ in adult mammalian cells on rigid substrates or without geometric confinements; while disorganized and diffusive F-actin structures are usually correlated with cytoplasmic localization and functional inactivation of YAP/TAZ in cells on soft substrates or confined in small adhesive islands. In addition, inhibition of actin polymerization using small-molecule drugs such as Latrunculin A and Cytochalasin D prevented nuclear accumulation of YAP/TAZ and abolished YAP/TAZ transcriptional activity [161]. Furthermore, through knocking down expression of proteins involved in F-actin severing and capping (*e.g.*, cofilin, gelsolin and cap Z), Aragona et al. demonstrated that ectopically reinforced actin CSK could override effects of extracellular physical cues such as cell confluence, geometric confinement, and substrate softness in functional inactivation of YAP/TAZ [162]. The results reported by Aragona et al. support that mechanosensitive YAP/TAZ activity may be regulated through F-actin dynamics and CSK integrity, which integrate signals from upstream mechanoresponsive Rho GTPase activities. Another interesting possibility is that mechanosensitive CSK integrity may help sequester proteins on F-actin that can interfere with YAP/TAZ (such as Angiomotin and Lats1/2) to prevent their nuclear translocation and transcriptional activation. Indeed, Lats1 has recently been identified as an actin-depolymerizing factor [125], supporting Hippo-independent regulation of Lats1 in controlling YAP/TAZ *via* F-actin dynamics and CSK integrity.

It should be noted that all above observations were obtained through pharmacological treatments and specific knockdown or ectopic overexpression targeting the actin CSK. It remains unclear whether mechanoresponsive F-actin dynamics and CSK integrity can regulate YAP/TAZ activity under physiological conditions. Endogenous mechanosensitive mechanisms mammalian cells employ to regulate F-actin dynamics and CSK integrity are still under intensive study. For example, do extracellular physical cues really modulate activities (mainly through phosphorylation) of actin severing and capping proteins such as cofilin, gelsolinm and cap Z endogenously, or there exist other unidentified endogenous mechanosensitive mechanisms acting in parallel? For example, mechanical tension in F-actin stress fibers could suppress binding of cofilin to F-actin and thus the actin-severing property of cofilin, potentially regulating CSK integrity without modulating the phosphorylation states of cofilin [163]. In our recent observation, during their response to changes of substrate rigidity, hPSCs would modulate the actin CSK with concomitant changes of YAP/TAZ activity [46]. This observation certainly corroborates the significance of F-actin and the CSK in regulating YAP/TAZ activity.

Cell mechano-sensing and -transduction involves a modulated delicate force balance between endogenous CSK tension and external mechanical forces transmitted across cell-ECM adhesions [155]. Recent evidence obtained from adult mammalian cells has implicated an important role of such external-internal force balance in mediating YAP/TAZ activity through key pathways discussed in the aforementioned YAP/TAZ signaling network, including EGF/JNK signaling [149] (Fig. 8). As another example, Palecek and colleagues applied external cyclic stretch to hPSCs and observed upregulated transcription of TGF-β signaling ligands (such as Activin, nodal, TGF-β) and phosphorylation of Smads, which could cooperate with TAZ to maintain hPSC pluripotency [164]. In addition, mechanical force could also promote the stability of actin stress fibers through unique catch-bonds between F-actin and anchoring cell-ECM adhesion proteins [165] as well as that between actin monomers [166], and thus might promote F-actin-association of Angiomotin and Lats1/2 and attenuate their effects in cytoplasmic retention of YAP/TAZ. CSK tension is also necessary for maintaining the integrity of AJs [167], loss of which results in release of β-catenin to the cytosol for formation of the GSK3β/Axin/APC/β-catenin destruction complex that prevents YAP/TAZ from nuclear translocation.

Together, mechanotransductive signaling pathways centering on YAP/TAZ activity have emerged as a master regulatory mechanism controlling mechanosensitive and -responsive cell behaviors. It will be exciting to fully examine, in hPSCs, the functional role of YAP/TAZ and different upstream mechanotransductive pathways that relay external mechanical signals to YAP/TAZ activity. Studying mechanosensitive properties of hPSCs is critical for understanding hPSC-biomaterial interactions and will chart a path for future investigations to unravel their full complexity, which will improve our understanding of hPSC biology and facilitate future developments of functional 3D biomaterials for large-scale culture of hPSCs.

5. Large-scale and integrated 3D hPSC engineering: challenges and opportunities

In the near future, scaling up fabrication of hPSCs and rapidly producing high-purity and -yield functional somatic cells from hPSCs are critically needed for meeting future demands of their biomedical and clinical applications. For example, about 10^9 functional CMs are needed for regeneration of one third of the left ventricle for replacing the damaged tissue after myocardial infarction [168]. Large-scale generation of functional human tissue and organ models *in vitro* with functionally mature hPSC-derived cells is also required for drug and toxicology screening for future precise, personalized medicine. Here, we speculate that a few key aspects of large-scale production and integration of 3D biomaterial systems for hPSC engineering need to be fully examined and understood before the promises of hPSCs for biomedical and clinical applications can be fully fulfilled: (1) Automated and well-controlled dynamic culture conditions for efficient hPSC expansion and differentiation. Such conditions include both soluble components as well as 3D scaffold properties (mechanical rigidity, pore size, 3D architecture, and degradation characteristics) that can be modulated precisely in space and time; (2) Extracellular biochemical and physical cues within 3D hPSC constructs conductive for establishing *in vivo*-mimicking tissue organization and function; (3) Large-scale culture and convenient harvesting of hPSCs and hPSC-derived cells or tissue and organ models; and (4) Integration of mass-production of hPSCs, hPSC-derived cells, and tissue and organ models with inline quality monitoring and *in situ* conditioning, characterization, and sample processing and analysis. These critical issues and challenges for large-scale and integrated 3D hPSC engineering provide opportunities for stem cell biologists, bioengineers, and materials scientists to collaborate closely to develop novel biomaterial systems critically needed for future fundamental biology and translational medicine applications.

6. Concluding remarks

Given their unlimited potential for self-renewal and differentiation into any type of human somatic cells, hPSCs constitute a very promising cell source for human tissue and organ regeneration, *in vitro* modeling of human diseases, as well as screening for patient-specific therapeutic options and drug responses. In the last 15 years, bioengineered 3D hPSC culture platforms have greatly improved their robustness, efficiency, and versatility for controlling hPSC fate and function *in vitro*, *via* precisely controlling biochemical and biophysical cues within the 3D cell microenvironment, even down to the single cell resolution. In addition, recent exciting researches using novel bioengineering methods have achieved hPSC patterning and organization in 3D microtissue constructs and organoids, serving as *in vitro* biomimicry recapitulating the structural blueprint of human tissues and organs and thus simulating their functional maturation and native responses to various physiological and pharmaceutical stimuli. Although still at its beginning, engineering functions and behaviors of hPSCs or hPSC-derived tissues and organs in 3D with appropriate *in vivo*-like multiscale structures have opened unlimited opportunities as well as posing significant challenges for future tissue- and organ-scale regenerative therapies and *in vitro* disease modeling.

The emerging field of hPSC mechanobiology brings a new perspective researchers can leverage to regulate and fine tune hPSC fate and function *in vitro* through multiple converging signaling pathways initiated by both soluble growth factors and insoluble biophysical cues. The multifaceted mechanotransductive signaling network centering on YAP/TAZ, which has been primarily established in adult mammalian cells, presents an exciting opportunity for bioengineering control of hPSC fate in 3D as well as for rational designs of 3D biomaterial systems with conductive properties targeting specific intracellular signaling events leading to desirable hPSC fate and function.

Last but not the least, combined with recently developed high-throughput hPSC assays, which provide well-controlled mass production, multiplexed and combinational screening, and *in situ* high spatiotemporal resolution characterization and analysis, highly integrated multiparametric functional 3D bioreactors will emerge

for future large-scale biofabrications for hPSC-based regeneration therapies and human disease studies.

Acknowledgments

We acknowledge valuable comments and suggestions on the manuscript from members of the Integrated Biosystems and Biomechanics Laboratory. Research in Dr. Fu's laboratory is supported by the National Science Foundation (CMMI 1129611, CBET 1149401, ECCS 1231826, and CBET 1263889), the National Institutes of Health (R21 HL114011, R21 EB017078, and R01 HL119542), the American Heart Association (12SDG12180025), and the Department of Mechanical Engineering at the University of Michigan, Ann Arbor. Finally, we extend our apologies to all our colleagues in the field whose work we are unable to discuss formally because of space constraints.

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