Grow with the Flow: When Morphogenesis Meets Microfluidics

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Developmental biology has advanced the understanding of the intricate and dynamic processes involved in the formation of an organism from a single cell. However, many gaps remain in the knowledge of embryonic development, especially regarding tissue morphogenesis. A possible approach to mimic such phenomena uses pluripotent stem cells in in vitro morphogenetic models. Herein, these systems are summarized with emphasis on the ability to better manipulate and control cellular interfaces with either liquid or solid materials using microengineered tools, which is critical for attaining deeper insights into pattern formation and stem cell differentiation during organogenesis. The role of conventional and customized cell-culture systems in supporting important advances in the field of morphogenesis is discussed, and the fascinating role that material sciences and microengineering currently play and are expected to play in the future is highlighted. In conclusion, it is proffered that continued microfluidics innovations when applied to morphogenesis promise to provide important insights to advance many multidisciplinary fields, including regenerative medicine.

1. Introduction

Developmental biology has long been motivated toward understanding fundamental morphogenetic processes based on investigation of in vivo embryonic development. This approach has undeniably led to an increase in our understanding of the intricate and dynamic processes that are involved in the formation of an organism from a single cell. However, important gaps in our knowledge relating to tissue morphogenesis remain, including the early morphogenetic events that control embryonic patterning, which have consequences for other multidisciplinary fields such as tissue engineering and regenerative medicine. For example, how do certain cells organize into tissues and organs with a particular shape and size? Which chemical and physical factors are important in the formation of an organ? Despite a promising start, creating viable organs in vitro has not been achieved because such fundamental concepts of mammalian development including cell patterning and morphogenesis remain elusive.

To better understand mammalian development, cells derived from an early phase of development, that is, pluripotent stem cells (PSCs), can be used to mimic embryonic development in vitro. PSC-derived models are an attractive alternative to in vivo embryonic studies, which lack accessibility and are fraught with ethical issues. To date, several biological models that mimic specific aspects of embryonic development have been established, including early embryonic patterning, gastrulation-like events, and neural tube formation. This review aims to particularly highlight microfluidic tools that can be used to interact with living systems on a single cell or even subcellular level.

Originally developed for the semiconductor industry in the early 1960s, microengineering includes the design and fabrication of structures and machines, usually on the same scales as cells and their functional entities. Over two decades ago, the application of microengineered tools to control fluid flow has paved the way for customizable microfluidic platforms. Compared to conventional cell culture in a dish, microfluidic cell-culture systems offer the potential to exert a high level of

 DOI: 10.1002/adma.201805764

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adma.201805764.
control over the above mentioned in vitro models by creating spatiotemporally controlled microenvironments including control of biological, (bio)chemical, and physical cues. Highly laminar fluid flow on the microscale allows to better predict and control flow patterns, an advantage that has led to widespread popularity of microfluidic platforms.

Here, we first detail relevant in vitro morphogenetic models and emphasize how a convergence of microengineered systems and microfluidic platforms with these biological models can help us to further improve control over the tissue formation processes. We will review how customized cell-culture systems for developmental biology and PSC research have supported important advances in the field of morphogenesis to date. We then discuss the current and future roles that microfluidic systems play in these advancements.

2. Embracing Complexity: From Stem Cell Culture toward Morphogenesis

In a developing mammalian embryo, the pluripotent cells of the inner cell mass give rise to epiblast (or primitive ectoderm) and hypoblast (or primitive endoderm). The embryonic epiblast cells differentiate and give rise to the three organized germ layers—ectoderm, mesoderm, and endoderm—finally leading to tissue morphogenesis. Such embryonic patterning processes are tightly regulated by (bio)chemical and mechanical factors originating from embryonic or extraembryonic tissues. Efficient spatiotemporal exposure to gradients of both soluble and cellular factors ensures that the PSCs differentiate and organize into different tissues during embryonic development. The factors that signal nearby cells to differentiate and organize in patterns remain unknown, mainly because accessing and analyzing morphogenesis in a growing embryo in vivo poses a major ethical and technological challenge. One might argue that a logical alternative is to follow the development of actual embryos in vitro. Here, it is important to note that the onset of the primitive streak (which forms immediately prior to gastrulation in the blastula) in human embryos occurs 14 d after fertilization, which is also the ethical limit up to which a human embryo is allowed to be cultured in vitro.[8] Hence, no patterning events involving the three germ layers can be currently observed and studied using actual human embryos in real time. Nevertheless, various microengineered platforms have been developed to allow mammalian embryogenesis in vitro, until the early blastocyst phase. Most platforms enabling embryonic growth have been developed to aid assisted reproductive technologies.[9–11]

PSCs represent an attractive source of cells for artificial organogenesis since they can be propagated indefinitely in the lab and can be differentiated into all cell types of the body under appropriate conditions.[12-14] PSCs include two types: (i) embryonic stem cells (ESCs) by extracting cells constituting the inner cell mass of a blastocyst[12,15] and (ii) induced PSCs (iPSCs), derived by overexpression of the pluripotency genes Oct4, Sox2, c-Myc, and Klf4 in somatic cells.[13,14,16] ESCs form an excellent platform for studying and understanding developmental processes[17] but they can form teratomas and their obtainment raises moral and ethical issues; both restrict their application in tissue engineering and regenerative medicine.

Because of the somatic origins of iPSCs, these cells could circumvent the moral and ethical issues involved with ESCs. A recent alternative to obtain stem cells for regenerative medicine purposes, which circumvents the use of PSCs, is the reprogramming of somatic cells to tissue specific stem cells using

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transient expression of YAP/TAZ signaling pathway.\cite{18,19} These reprogramed tissue specific stem cells, which are non-tumorigenic, can also be used to form self-expanding organoids.\cite{19}

The standard method to differentiate PSCs involves a 3D culture step to produce embryo bodies (EBs), followed by exposure of the aggregate to the required growth factors.\cite{20,21} Using protocols mimicking pathway activations during embryogenesis, PSCs have already been differentiated into various cell types, including pancreatic, kidney, and cardiac cells.\cite{22–25} General aggregate formation techniques include culture in low-attachment U-bottom 96-well plates, hanging drops, suspension, and spinner flasks. Despite this success, the size and shape of resulting EBs have variable homogeneity in suspension or spinner flask cultures. This lack of reproducibility can pose a problem because EB size can affect differentiation and patterning events.\cite{26,27} Techniques resulting in improved homogeneity such as the hanging drop method are more labor intensive.\cite{20}

Approaches based on artificial systems that mimic embryonic development are increasingly being used to study morphogenetic processes. The self-organization of PSCs gives rise to organoids, 3D microtissues similar to organs. Corresponding PSC-derived organoid models have been described for

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**Figure 1.** Convergence of in vitro morphogenesis, material science and engineering principles. (Top) PSCs have recently been used to mimic important morphogenetic events—such as early embryonic patterning and gastrulation in vitro, demonstrating the astonishing self-organizing capacity of these cells. The ability to control and manipulate these morphogenetic events in vitro is critical for attaining deeper insights into pattern formation and stem cell differentiation during organogenesis. (Left column) The development and application of an engineering toolbox, including microengineered tools, has become increasingly relevant because these tools are well suited for understanding and controlling these morphogenetic models.
several tissue types including gut,[28,29] lung,[30–32] kidney,[23,24] stomach,[31] liver,[34] brain,[35,36] and optic cup.[37]

Several studies have reported that PSCs have the ability to self-organize. Both human PSCs and progenitor cells derived by differentiating PSCs have demonstrated self-organization. For example, in a recent study, renal cells obtained by differentiation of hESCs using soluble factors on a monolayer culture gave rise to an organized structure that evidently showed the presence of nephrons.[23] The group published additional results showing the formation of kidney organoids from hiPSCs, which matched the transcriptome profile of fetal kidney[24] with segmented nephrons. Endocytosis of dextran by proximal tubular cells further demonstrated partial functional maturity,[24] and the formation of this organoid model was shown to mimic in vivo kidney tissue patterning and organization. Self-organization and patterning have been especially well-illustrated with mouse ESCs (mESCs) differentiated to form structures resembling postnatal neural retina[37,38] or polarized cerebellar tissue.[39] These processes were guided by an “intrinsic self-organizing program involving stepwise and domain-specific regulation of local epithelial properties.”[37,38]

Although the conditions required for the formation of some organoids and self-organizing 3D aggregates have been partly deciphered, many of the processes involved in differentiation and patterning of PSCs in such structures remain unknown. Further, these processes mainly rely on self-organization and are a consequence of stochastic rather than deterministic patterning.[40] To what extent the organoids mimic the in vivo development processes needs to be further determined.[40] Different organoids can also portray random positioning of microtissues relative to each other leading to a certain degree of heterogeneity. It has been reasoned that this randomness is caused “possibly because of a lack of embryonic axis formation.”[40] Hence, there is an increasing need to study biological systems that more closely mimic tissue development and patterning during embryonic development, without involving actual embryos.

A widely discussed question is how morphogens can signal to nearby cells to differentiate and organize in patterns, both in vivo and in vitro. Perhaps one of the most influential mathematical model concerning the pattern forming effects of chemical moieties known as morphogens was the one suggested by Turing[41] as early as 1952. Also known as the reaction-diffusion model, it describes how two diffusible substances that interact with each other through local activation and long-range inhibition can lead to pattern formation in an initially homogenous system.[41] The model has been used to describe pattern formation for certain aspects of embryonic development.[42] For example, the development and patterning of digits in mouse limb were proven to be a result of a Turing network occurring between the wingless/integrated (Wnt) proteins, bone morphogenetic protein (BMP), and Sox9.[43] Nevertheless, this model does not provide a complete explanation for patterning of more complex organs, such as lungs or kidneys.[44]

A comparatively simpler model was proposed by Wolpert,[45] also known as the French flag model, where the diffusion of chemical moieties from a fixed cell source can lead to formation of a morphogen gradient that defines cell fate. Cells in contact with different concentrations of the morphogen (due to physical distance from the morphogen-secreting source) can acquire different outcomes. Stem cell differentiation and patterning cannot be described completely by one of these models alone, and development of different types of tissues might require different mechanisms of cell–morphogen interaction. More sophisticated biological systems that mimic embryonic tissue morphogenesis in vitro could help researchers better understand these mechanisms.

3. Embryonic Tissue Morphogenesis In Vitro

Several in vitro models that mimic embryonic morphogenesis using PSCs have been established in the past decade (Figure 2). As early as 2008, activation of Wnt signaling in mESC aggregates was shown to result in symmetry breaking (establishment of tissue polarity), axis formation, mesodermal differentiation, and the epithelial–mesenchymal transition, processes that occur during gastrulation (Figure 2a).[46] Axial elongation and cell differentiation events similar to gastrulation were also shown to occur in aggregates composed of mouse embryonic carcinoma cell line P19 (Figure 2b).[47] A recent study reported that these events could be stimulated in 3D aggregates derived from mESCs by optimizing the cell seeding density in a U-bottom, 96-well plate, followed by a pulse of CHIR99021 (agonist of the Wnt signaling pathway) between 48 and 72 h (Figure 2c).[48] The rearrangement and differentiation of mESCs led to symmetry breaking and formation of polarized EBs, which were termed “gastruloids.”[46,48] These studies demonstrated that early embryonic pattern formation can be initiated in “free-floating” 3D cell cultures of mESCs in vitro. Symmetry breaking and axis formation events were also reported in mESC aggregates when attached to a gelatin-coated culture dish (Figure 2d).[49] Another study showed differentiation and patterning of 2D colonies of hESCs, which were spatially restricted to micropatterned cell adhesion islands of 1000, 500, and 250 μm in diameter on a glass substrate (Figure 2e).[1] By controlling the initial size of the 2D colonies and exposure to different biochemical cues, the authors were able to obtain structures with distinct patterns of cells from all three germ layers; the outer layer showed trophoderm-like cells when the 2D colonies derived from hESCs were spatially restricted and exposed to BMP4.

More recently, in vitro studies to mimic morphogenesis have produced embryo-like structures “whose morphogenesis, architecture, and constituent cell-types resemble natural embryos” (Figure 2f).[2] In the so-called “ETS-embryo” model, self-assembly of mESCs and mouse trophoblast stem cells (mTSCs) cultured in a 3D extracellular matrix (ECM) scaffold in Matrigel[53] resulted in a structure highly similar to a mouse embryo during early development, including formation of a prostomiotic cavity at ≈96 h and regionalized mesoderm expression leading to symmetry breaking at 110 h. However, this model lacks cells from primitive endoderm lineage, resulting in the absence of primitive endoderm-derived distal and anterior visceral endoderm cells.[50,51] Another study produced a blastocyst-like structure using a combination of 8 mESCs and 20 TSCs in nonadherent agarose microwells (Figure 2g).[52] The resulting structures, called “blastoids,” show similar morphogenetic
characteristics to embryonic day 3.5 mouse embryos including similar structural features like cavitation as well as transcriptome profiles. Although the blastoids did not mature into embryos when implanted into mice, it was found that they did undergo implantation and gave rise to patterned decidua.[52]

Another in vitro model that mimics neural tube patterning, a specialized aspect of embryonic morphogenesis, was recently established using mESCs.[5]

In summary, these developmental models have remarkably recapitulated early embryonic self-organization and demonstrated the morphogenetic potential of PSCs in vitro. However, they do not perfectly replicate in vivo morphogenesis. For example, some features like the movement of mesoderm-like cells in the “gastruloids” (Figure 2c) were found to be different compared to actual mouse gastrulation.[4,48] The micropatterned colonies (Figure 2e) also did not completely resemble the morphological structure of early human gastrulation as it occurs during embryogenesis; for example, Brachyury expression in the colonies begin to appear as a ring compared to a caudal midline in an implanted embryo.[3,53]

A very recent commentary regarding the ethical and experimental implications of in...
vitro embryonic tissue morphogenesis models can be found elsewhere.\[54\]

It is important to note that the aforementioned observations were obtained using culture systems that only allow control of initial experimental conditions like cell number or colony size, and it was not possible to manipulate and control them at a cellular resolution. Furthermore, the cells are either in contact with a homogenous environment partly consisting of materials like pristine plastic substrate (tissue-culture-grade polystyrene), protein-coated substrate (laminin-coated glass), free-floating aggregates on nonadherent substrates (in hanging drops or agarose microwells), or completely embedded in a hydrogel (Matrigel). This inherent simplicity, although advantageous in many situations, can give rise to culture conditions that do not completely mimic embryonic development and also make analysis of morphogenetic phenomena difficult. For example, in a free-floating aggregate culture, there is no control over the orientation of the aggregate or the direction of axis formation, which makes it impossible to probe only a particular region of the aggregate over longer periods of time. The current model for in vitro gastrulation-like events is also not capable of mimicking in vivo signaling centers, which are otherwise known to control localized exposure of the developing embryo, for example, to BMP, Nodal, and Wnt agonists\[4\] (Figure 3a,b). Controlling the microenvironment, and hence manipulating morphogenesis within these systems, could help to improve the relevance of these models by accurately reflecting developmental processes. This aforementioned ability to control the microenvironment can be bestowed by developing advanced microengineered platforms using materials that can sustain morphogenesis and aid downstream analysis. Without the application of more sophisticated microengineered materials and systems that can, for example, serve as local sources of growth factors (to mimic signaling centers) or apply forces locally to a cell aggregate, these in vitro models might not be able to completely mimic in vivo tissue formation processes.

We contemplate that the application of currently available and more advanced microengineered and microfluidic systems in future might offer a solution to overcome existing bottlenecks and limitations of the abovementioned morphogenetic models. Current microfluidic systems can already create in vitro gradients to mimic structures and signaling centers important for embryonic development such as extraembryonic ectoderm and posterior visceral endoderm, which are not limited to two dimensions but form a complex interplay of temporally controlled parameters in three dimensions (Figure 3a). To unleash the full potential of microfluidics to unravel morphogenesis,
In the last decades, the so-called "additive manufacturing" (3D printing) has become prominent, particularly with the soft-lithography method devised for the fabrication of microfluidic devices for biological applications. However, these techniques required further adaptation to fabricate microfluidic devices and to achieve patterning of soft, biocompatible materials. Thereby, the establishment of a platform for developing advanced microfluidic tools that can be used for analyzing and controlling tissue patterning in the future. As examples to portray the full potential of these emerging technologies, we also detail certain promising engineered systems that are currently used with adult stem cells.

4. Materials and Microfluidic Tools for Developmental Biology

4.1. Microfluidic Systems

Microfluidics deals with the fabrication and application of devices and scientific principles to control extremely small volumes of fluids in the range of $10^{-9}$ to $10^{-18}$ L.[56] The lower limit of $10^{-18}$ L corresponds to a cubic fluid volume of 0.1 µm edge length (or of $10^{-1}$ µm³; in comparison, a HELE cell is typically about $2 \times 10^3$ µm³). Hence, the usual cross-sectional dimensions of micro- and nanofluidic devices range from a few hundred micrometers down to some hundred nanometers, a range that enables the manipulation of single cells and the control of architecture and composition of the cells’ microenvironment at subcellular resolution.

Microengineered tools have been adapted to create controlled microenvironments suitable for cell culture (Figure 4). The most commonly used technologies used to fabricate microfluidic devices are derived from techniques developed for the semiconductor industry. Consequently, most of the derived techniques allow for high-resolution patterning of the so-called “Manhattan” or 2D/3D structures similar to integrated circuits on planar surfaces but struggle with patterning of more complex 3D structures, or structures on or with curved surfaces and with patterning of soft, biocompatible materials. Therefore, these techniques required further adaptation to fabricate microfluidic platforms for biological applications, with the most prominent one being the soft-lithography method devised by the Whitesides lab.[56-59] In recent years, multiple other methods have also been shown to be suitable for this purpose, including various forms of lithography,[57,60-62] vacuum hot embossing,[63-66] microinjection molding for replication,[67,68] microthermoforming,[69-72] sacrificial templates,[73] and (hydrogel) casting.[74] In addition to subtractive and additive patterning methods on the basis of lithography and structuring techniques based on micro- and nanomolding applied in the last decades, the so-called “additive manufacturing” (3D printing) is increasingly used.[75,76]

Many microfluidic systems have already found their way into biological research environments, although their use is largely restricted to platforms for performing analytical techniques like cell sorting,[77] polymerase chain reaction (PCR),[78] Western blotting,[79,80] Raman spectroscopy on-chip,[81] absorption spectroscopy on-chip,[82] electrical conductivity,[83] and impedance measurement on-chip[84] as well as drug, diagnostic, and pharmaceutical testing (reviewed in detail by Neužil et al.[85]). Recently, organ-on-a-chip[86] devices have been established, which can mimic certain functional aspects of organs or organ systems including lung,[86] kidney proximal tubule,[87] and gut[88] using miniaturized biomimetic systems. Microfluidic analytical techniques can also be used for on-chip, single-cell gene expression profiling for hESCs.[89]

Microfluidic regimes are usually dominated by viscous forces, leading to a more laminar flow that prevents intermixing, whereas macroscale fluidics is dominated by inertial forces, leading to a more turbulent regime and volume mixing. This property ensures that microfluidics can be utilized to create local gradients of concentrations that do not mix easily. The main physics governing fluid flow in a microfluidic channel is given by a set of time-dependent differential equations concerning conservation of momentum, mass, and energy for a moving fluid, called the Navier–Stokes equations, which are reviewed in detail elsewhere.[90] The dominance of convection or diffusion during flow is dependent on the extent of turbulence. This behavior can be represented quantitatively using mainly two numbers, namely, Reynolds and Peclet numbers,[91] which can be described by the following Equations (1) and (2), respectively.

$$N_{Re} = \frac{\text{inertial forces}}{\text{viscous forces}} = \frac{\rho dv}{\mu} \quad (1)$$

$$N_{Pe} = \frac{\text{convectional transport rate}}{\text{diffusion transport rate}} = \frac{\nu L}{D} \quad (2)$$

where $\rho$ denotes the fluid density, $d$ the microchannel diameter, $\nu$ the velocity, $\mu$ the viscosity, $L$ the characteristic length, and $D$ the diffusion constant. A low Reynolds number (for a microfluidic device, usually $N_{Re} \ll 100$) signifies that viscous forces become dominant, which means a laminar flow and difficulty mixing liquids.

Microfluidic platforms can generally be categorized into two classes: closed systems with fully confined microchannel networks for continuous or segmented flow (e.g., droplet-based) or open systems (e.g., liquid-handling robotics in conjunction with microowell arrays). Because flow and pressure are important parameters that can elicit cellular response, it is crucial to make a proper choice and eventually monitor both parameters during cell culture.[90,92] The main methods to create an active flow of liquids in closed microfluidic channels are pressure-driven flow, electrokinetic flow,[90,92] and electrowetting-based flow.[93]

4.1.1. Continuous-Flow Microfluidics

For controlling continuous flow, there are pressure-controlled microfluidic systems such as when the fluid is pneumatically pressurized by a gas cushion on top of it (e.g., Elveflow, ibidi), and flow-controlled systems such as when the fluid is displaced by a syringe pump (e.g., Cetoni). Gravity can be used to easily implement pressure-driven flow by establishing a differential height between the surface of a fluid reservoir and the interconnected inlet of the system. Electrokinetic flow is based on the...
flow of a polar fluid by application of an electric field. However, for electrokinetically controlled systems, only low flow velocities (of several mm s\(^{-1}\)) are usually possible, mainly because of an electric field limit of 1 kV cm\(^{-1}\), above which cell lysis occurs.\(^{[92,94,95]}\)

Continuous flow microfluidic systems can expose cells to a stable and highly controlled concentration gradient. Fluid mixing becomes an important parameter in order to obtain such concentration gradients. As mentioned earlier, this can be quite challenging due to the laminar flow. To mix fluids within these closed systems, researchers have developed designs where mixing is typically achieved by using either passive methods, such as a Zigzag mixer, Vortex mixers, and microfluidics network mixers, or active means like acoustic mixers and magneto-hydrodynamic mixers, all of which have been well described elsewhere.\(^{[96]}\) For biological applications, these techniques can thus be used to generate well-defined concentration profiles of soluble compounds within microchannels wherever required. However, for stem cell cultures, the highly laminar flow also opens the possibility to create a parallel flow of liquids with various concentrations comparable to a digital concentration profile. Hydrodynamic flow focusing, which is injection of at least two streams of fluids with different flow rates into a microchannel, is a powerful

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**Figure 4.** Timeline: Major microengineering advances over the last seven decades (green column) and advances in morphogenesis and PSC-derived microtissues (yellow column). The lithography resolution (red column) denotes the surface resolution, which semiconductor industry has been developing for lithography, with corresponding sizes of biological entities. At the current rate of convergence between technological progress and progress in biological sciences, controlling morphogenesis will become a reality in the near future.
method to precisely control such concentration profiles and to continuously change or adapt them over time.\textsuperscript{[97]} Such profiles can be used, for example, to expose only dedicated cells in an aggregate to a certain morphogen. The instrumentation and standard parts as well as the underlying designs to create such continuous or digital gradients are widely available. Corresponding strategies—including “Christmas tree” networks, hydrogel or ECM-based networks, flow splitters, and T junctions—and their underlying principles are reviewed extensively by Toh et al.\textsuperscript{[98]}

4.1.2. Segmented-Flow Microfluidics

Segmented-flow microfluidics enables the formation of droplets, which act as separate entities, leading to increased throughput and a high degree of control.\textsuperscript{[99]} These benefits make segmented flow microfluidics a powerful tool for the PSC niche. For example, Agarwal et al. described a device that formed microdroplets with an alginate hydrogels outer shell and an inner aqueous core consisting of mESCs.\textsuperscript{[100]} Microdroplets can be used to encapsulate cells and are formed mostly by shear force\textsuperscript{[101]} or flow focusing\textsuperscript{[102]} of immiscible liquid reagents.

In another method, known as digital microfluidics, droplets are controlled, split, merged, and dispensed using potentials applied to insulated electrodes.\textsuperscript{[103]} Digital microfluidics is performed based on several techniques, including dielectrophoretic or electrowetting-on-dielectric technique, which have been well-reviewed elsewhere.\textsuperscript{[103,104]} Advantages of digital microfluidics include reduced clogging and the high range of volume that can be controlled without valves or pumps. However, it suffers from drawbacks including evaporation in low-volume drops in open systems. Further, gas exchange can be limited if oil encapsulation is used to create the droplets, which can further inhibit the use of this method in conjunction with cell culture. The use of liquid-marble droplet-based methods, which can function as 3D bioreactors,\textsuperscript{[105,106]} can help in circumventing these problems. Liquid-marble droplet-based micro-bioreactors, using hydrophobic polytetrafluoroethylene particles, have been shown to induce cardiogenesis in mESC EBs.\textsuperscript{[107]}

4.2. Materials

The materials generally used for manufacturing microfluidic platforms can be classified into three main categories: elastomers, thermoplastics, and hydrogels. Glass was used originally for devices for analytical purposes like chromatography, but with the adaptation of microfluidics to the field of biology, elastomers (especially polydimethylsiloxane (PDMS)) have become the material of choice.\textsuperscript{[56]} Advantages of PDMS include bio-compatibility, easy bonding to glass or other PDMS substrates, low Young’s modulus (1.32–2.97 MPa),\textsuperscript{[108]} at the expense of unsuitability in some applications involving actuation where also the housing deforms), high transparency, and low autofluorescence.\textsuperscript{[109]} The disadvantages of PDMS are the absorption of small molecules,\textsuperscript{[110]} such as drugs or growth factors, as well as lack of automated fabrication. Another drawback is the change in volume of the cell-culture medium and concentration of various components, resulting from evaporation as a result of PDMS’s high permeability to water vapor.\textsuperscript{[111,112]} This change can disrupt long-term stem cell culture, especially in small microchannels. Although direct PDMS microstructuring techniques avoiding the use of a master template are available with a resolution of 5–10 µm,\textsuperscript{[113]} they have still not been widely adapted by biologists.

The second common material for microfluidics fabrication is thermoplastic polymers, which include polystyrene (PS), polymethylmethacrylate (PMMA), cycoolefin copolymer and polymer (COC/COP), or polycarbonate (PC). These materials have excellent biocompatibility, are nonabsorbent/adsorbent for biomolecules, and optically clear, which make them ideal for long-term stem cell culture and imaging. A drawback for thermoplastics is that the materials have a high Young’s modulus in the lower GPa range, approximately three orders of magnitude higher than PDMS and several orders of magnitude higher than most biological tissues.\textsuperscript{[76]} However, this can be resolved using thin hydrogel coatings or hydrogel plugs as a substrate for culturing cells within these thermoplastic chips. Thermoplastic chips are manufactured by variations of embossing,\textsuperscript{[114]} molding,\textsuperscript{[115]} micromilling,\textsuperscript{[116]} and laser ablation.\textsuperscript{[117]}

Fabrication of microstructures with hydrogels is challenging since their resolution is often in the micrometer range (≈10 µm) compared to the nanometer range of thermoplastics (≈100 nm) and elastomers (<1 µm).\textsuperscript{[112]} They also exhibit high absorption and adsorption of small biomolecules and typically have lower optical transparency compared to the other classes of materials described above.\textsuperscript{[112]} Nevertheless, there have been instances of hydrogel (gelatin- and agarose-based) microfluidic devices for mESC culture and neuronal differentiation.\textsuperscript{[118]} Since hydrogels have a considerably low Young’s modulus (in kPa range), they are well suited to mimic extracellular matrix and the cellular environment of soft tissues.

4.2.2. Scaffold Materials

The most widely used scaffold material in conjunction with PSCs are hydrogels. Microfluidic structures have also been devised using hydrogels to study cell migration and mimicking matrix elasticity of soft tissues, for example, engineered
Hydrogels can be of either synthetic or plant/animal origin; the more common ones are prepared from agarose (plant origin), collagen (animal origin), or polyethylene glycol (PEG, synthetic). Hydrogels are characterized by a high water content (often 90–99%). These classes of materials have a considerably low Young’s modulus (in kPa range), which is one of the reasons why hydrogels are well suited to mimic extracellular matrix and the cellular environment of soft tissues. Commercially available hydrogels form an especially dominant class of materials used for culturing PSCs. However, many of these hydrogels are derived from a biological source (e.g., Matrigel, which originates from a mouse sarcoma cell line), which leads to problems such as batch-to-batch variations, long-term instability, and difficulties in accessing entrapped cells.

Figure 5. Diverse materials are used for fabrication of microfluidic chips, and the selection of these materials is usually based on the required application. Along with biocompatibility, one of the main criteria for selection is material stiffness (the options for which can range from kPa to GPa range, top) because changes in gene expression are directly associated to material stiffness. For stem cell biology, it is further desirable to have a material that does not adsorb and absorb any biomolecules (middle). Unfortunately, a drawback of using soft materials like PDMS and hydrogels is that they can take up the biomolecules and desorb them during a later stage, thereby eliciting unwanted responses from cells. Optical properties as well as ease of fabrication (bottom) also play a major role in material selection \( (Y = \text{Young's modulus, PMMA} = \text{poly(methyl methacrylate)}) \).

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In tandem with current microengineered tools to control the cell-culture microenvironment, using microfluidics for studying developmental processes offers major advantages. Microfluidics enables the rapid exchange of medium in small volumes, which can be used to control the exposure to growth factors over rapid intervals of time, thus leading to high spatiotemporal resolution.

The flow rate can be tuned to exert shear forces on biological systems, thereby giving control over an additional parameter that is known to influence, for example, mESC behavior by transduction of heparin sulfate proteoglycans. Active flow for 3D cell culture removes metabolites and provides a better supply of nutrients and gas, thereby enabling the growth of bigger aggregates (usually determined by the diffusion rate of oxygen and other nutrients/metabolites and in the range of a few hundred micrometers), without having a functional vasculature in place. Flow behavior can be measured using several tools, such as particle flow velocimetry, standard flow sensors (which do not show the velocity profile), and Doppler effect-based techniques (optical Doppler tomographic imaging).
Microfluidics requires lower volumes of growth factors, thereby reducing the overall consumable costs.

In addition, flow behavior and concentration profiles in microfluidics can be computationally modeled and predicted using commercial software, such as MATLAB (script-based mathematical analysis), COMSOL (graphical user interface (GUI)-based multiphysics with a specialized microfluidics module), and ANSYS (GUI-based multiphysics, with a specialized computational fluid dynamics module). Other computer-aided design software, such as Autodesk Inventor and SolidWorks, are also offering increasingly intuitive simulation capabilities. These advantages of microengineered tools, in particular microfluidics, have facilitated its application to in vitro stem cell biology, which is discussed in detail below.

5. Materials and Microengineered Systems for 3D Pluripotent Stem Cell Cultures

Generally, 2D cell-culture platforms offer better accessibility of surfaces for functionalization, the possibility for modification by subcellular topographies, and simplified read-out (including imaging). However, culturing cells in a 2D environment can lead to changes in cellular and nuclear shape compared to physiological conditions, which in turn can change the expression of certain genes in mammalian cells.[121,124] Cells cultured under similar experimental conditions can give varied results depending on whether they are cultured in 2D or 3D. For example, certain mammalian cells grown in 2D have been shown to be more susceptible to cytotoxic agents compared to those grown in 3D.[125,126] Due to increased cell–cell contact as well as interaction with endogenously produced ECM, 3D cell cultures are more physiologically relevant.[127,128] Certain proteins are mainly present on the ventral surface (basal surface in contact with the substratum) of a cell when grown in a monolayer but show a more uniform distribution in a 3D culture.[126] It has been shown that this can aid in mimicking developmental processes and allows for differentiated structures with a more complex pattern.[127] Enhanced differentiation of PSCs along certain lineages has also been shown using 3D culture, for example, mESCs underwent enhanced chondrogenesis when cultured as EBs, compared to cultures grown on 2D petri dishes.[129] For these reasons, 3D cell cultures are preferred as model systems for stem cell biology, cancer biology, and tissue engineering.[128]

Application of currently used materials for 3D PSC culture can be broadly classified into cellular interfaces with either liquid or solid-based materials. While the former refers to the use of engineered materials to control and manipulate the fluidic microenvironment surrounding an aggregate, the latter includes interactions of the aggregate or cells with a material either by: (a) incorporation of the material within a cellular aggregate or (b) incorporation of the cells or aggregates within the material. Encapsulated microbeads have been used within the field of developmental biology in the past, especially to study developmental effects of growth factors on appendage development.[130,131] For example, FGF-coated heparin acrylic beads[131] and TGFB1-coated agarose beads[130] were implanted in chicks to study limb development. Because the incorporation of morphogen-soaked microbeads can lead to a spatial gradient of the morphogen within the aggregate, studies have been conducted to determine the effects of gelatin, poly(lactic-co-glycolic) acid (PLGA) and agarose microbeads on mESC aggregates, and incorporation of these materials did not interfere with PSC differentiation.[132] Building on these results, the group further showed that by using 12-fold less BMP4 and noggin delivered through gelatin microparticles compared to solubilization in media, similar gene expression of mesoderm and ectoderm was obtained in mESC aggregates.[133]

The potential of microengineered tools for growth factor-containing microparticles was showcased in another study where gelatin microparticles laden with BMP4 were incorporated into mESC aggregates and fused with aggregates containing no microparticles using a PDMS-based microfluidic trap, which could generate “controlled differentiation patterns in fused multicellular assemblies” (Figure 6a).[134] The fused aggregates resembled certain aspects of processes similar to mouse embryonic day 6.5 and expressed the mesodermal protein Brachyury.[134]

The second method of inducing PSC–solid material interaction is via encapsulation of the cells within a material, usually a hydrogel. The typical characteristics of hydrogels used in conjunction with PSCs have been described earlier in this review (Section 4). Hydrogels that mimic chemical, physical, and biological properties of native tissue have been increasingly used with PSCs to direct differentiation and morphogenesis.[140,141] Advantages of using hydrogels for PSC encapsulation include the ability to incorporate ECM proteins, control of elasticity, and ability to adapt hydrogels for processes like 3D printing.[141]

In addition to the materials for 3D PSC cultures, microengineering has led to advanced systems that provide higher reproducibility and greater accuracy. A micro- and nanoscale forming and functionalization technology for 3D polymer film, termed substrate modification and replication by thermoforming (SMART), may enable spatiotemporal control over 3D stem cell cultures.[72] Cell-based studies have shown that SMART can be used to form complex film-based microstructures for 3D in vitro studies or to develop corresponding models.[70,71]

Current microengineered systems can also be adapted to be used with traditional well plate systems, as demonstrated with microthermoformed cycloolefin copolymer films containing 144 microcavities (with inner diameter of 300 μm and depth of ≈260 μm) per well of a 96-well plate, for mESC-derived organoid culture.[142] The system was used for high-throughput screening and high-content imaging to check for primitive endoderm differentiation using a nuclear fluorescent reporter for PDGFRα, a marker for the primitive endoderm. Square microwells with side lengths of 800, 400, 200, and 100 μm have been utilized for hESC culture, and EBs described in the study showed self-organization and tissue-level morphogenesis, in certain aspects analogous to embryogenesis.[143] In another study, agarose microwells were used to culture EBs derived from hiPSCs and hESCs, which showed differentiation into the three germ layers.[164] The use of PEG microwells to initiate formation of embryoid bodies can lead to a spatial gradient of morphogen within the aggregate, studies have been conducted to determine the effects of gelatin, poly(lactic-co-glycolic) acid (PLGA) and agarose microbeads on mESC aggregates, and incorporation of these materials did not interfere with PSC differentiation.[132] Building on these results, the group further showed that by using 12-fold less BMP4 and noggin delivered through gelatin microparticles compared to solubilization in media, similar gene expression of mesoderm and ectoderm was obtained in mESC aggregates.[133]
agarose wells of different shapes and sizes,[146] used bottom-up engineering to develop centimeter-scale complex 3D tissues using human mesenchymal stromal cells (hMSCs) and mESCs. In another interesting study, a hanging drop method adapted to a 384 hanging-drop array facilitated spheroid formation from African green monkey kidney fibroblast cells, mESCs, and human epithelial carcinoma cells.[147] The platform was created from PS via injection molding and was used for high-throughput screening of anticancer drugs on human epithelial carcinoma cells.[147] a methodology that could be adopted to test differentiation or proliferation potential of various morphogens.

Building on the advantages of available microengineered platforms, several microfluidic systems have been developed to expose 3D aggregates to highly defined gradients in order to control cell fate. The usual material used to fabricate such devices is PDMS. Kim et al. used a “(flow) resistance network” using a PDMS device to control the size of EBs derived from embryonal carcinoma cells and a microwell-based system for differentiation (Figure 6b).[135] The authors found that the number of cells trapped per microwell and size of EBs were dependent on the time and flow rate of the suspension over the wells. On-chip differentiation of the cells toward the neuronal lineage was shown to be possible after exposure to retinoic acid.[135] Gradients of retinoic acid delivery also led to neural differentiation of mESC EBs.[135] Another microfluidic system was shown to be capable of capturing mESCs and hESCs in PDMS traps to form EBs, followed by polarized differentiation using multipie segmented traps (Figure 6c).[136] In another recent microfluidics application, a PDMS-based chip capable of mimicking the in vivo spatiotemporal (chemical) microenvironment induced in vitro neural tube patterning using mESCs embedded in a 3D Matrigel or Geltrix matrix (Figure 6d).[137] A platform consisting of PDMS microcavities with width of 300, 500, or 700 µm was used to form EBs from mESCs, upon which the EBs were shown to be capable of expressing genes from all three germ layers upon further culture.[138]

Chips made using thermoplastic have also been used for stem cell culture. After merging PMMA, PDMS, and glass to form a multilayered chip, it was shown that laminar flow could be induced in the chip to control two separate flow streams within the same microchannel, with EBs in the center region (Figure 6e).[138] The authors were able to differentiate half of the EBs by exposure to differentiation media, while maintaining pluripotency of the other half using basal media. A microfluidic bioreactor “3D-KITChip” was manufactured using a combination of microinjection molding/microhotembossing and microthermoforming (Figure 6f).[139] The modular bioreactor consisted of a polyether ketone (PEEK) housing, PDMS gaskets, and integrated glass coverslips while the microcavity chip was made from PC. Using the platform, hMSCs were cultured along with umbilical cord blood hematopoietic progenitor cells (HPCs) in microwells of diameter 300 µm for 14 d. The use of 3D coculture platform led to better preservation of hematopoietic stem cell plasticity and provided a better physiological microenvironment for CD34 expressing human hematopoietic progenitor cells compared to monolayer coculture.[139] The flexibility of this bioreactor platform also enables its use with 3D culture of different cell types including PSCs.

Generation of gradients across a hydrogel plug has been shown to be possible using a “ladder chamber,” where hydrogel grooves are placed between a source and a sink microchannel to create diffusion-based gradients.[149] The platform was especially important to study the chemotaxis of cells along a gradient of any signaling molecules, without exposing the cells to fluid flow and shear stress. The drawback of such gradients is that they are inherently unstable and change over time, leading to unpredictable cell response. A different approach also using a hydrogel-based microfluidic setup in a 24-well tissue culture plate[118] could be used for both adherent (gelatin-based chip) and nonadherent EB culture (agarose-based chip) (Figure 6g).

In another microfluidic system, porous polycarbonate films between PDMS channels were used to form mESC-derived EBs.[130] Uniformly sized EBs were reproducibly obtained, and the size was shown to be dependent on the cross-sectional area of the (upper) microchannel. To generate better control over 3D cell cultures, agarose hydrogels have been modified to include photolabile substrates to guide patterning and migration of mammalian neural cells.[151] Spatial photopatterning of PEG hydrogels was shown to be possible and was used to control hMSC invasion in 3D.[152] Both studies were conducted using single-photon lasers, and adapting such systems with multiphoton lasers is expected to increase the resolution in such systems.

Despite the advantages of the described 3D cell-culture platforms, it is important to note that the cell differentiation and patterning processes in these systems, although predictable to a certain degree, are still highly heterogeneous and stochastic at a cellular level and difficult to control precisely. This makes it difficult to translate results from these systems to physiological states. Differentiation of 3D stem cell cultures in microfluidic systems is usually performed by exposing either the whole or only large regions of the 3D aggregate to soluble chemical factors, which is not analogous to natural organogenesis.[134,135,138] Gradients in most microfluidic systems also often consist of only one morphogen, whereas a series of parallel or antiparallel gradients of several morphogens control morphogenesis in vivo. As the field progresses, we foresee the development and application of more advanced systems in conjunction with in vitro morphogenesis.

Figure 6. Microengineered platforms using (a combination of) varied materials for stem cell culture. a) Elastomer-based microfluidic device for pattern formation by fusing mESC EBs. Reproduced with permission.[136] Copyright 2013, Royal Society of Chemistry. b) Microfluidic resistance network in elastomer chip for p19 EB differentiation. Reproduced with permission.[135] Copyright 2011, Royal Society of Chemistry. c) Microfluidic trap system in elastomer chip for prolonged culture of mESC and hESC EBs. Reproduced with permission.[130] Copyright 2010, Springer Nature. d) Reconstructing in vitro neural tube patterning using mESCs in an elastomer-hydrogel-based microfluidic device. Reproduced with permission.[137] Copyright 2016, The Company of Biologists Ltd. e) Multicomponent thermoplastic/elastomer chip for mESC differentiation using laminar flow across EB. Reproduced with permission.[134] Copyright 2009, Royal Society of Chemistry. f) Thermoplastic microfluidic bioreactor for coculture of HPCs and MSCs. Reproduced with permission.[139] Copyright 2016, Springer Nature. g) Hydrogel microfluidic chip for spatiotemporally controlled biomolecule gradients to mESC EBs. Reproduced with permission.[138] Copyright 2014, Springer Nature.
tissues or organizers that are absent or cannot be induced in vivo. Chick signaling and forces from maternal and extraembryonic tissues that cannot easily be recapitulated in vitro. Mimicking rudimentary stem cell-based models with structures as bioartificial “form givers” (Greek, “Morphogen”) and complement growing microfluidic platforms will increasingly serve the concept of “morphogenetic engineering” introduced by Renee Doursat.[153] Microengineered platforms will increasingly serve the development provides stimulating prospects thus far, and progress in the field shows that mimicking morphogenesis in vitro is closer than ever. We expect the development of exciting bioengineering models, which better mimic embryonic development, will help in elucidating key factors, which control tissue and organ development. Using gene editing techniques like CRISPR-Cas9 can further accelerate the discoveries of such factors. Advanced high-throughput molecule screening platforms will complement this approach by systematically assessing the performance of small molecules on morphogenesis without the need for large animal studies and also help in solving problems like infertility. As technology and biology develop, we foresee platforms that can automatically culture functional tissue without human intervention, thereby reducing labor and costs involved in fabricating artificial organs. This would greatly benefit areas like regenerative medicine and aid in increasing the quality of life of patients.

6. Future Perspective

The combination of advanced materials and microengineering techniques creates new avenues for the fabrication of microfluidic platforms, which will help support and further unravel embryonic development in vitro. Recapitulation of embryonic development provides stimulating prospects thus far, and progress in the field shows that mimicking morphogenesis in vitro is closer than ever. We expect the development of exciting new microfluidic tools in an emerging field we term “morphogenetic microengineering.” (Figure 7) inspired by the broader concept of “morphogenetic engineering” introduced by Renee Doursat.[153] Microengineered platforms will increasingly serve as bioartificial “form givers” (Greek, “Morphogen”) and complement rudimentary stem cell-based models with structures and tissues that cannot easily be recapitulated in vitro. Mimicking signaling and forces from maternal and extraembryonic tissues or organizers that are absent or cannot be induced in the biological models, for example, through creating bioartificial signaling centers, will help to determine reliable and fixed extrinsic coordinate systems and will enable us to take these models to the next level. For example, a recently published study has shown that using engineered gradients of two morphogens, BMP and Nodal, created using mRNA microinjection, was sufficient to form a zebrafish embryo from uncommitted cells.[154] Formation and manipulation of such gradients consisting of multiple morphogens is an example where microfluidic platforms could play a vital role in the future. By creating spatiotemporally controllable artificial signaling centers and fate-determining morphogenetic gradients, these tools can serve as programmable and adaptive coordinate systems to initiate and guide critical developmental events, such as self-organization/self-assembly of cells, (synthetic) morphogenetic processes, symmetry breaking, and/or establishment of stable tissue boundaries in advanced stem cell-based 3D in vitro models. Further knowledge about tissue morphogenesis and development can be gained by application of gene editing tools like CRISPR-Cas9[155–157] (Figure 7). For example, CRISPR-Cas9 can be applied to in vitro morphogenetic models to perform loss-of-function or gain-of-function studies and elucidate the role of genes in development without utilizing embryos.

This rapidly developing discipline will not only help to gain deeper insights into fundamental morphogenetic processes, such as gastrulation, but also envisaged to pave the way for developmental biology to high-throughput testing platforms (Figure 7). This becomes even more relevant considering mouse iPSCs and mESCs were recently shown to be capable of differentiating into viable totipotent oocytes, which upon in vitro fertilization and implantation in mice, led to the birth of viable progeny.[158,159] This process could lead to potentially infinite egg cell production, an attractive source of early embryonic microtissues after artificial fertilization, although the ethical and moral implications of the method will first need to be resolved. Reproducible and reliable patterning of such microtissues in vitro on a large scale, for example, with accurately aligned polarity, spatiotemporally defined organization of germ layers and “body” axes, will grant access to new developmental and pharmaceutical/toxicological studies, and might even lead to clinical applications (Figure 7).[40] The knowledge and tools to eventually control pattern formation and differentiation in vitro will in turn facilitate researchers to develop new and more sophisticated tools and automated culture platforms for biomedical applications to better support and improve regeneration in adult organisms. Lack of control over such differentiation and pattern-formation events post-trauma or disease is one of the major reasons why functional tissue cannot be fully restored in clinics today. Newly devised materials and tools to contain inflammation at the site of an injury and to enable modulation of local morphogen gradients may increase restoration of function by inducing regionally controlled stem cell migration, proliferation, and differentiation. The development and application of new platforms to exert control with increasing spatiotemporal resolution will therefore be an indispensable tool for tissue engineering and regenerative medicine in the future. This epic voyage has just begun!
Acknowledgements

R.T. and S.G. contributed equally to this work. The authors acknowledge financial support by the Dutch Province of Limburg (program “Limburg INvesteert in haar Kenniseconomie/LINK”).

Conflict of Interest

The authors declare the following competing interests: R.T. and S.G. are founders and shareholders of 300MICRONS GmbH.

Keywords

artificial organogenesis, cell–material interactions, microfluidics, morphogenetic microengineering, pluripotent stem cells

Received: September 5, 2018
Revised: January 4, 2019
Published online: February 14, 2019
