ANNUAL REVIEWS

Annual Review of Biomedical Engineering

Derivation and Differentiation of Human Pluripotent Stem Cells in Microfluidic Devices

Camilla Luni,[1] Onelia Gagliano,[2,3] and Nicola Elvassore[2,3,4]

[1]Department of Civil, Chemical, Environmental and Materials Engineering (DICAM), University of Bologna, Bologna, Italy; email: camilla.luni@unibo.it

[2]Department of Industrial Engineering, University of Padova, Padova, Italy; email: onelia.gagliano@unipd.it, nicola.elvassore@unipd.it

[3]Veneto Institute of Molecular Medicine (VIMM), Padova, Italy

[4]Stem Cell and Regenerative Medicine Section, Great Ormond Street Institute of Child Health, University College London, London, United Kingdom

Annu. Rev. Biomed. Eng. 2022. 24:231–48

First published as a Review in Advance on April 4, 2022

The *Annual Review of Biomedical Engineering* is online at bioeng.annualreviews.org

https://doi.org/10.1146/annurev-bioeng-092021-042744

Copyright © 2022 by Annual Reviews. This work is licensed under a Creative Commons Attribution 4.0 International License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited. See credit lines of images or other third-party material in this article for license information

ANNUAL REVIEWS CONNECT

www.annualreviews.org

- Download figures
- Navigate cited references
- Keyword search
- Explore related articles
- Share via email or social media

Keywords

miniaturization, microtechnology, microfluidics, cytological techniques, cellular reprogramming techniques, stem cell research

Abstract

An integrative approach based on microfluidic design and stem cell biology enables capture of the spatial-temporal environmental evolution underpinning epigenetic remodeling and the morphogenetic process. We examine the body of literature that encompasses microfluidic applications where human induced pluripotent stem cells are derived starting from human somatic cells and where human pluripotent stem cells are differentiated into different cell types. We focus on recent studies where the intrinsic features of microfluidics have been exploited to control the reprogramming and differentiation trajectory at the microscale, including the capability of manipulating the fluid velocity field, mass transport regime, and controllable composition within micro- to nanoliter volumes in space and time. We also discuss studies of emerging microfluidic technologies and applications. Finally, we critically discuss perspectives and challenges in the field and how these could be instrumental for bringing about significant biological advances in the field of stem cell engineering.

OPEN ACCESS CC BY

Contents

1. INTRODUCTION 232
2. SOMATIC CELL REPROGRAMMING AT THE MICROSCALE 233
   2.1. Microfluidic-Based Methods for Delivery of Reprogramming Exogenous Signals 234
   2.2. Reprogramming in a Confined Environment 234
3. HUMAN PLURIPOTENT STEM CELL DIFFERENTIATION AT THE MICROSCALE 238
   3.1. Embryo Development–Inspired Differentiation 239
   3.2. Forward Programming 241
4. TOWARD A FULLY INTEGRATED PROCESS 242
5. TECHNOLOGICAL DIRECTIONS 243
   5.1. Toward GMP Compliance in hiPSC Derivation 243
   5.2. Role of PDMS in Microfluidics 243
   5.3. Microfluidics for hPSC Microencapsulation 244
6. CONCLUSIONS AND PERSPECTIVES 244

1. INTRODUCTION

Human pluripotent stem cells (hPSCs) are cells that have the potential to produce any tissue of the human body (1). Thus, there is a strong interest in using them for clinical applications (2) and to derive human tissues in vitro (3, 4). In vitro models of human tissues have a number of applications: fundamental studies of diseases and embryonic development, drug screening in preclinical trial stages, and personalized medicine (4). Two main challenges to fulfilling these expectations are developing trackable, reproducible, and cost-effective methods to produce high-quality hPSCs and finding the conditions to make hPSCs express their developmental potential in vitro.

The first method used to obtain hPSCs was their derivation from the inner cell mass of the human embryo at the blastocyst stage, as human embryonic stem cells (hESCs), by Thomson et al. (1) in 1998. These hESCs can be expanded in vitro almost indefinitely and have the potential to develop all tissues of the human body. They have been extensively used for two decades in more than 10,000 research studies, and the research community has greatly benefited from several available hESC lines. However, derivation of hESCs involves human embryos, and thus their availability is restricted. Almost 10 years after the first hESC line derivation, a process for inducing pluripotency into adult somatic cells, called reprogramming, was developed by Yamanaka and colleagues (5). The cells obtained by reprogramming are called human induced pluripotent stem cells (hiPSCs). The use of hiPSCs is devoid of ethical concerns, and they can be obtained from an adult patient to recapitulate in vitro a model of a specific tissue with a matched genetic background. Thus, hiPSCs have a definitive advantage over hESCs for personalized medicine applications. On the other hand, their derivation by reprogramming is still poorly understood in its molecular mechanisms and is difficult to control, with the consequences of variable quality, success rate, and efficiency (6).

Both hESCs and hiPSCs have the potential to produce any tissue of the human body through a process called cell differentiation (4). However, to this aim, even if evolving at a fast pace, differentiation protocols still require additional development to be further improved (4, 7). hPSCs on their way to becoming a fully differentiated tissue are very sensitive to environmental cues. This intrinsic plasticity of hPSCs allows us to fully capture their developmental potency in vitro, but

it also requires technological advances for dynamic control of their behavior during differentiation. At the same time, they display a high level of self-regulation that coordinates the phenotypic changes within the cell population and organizes the spatial arrangement of different types of cells in tissues (8).

Overall, there is a technical challenge in improving the reprogramming and differentiation processes that is represented by our ability to control the environment where the cells reside, at the micrometer scale (cell diameter ranges from ~10–30 μm) (7). More specifically, the culture system should be able to control the delivery of exogenous stimuli without completely overwriting the cellular self-regulation that occurs by autocrine and paracrine communication. Moreover, the culture system should be able to provide spatial gradients of regulatory molecules with appropriate timing.

The ensemble of technological solutions able to manipulate the fluid velocity field, mass transport regime, and controllable composition within micro- to nanoliter volumes is collectively indicated as microfluidics (9). It has been shown that microfluidics allows culture of stem cells, including hiPSCs, in simple and complex configurations (10). In all of these microfluidic systems, the fundamental unit, which has quite similar design across applications, is the microfluidic cell culture chamber. This chamber typically has a volume of a few microliters and a height of a few hundred microns. In this geometrical configuration, compared with conventional culture systems, cell-secreted molecules are accumulated, whereas the large amount of medium strongly dilutes endogenous molecules. Thus, the relative balance between exogenous and endogenous molecules in the culture medium can be finely controlled in the microfluidic experimental setup. This control can be accomplished by optimizing the perfusion in terms of (*a*) flow rate, if perfusion is continuous (11), and (*b*) frequency of medium change, if perfusion is discontinuous (12). A further advantage of using perfused microfluidic systems is that the established fluid flow regime therein is laminar flow, with a completely defined velocity field (13). Thus, exogenous biochemical stimuli can be provided with high spatial resolution, combining geometrical configurations and tuning diffusion rate relative to convection rate.

Here, we examine the body of literature that encompasses microfluidic applications where hiPSCs are derived starting from human somatic cells and where both hiPSCs and hESCs are differentiated into different cell types. We focus on recent studies where the intrinsic features of microfluidics, described above, have been exploited to control the reprogramming (from somatic cells to hiPSCs) and differentiation (from hPSCs to differentiated progenies). In this review, we first summarize studies that performed reprogramming and differentiation at the microscale, including emerging microfluidic applications. Then, we critically discuss perspectives and challenges in the field and how these could be instrumental for deriving a seamless process from somatic cells to hiPSC-derived differentiated cells. The focus is primarily on works that have brought significant biological advances rather than technological advances but that could not have been performed without the contribution of microfluidic technology.

2. SOMATIC CELL REPROGRAMMING AT THE MICROSCALE

Reprogramming is the process that converts human somatic cells, such as fibroblasts, blood-derived cells, and urine-derived cells, into an embryonic-like pluripotent state (5, 14, 15). For this conversion to occur, cells are induced to express a combination of transcription factors (originally, OCT4, SOX2, KLF4, and MYC, collectively known as OSKM, or Yamanaka's factors) (5) delivered by retroviruses, lentiviruses, plasmids, mRNAs, or proteins (16). The effective delivery of these vectors is critical for successful reprogramming (17). Thus, we first review applications where microfluidics was applied only to increase the efficiency in the delivery of

pluripotency-related transcription factors. Next, we discuss studies where microfluidic technology was used to support the full reprogramming process.

2.1. Microfluidic-Based Methods for Delivery of Reprogramming Exogenous Signals

The literature on microfluidic setups for optimal vector delivery is quite vast and has been reviewed already (18). Here, we focus on applications more closely related to the somatic cell reprogramming field.

Microfluidic devices have shown a few advantages for intracellular delivery of exogenous material, compared with conventional culture systems. First, this delivery is based on diffusion-reaction or diffusion-adsorption mechanisms, which take place at the cellular membrane. Given the small height of the microfluidic channel, the exogenous signals need to diffuse a shorter distance from the bulk medium to hit the cell membrane (**Figure 1*a***). Indeed, the probability of intracellular delivery is highly increased. This is an advantageous aspect in that it reduces the amount of the often very valuable exogenous material to be delivered, and it makes the overall process particularly cost effective. An example has been shown for the delivery of mRNAs for somatic cell reprogramming, as discussed below (19). Furthermore, this delivery can be improved by adding convective transport, accomplished by perfusion of the microfluidic chambers, to obtain high temporal resolution (**Figure 1*b***).

Microfluidics can be coupled with other technologies, such as electrofusion or electroporation, to deliver exogenous material inside the cells, according to different physical mechanisms. In these applications, the use of microfluidics is due to its defined fluid dynamics, and the laminar flow that develops within microfluidic devices is used for localizing processes with micrometer resolution (**Figure 1*c***). Skelley et al. (20) applied this feature to localize two cell types, murine fibroblasts and embryonic stem cells, in contact with each other inside micrometer traps. Then cell fusion was promoted by the application of an electric field. The resulting tetraploid cells were subsequently cultured in conventional wells, forming pluripotent colonies, with fibroblast nuclei reprogrammed by factors from the embryonic stem cells. Okanojo et al. (21) showed the possibility of reprogramming somatic cells via the delivery of the cytoplasm of an hiPSC, without nuclear sharing. The mechanism was grounded on the coupling of microfluidic-based cell localization and membrane electrofusion. The resulting diploid cell could potentially undergo reprogramming thanks to the reprogramming factors provided by the pluripotent stem cell cytoplasm, although the study does not demonstrate the actual feasibility of the full reprogramming process. This system has much lower throughput than the previous one but may be relevant for fundamental studies. Microfluidics has been used also to create physical constrictions and flow human fibroblasts through a microchannel one by one (22, 23). In the first study, OSKM proteins were delivered into the cells by mechanically inducing transient membrane disruption when cells passed through constrictions (22); in the second study, DNA minicircles were delivered by inducing transient membrane disruption by ultrashort laser pulses in a specific section of a microfluidic channel (23). In both studies, the actual cell reprogramming culture occurred in conventional systems afterward, and microfluidics was used only as an exogenous material delivery tool.

2.2. Reprogramming in a Confined Environment

In 2016, for the first time, microfluidics was used not only for vector delivery but also as a reprogramming environment (19). This work unexpectedly revealed that microfluidic culture strongly promotes the reprogramming process, leading to high-efficiency conversion (~50-fold higher compared with conventional methods) of somatic cells into hiPSCs. In this work, an mRNA-based method was adopted, and microfluidics was used to enhance the efficiency of

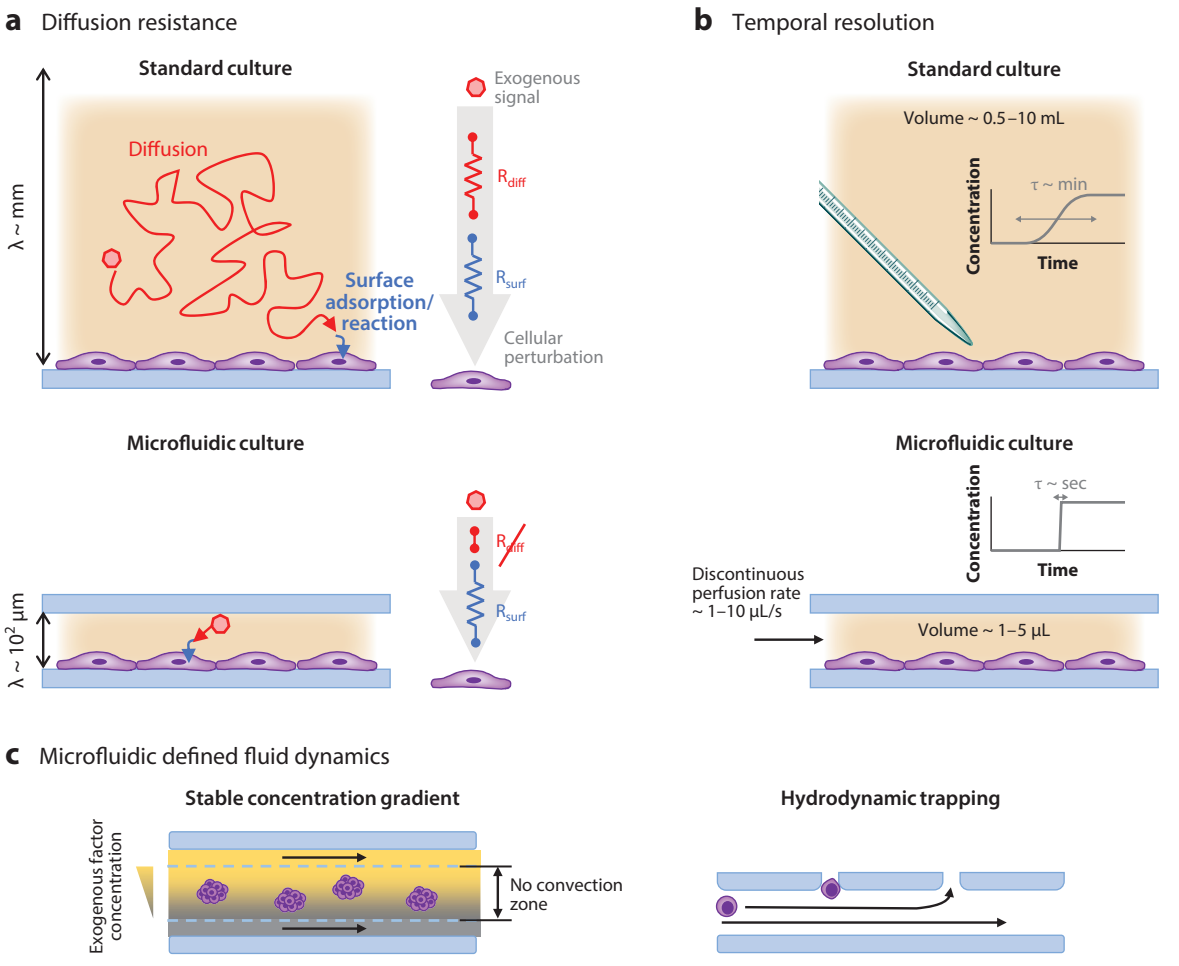


Figure 1

Schematic representation of microfluidic-specific physical phenomena that have been already applied in human pluripotent stem cell reprogramming and differentiation. (*a*) Relative rate of diffusion and surface adsorption or reaction. Due to the small microfluidic chamber height, the diffusive resistance to mass transport is almost completely abolished compared with what occurs in a standard large-scale culture system. On the right, the analogy with an electrical circuit is displayed: The resistance to diffusion, R_{diff}, and the resistance to adsorption or chemical reaction occurring at the cell surface, R_{surf}, are in series. (*b*) Different temporal resolutions achievable in the two culture systems. To provide a specific exogenous stimulus at a defined time point, the medium is changed. In standard large-scale culture systems, medium change is often accompanied by one or two washes with buffer, for an overall time of procedure of a few minutes. In microfluidics, flowing with three or four times the volume of the culture chamber to wash and replace the medium takes a few seconds. Insets show how much steeper the change in concentration of a specific stimulus in microfluidics is compared with a standard system. (*c*) Microfluidic fluid dynamics. The velocity and concentration fields are defined and predictable due to the laminar flow. Two examples of how laminar flow can be advantageously used are shown. On the left, the generation of a stable concentration gradient of gases or soluble signals is achieved by confining the convective flow in the two lateral channels. The middle channel, where the gradient develops, can host a 2D culture or 3D cell aggregates, either in stagnant medium or in hydrogel. On the right, the principle of hydrodynamic trapping is shown. Microfluidic geometry can be designed to localize cells or cell aggregates in specific positions with micrometric precision.

transfection of mRNAs encoding for Yamanaka's factors and the NANOG and LIN28 transcription factors. This approach, which used a nonintegrating system, allowed for the smooth transition from a somatic state to pluripotency of fibroblast lines as well as other primary cells from patients.

Interestingly, using culture chambers with different heights and different mRNA concentrations, the authors showed that this enhancement was due to a scale effect. The confined environment was able to accumulate still unidentified reprogramming-promoting cell-secreted factors (**Figure 2*a***). The resulting hiPSC colonies were transcriptionally undistinguishable from their multiwell-plate counterpart after only four passages. Overall, this study provided a tool to perform many parallel reprogramming experiments in a cost-effective manner with high efficiency and also shed light on the importance of self-regulation (autocrine and/or paracrine) in this process (**Figure 2*b***). The optimized protocol was recently reported (24) and a comparison of its outcome with nonmicrofluidic works in the literature is shown in **Figure 2*c***.

Conventionally reprogrammed human cells acquire a phenotype resembling a stage of so-called primed pluripotency, which is developmentally more advanced than the corresponding state emerging from murine reprogramming, called the naive state (25). Primed hiPSCs have been shown to have a differentiation bias toward some germ layers, compared with their naive counterparts (26). Thus, current reprogramming efforts focus on obtaining naive hiPSCs, for their relevance in subsequent applications. Giulitti and colleagues (27) demonstrated that the same microfluidic device used in their previous study (19) also promotes the acquisition of naive pluripotency under modified reprogramming conditions. This study showed that, as in primed reprogramming, the process is promoted by the positive effect of autocrine and paracrine signaling, which are enhanced in a microfluidic confined environment.

Not only microfluidics but also hydrogel-based strategies have been used to create a confined environment that is able to enhance the reprogramming process, as shown in a study by Lin et al. (28). Human fibroblasts were first electroporated with a reprogramming plasmid mixture and then cultured in alginate hydrogel tubes. hiPSCs emerged with high efficiency and high purity inside the alginate tubes and expanded, filling the microtube to a 400-μm diameter. The increased efficiency in cell reprogramming inside hydrogel is likely due to the selective permeability of these hydrogels and their ability to regulate molecular diffusion of signals between the external medium and the inner part of the microtubes.

Overall, the field of microfluidic reprogramming is still young. However, it has high potential for good manufacturing practice (GMP)-conforming applications, by taking advantage of setups that can be easily run in full automation and in a closed loop. This allows continuous control over the cell process within a closed environment, minimizing risks of contamination. However, there are still some aspects of cellular reprogramming that need to be changed before moving to a chemically defined and GMP-grade system (i.e., use of fetal bovine serum and episomal vectors). From this perspective, the use of episomal vectors requires prolonged hiPSC culture for the complete removal of exogenous materials (6). Alternatively, multiple mRNA transfections have the advantage of delivering exogenous materials with the possibility of their complete clearance 48 h after the last transfection (29). Reprogramming by mRNA transfections also has faster dynamics (29): Reprogramming lasts 14 days in the perfused system (19, 24) and 30 days in alginate microtubes (28). The perfused system is generally made of polydimethylsiloxane (PDMS), which allows optical monitoring due to setup transparency, gas exchange for high gas permeability, and highly reproducible fluid dynamics between different channels due to a laminar flow regime. On the other hand, in hydrogel microtubes, local concentration gradients are possible outside, and consequently inside, the microtubes; some parameters need to be considered in the design to avoid such changes, including the volume size, geometry, and mixing conditions of the medium where the microtubes are floating. The perfused system has an advantage in the parallelization of

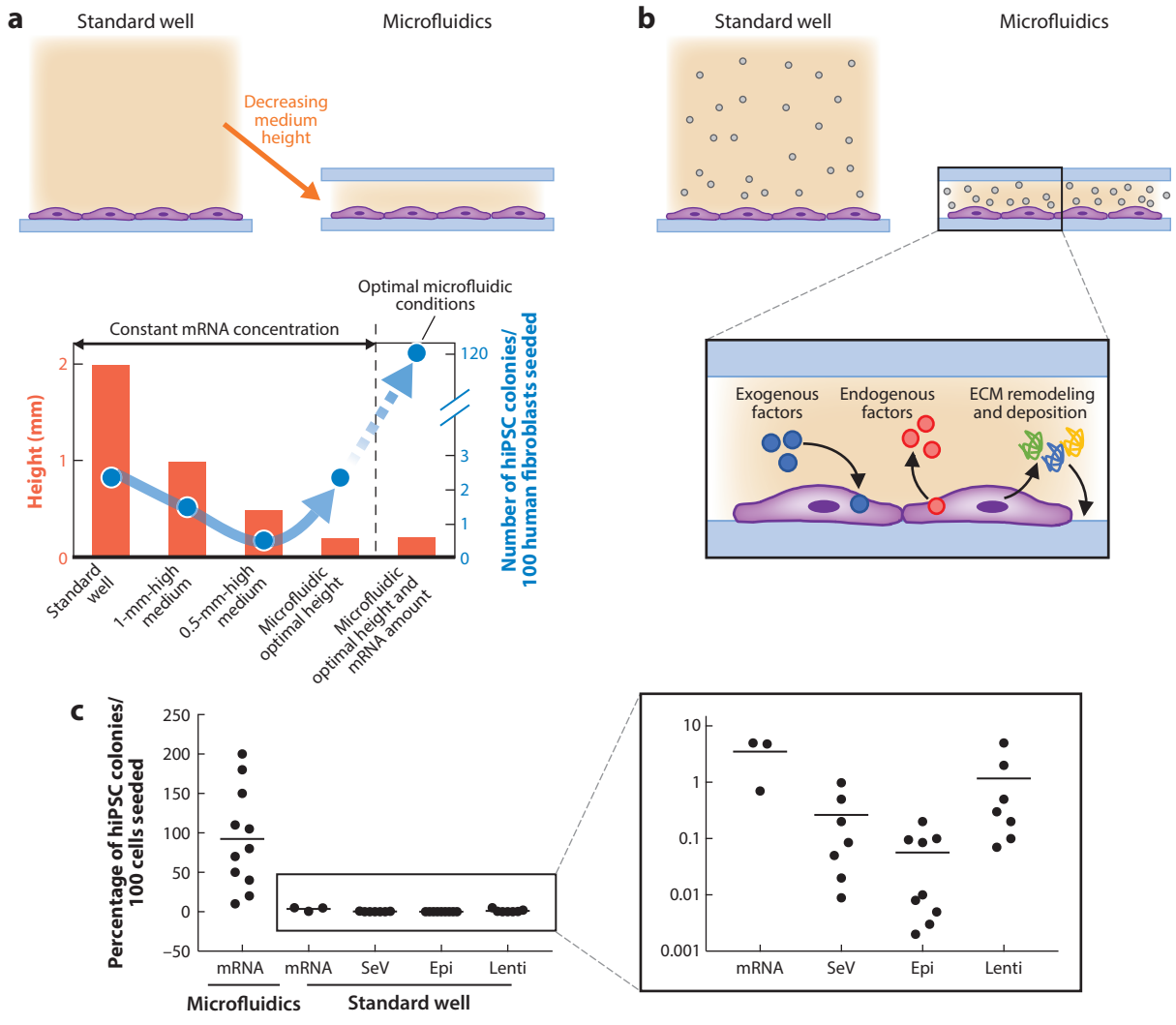


Figure 2

Microfluidic somatic cell reprogramming. (*a*) Reprogramming performed in microfluidics under discontinuous perfusion with ~50-fold-higher efficiency than in a standard well. The main effect of miniaturization is the reduction of the medium height on the cells. For a given frequency of medium change (approximately every 12 h), the medium height affects the accumulation of endogenous cell-secreted molecules in the extracellular space. For constant mRNA concentration across conditions, reprogramming efficiency, in terms of the number of hiPSC colonies per 100 fibroblasts seeded, has a nonlinear dependence on the medium height. Only when the medium height is small enough (~200 μm) does the accumulation of endogenous molecules become relevant in promoting cell reprogramming by self-regulation. Optimizing mRNA delivery conditions at this critical height then unveils the full potential of microfluidic reprogramming, with the emergence of ~120 hiPSC colonies per 100 cells seeded. (*b*) Schematic representation of culture environment of standard well and microfluidics. (*c*) Comparison between microfluidic mRNA reprogramming (19, 24) and standard well processes with different vectors (6). Abbreviations: ECM, extracellular matrix; Epi, episomal: hiPSC, human induced pluripotent stem cell; Lenti, lentiviral; SeV, Sendai-viral.

Table 1 Summary of advantageous microfluidic features for hPSC differentiation

Features	Standard well	Microfluidics	Reference(s)
mRNA delivery	Low efficiency	High efficiency	19, 39, 40
Endogenous signal feedback	Low due to dilution	Strong	33, 38
ECM remodeling	Slow	Fast	38
Temporal control of signals	Buffered	Tunable	33, 40
Spatial control of signals	Not tunable	Tunable	37

Abbreviations: ECM, extracellular matrix; hPSC, human pluripotent stem cell.

multiple reprogramming from a few cells of different patients. Finally, it is hard to understand if confinement plays a role in the hydrogel microtubes or if the alginate hydrogel applies a selective pressure; nonetheless, the system has been demonstrated to be very hiPSC selective, achieving almost 100% hiPSC purity versus 85% in the perfused system. Putting this all into perspective, the throughput of the alginate tube system may be suitable for cell production intended for clinical applications, while the perfused microfluidic system may be suitable for the development of in vitro models for disease studies or drug screening.

3. HUMAN PLURIPOTENT STEM CELL DIFFERENTIATION AT THE MICROSCALE

Under suitable culture conditions, hPSCs can be differentiated to acquire a phenotype typical of cells from the three germ layers (endoderm, mesoderm, and ectoderm) and can subsequently mature into tissue-specific cell types (4). The advantages of performing hPSC differentiation in microfluidics are related to the general features of this technology described in **Figure 1**. Studies that took advantage of these phenomena in the field of hPSC differentiation are reported in **Table 1**.

At the microscale, differentiation has been accomplished according to two complementary biologically inspired strategies (**Table 2**). The first approach uses microfluidics to mimic the physiological environment during embryonic development and morphogenesis (19, 30–38). It is based on providing exogenous molecules in the culture medium that sequentially activate the developmental intracellular signaling pathways. The second direction, also known as forward programming, is based on forcing the phenotypic change by acting on transcription factors to acquire a defined cellular epigenetic state. This approach is achieved by inducing expression of transcription

Table 2 Summary of literature studies on microfluidic differentiation from human pluripotent stem cells

Target phenotype	Differentiation strategy	Reference(s)
Early embryogenesis	Development mimicking	30, 31
Endoderm		
Hepatocytes	Development mimicking	19, 33, 37, 38
Pancreatic islet cells		35, 36
Intestinal cells		34
Mesoderm		
Cardiomyocytes	Development mimicking	19, 33
Skeletal muscle	Forward programming	40
Ectoderm		
Neuronal cells	Development mimicking	32
	Forward programming	39

factors that enable epigenetic remodeling into the target phenotype (39, 40). These two extreme strategies do not need to be mutually exclusive, and the second one is accompanied by exogenous administration of growth factors and small molecules. Here, we review studies that applied these concepts for differentiation in microfluidics starting from hPSCs.

3.1. Embryo Development–Inspired Differentiation

Embryo development–inspired differentiation is achieved in microfluidics by mimicking different aspects, from biomaterials to morphological and mechanical features. On the other hand, the so-called organoids (41) reproduce in vitro the functional units of tissues mainly by cellular self-regulation within 3D cultures. These two approaches are coming together in the most advanced microfluidic systems for hPSC differentiation where a 3D cell arrangement is achieved (34–36).

3.1.1. Studies of early human embryogenesis. Due to the limited accessibility of the human embryo, early human embryonic development has been often inferred from studies in other species (42). hPSCs have changed this perspective toward the study of human embryo development by in vitro models (4).

In a study of hPSCs undergoing the first stages of differentiation, Warmflash and colleagues (43) demonstrated the importance of spatial organization of signals. They geometrically confined hPSCs in disk-shaped areas of precise size, and, under bone morphogenetic protein 4 (BMP4) stimulation, they obtained a reproducible spatial pattern of differentiation. This study was not performed in microfluidics, but, due to the high cell density within hPSC colonies, endogenous molecules could still accumulate and radially diffuse for paracrine signaling within the colony, defining germ layer territories of fixed size.

The radially symmetric and bidimensional pattern of differentiation obtained in the Warmflash et al. (43) study was, however, limited in its ability to reproduce the in vivo embryo path toward an apical-basal polarization. Taking advantage of microfluidics and hydrogel technologies, Manfrin et al. (30) added a new layer of spatial control to the disk-shaped hPSC colonies. They applied stimulation via a morphogen (BMP4) and its inhibitor (NOGGIN), building stable gradients across an hPSC colony. This study showed a breaking symmetry in the round hESC colonies and the dynamics of the diffusion-regulated interplay between gradients of exogenous and endogenous soluble signaling molecules.

Almost contemporarily, starting from single hESCs or hiPSCs, Zheng et al. (31) recapitulated in vitro developmental events reflecting epiblast and amniotic ectoderm development in the postimplantation human embryo. Although they used a different technological configuration compared with that of Manfrin et al. (30), they still played on the same chemophysical phenomena to localize stable gradients of stimulants across the hPSC colonies, by microfluidic controlled fluid dynamics and accumulation of cell-secreted factors, coupled with hydrogel structures that spatially confine the region of diffusion-only molecular transport.

3.1.2. On-chip differentiation toward more mature phenotypes. It is often of interest to recapitulate in vitro a model of a single tissue, for studying biological phenomena in a simplified context. Toward this aim, the cell culture system does not need to recapitulate the whole embryo development but should rather provide the environmental cues that promote a single lineage specification. Here, we review studies that took advantage of different aspects of microfluidics to induce hPSC differentiation by mimicking lineage specification processes in embryo development.

Microfluidic systems are characterized by being perfused, either continuously or discontinuously. Giobbe et al. (33) studied how perfusion impacts hPSC differentiation. Continuous perfusion was shown to induce spatial heterogeneity in culture, with differences between downstream

and upstream regions more or less exposed to endogenous secreted factors, respectively, and differentially affected by exogenous factors, progressively depleted along the microfluidic channel. On the other hand, in discontinuous perfusion, medium change frequency was a means of tuning the balance between the accumulation of exogenous factors and cell-secreted factors. They showed how changing perfusion frequency alone was enough to direct early germ layer commitment, with, for example, endoderm differentiation favored at high frequencies. Giobbe et al. (33) also developed the first models on a chip of cardiac and hepatic lineages derived from hPSCs. Both protocols included multiple stages, where different promoting factors were exogenously provided to the cells in the culture medium and medium change frequency was optimized. Overall, they achieved mesodermal differentiation to produce functional cardiomyocyte-like cells, with 65% $CTNT^+$ cells, displaying sarcomeric organization, spontaneous contractility, and calcium transients, functionally responding to Ca^+ channel perturbations. Moreover, they obtained human hepatocyte-like cells displaying functional features, such as albumin secretion, glycogen storage, and indocyanine green digestion.

Due to microfluidic confinement, endogenous molecules are accumulated. This higher concentration is the case not only for soluble autocrine and paracrine signals but also for molecules that will build up the extracellular matrix (ECM) and for enzymes deputed at ECM remodeling. The relationship between cells and the ECM is responsible for a reciprocal and dynamic regulation, with important functional implications in development (44) and tissue functionality (45). Michielin and coworkers (38) explored this concept during human hepatic differentiation on a chip. The resulting phenotype showed a more advanced maturation in microfluidics than in wells, displaying the functionality of the urea cycle not only for urea production but also for ammonia detoxification. By quantitatively analyzing the proteomic composition of conditioned media in microfluidics and in conventional wells (46), they demonstrated that the highly concentrated microfluidic soluble environment promoted an endogenous ECM and, in particular, collagen I and fibronectin deposition and remodeling. The maturation-promoting role of ECM endogenous composition was then confirmed in an organoid hepatic model (38). The relevance of ECM was shown also in endoderm differentiation toward the intestinal phenotype in a study by Naumovska and colleagues (34). They designed a microfluidic in vitro model of a human intestinal tubule that benefited from exogenous collagen I addition. Interestingly, hiPSCs that progressed through the differentiation process ended up covering the whole wall of the cell channel, building a gut-like 3D tubule. After 14-day differentiation, tubule permeability reached levels similar to those of the primary tissue.

Gas diffusion across tissues is an important process occurring in vivo, wherein cells exchange oxygen and carbon dioxide with the circulatory system. Microfluidic culture has a characteristic dimension that has a magnitude similar to the maximum distance of cells from capillaries in vivo, that is, 50–100 μm (47). Thus, this technology can better capture the gas gradient concentrations occurring in tissues. Tonon et al. (37) designed a microfluidic device to produce a stable oxygen gradient during hESC hepatic differentiation. This technology mimics what occurs in the liver, where an oxygen gradient develops around the portal vein and induces a metabolic zonation within the structures called hepatic lobules. The results of this work showed a corresponding gradient in the hepatocyte maturation phenotype. As the authors speculated, it is possible that biochemical gradients of other secreted soluble molecules emerged as a consequence of the differential response to oxygen concentration, with an overall amplifying effect favored by microfluidic confinement.

To reproduce in vitro gradients of soluble molecules provided exogenously, different strategies have been applied in the hPSC field. As discussed in the previous section, gradients of soluble molecules can be obtained in microfluidics by integrating hydrogels that define regions where molecular transport occurs almost exclusively by diffusion (30, 31). A second approach uses the

so-called microfluidic gradient generator developed by the Whitesides group (48). Rifes et al. (32) followed this second line and made a microfluidic culture system with a stable linear gradient of a glycogen synthase kinase 3 (GSK3) inhibitor, a known activator of the WNT pathway. They used this system to develop an in vitro model that mimics the events occurring during early neural tube development, specifically the regionalization in the rostro-caudal neural patterning axis. hESCs were cultured in a neural induction medium with dual SMAD inhibition under gradient. Cells in the 2-cm-long culture chamber showed a remarkable spatial pattern of differentiation after 14 days, with OTX2 and GBX2 (rostral and caudal markers, respectively) expression having opposite spatial trends, separated by an area of $PAX8^+$ cells that mimics the midbrain–hindbrain boundary. Interestingly, increasing GSK3 inhibitor concentration shifted the phenotypic change toward the lower concentration side of the gradient, demonstrating that the phenotypic switch was concentration dependent.

Microfluidic systems were shown to be suitable for obtaining 3D cell aggregates, such as human pancreatic islets from hPSC (35, 36). The two studies had a similar microfluidic configuration where hPSCs were seeded within microwells at the bottom of a microfluidic chamber to confine the cellular aggregates, control their size, and avoid direct exposure to medium continuous perfusion. Hirano et al. (35) performed a seven-step growth-factor-based protocol that produced 95% of PDX^+ cells by day 13, 35% of INS^+ cells by day 20, and the presence of alpha and delta cells at day 27. Two years later, Tao et al. (36) developed a microwell system in microfluidics that exposed the differentiating hiPSC aggregates to the culture medium more uniformly thanks to an additional fluidic bottom. Their 23-day four-step protocol produced alpha and beta cells, while no information was given about the presence of delta cells. They also found that the resulting islet organoids were functional in terms of glucose-stimulated insulin secretion and Ca^+ dynamic response to cyclic glucose concentrations.

3.2. Forward Programming

Performing hPSC differentiation by inducing the expression of critical transcription factors has been shown to be an alternative strategy with its own advantages. For example, in neural differentiation, benefits have been found in the reduction of time required for the conversion, simpler protocols, and a more precise control of the cell type obtained (49). In addition, microfluidics can act favorably on the delivery efficiency of the transcription factors and enhance the cellular self-regulation by the accumulation of cell-secreted endogenous factors at discontinuous low-frequency perfusion.

Tolomeo et al. (39) developed a neuron differentiation protocol in microfluidics that forced the acquisition of a neuronal phenotype by inducing the expression of the neurogenin 2 (NGN2) transcription factor in hiPSCs. NGN2 expression was induced by multiple mRNA transfections, as exogenous mRNA persists in the cells for approximately 48 h. Unexpectedly, they found that inducing NGN2 expression by mRNA produced, besides neurons, 20–50% of neural stem cells, a subpopulation that was absent using other delivery methods. It was hypothesized that repeated transfections of the short-lived mRNA could induce an oscillatory NGN2 expression distributed across the cell population, enabling the production of the stem cell phenotype by a mechanism of lateral inhibition (50). Although this explanation needs further confirmation, it is an interesting preliminary result in the direction of exploiting mRNAs for the dynamic control of the expression of transcription factors in culture, taking advantage of their short life span.

Forward differentiation may not be applied directly if the epigenetic state of hPSCs is not permissive for the binding of the transcription factor of interest to the DNA. For example, Selmin et al. (40) recently reported the first skeletal muscle model on a chip derived from hPSCs by myogenic differentiation 1 gene (*MYOD*) induction. However, delivery of *MYOD* mRNA

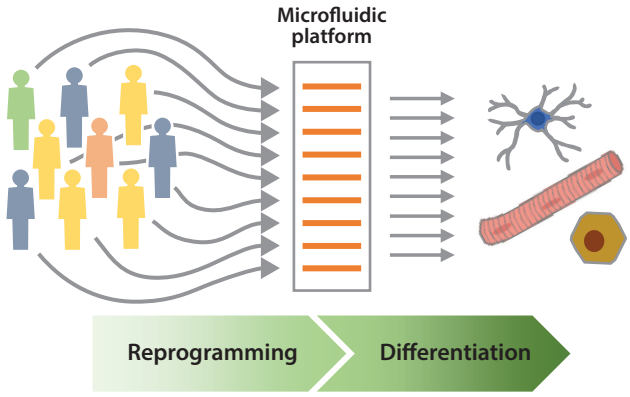


Figure 3

Concept of microfluidic use for an integrated process of reprogramming and differentiation. Each biopsy from a large number of patients can be reprogrammed within a single independent microfluidic chamber. Due to the small size of this culture system, as low as ~1,000 somatic cells per biopsy can be used. Integration of human induced pluripotent stem cell (hiPSC) differentiation at the end of cell reprogramming can be performed without hiPSC passaging or expansion, if certain conditions are met: reprogramming factors have been eliminated from the cells (if nonintegrating short-lived vectors, such as mRNAs, are used) and reprogramming efficiency and purity are high.

alone was not sufficient to induce hPSC phenotypic change. A preliminary step was required to first induce the exit from pluripotency using a small-molecule protocol, and then the forward programming strategy could be pursued. Interestingly, a myogenic phenotype was observed after only 11 days, compared with traditional protocols that can take more than 30 days.

To the best of our knowledge, no microfluidic differentiation methods have been reported to use forward programming toward the endoderm lineage.

4. TOWARD A FULLY INTEGRATED PROCESS

Integrating the processes of reprogramming into hiPSCs and differentiation into specific lineages within the same microfluidic device is an important step forward toward higher throughput, decreased manual handling of cells, shortened processing time, and easier automation (**Figure 3**).

Besides these practical considerations, there is also a more important benefit in reducing the expansion of hiPSC between the two steps. It has been demonstrated that prolonged expansion of hiPSC introduces genetic abnormalities (51). Moreover, hiPSCs that start the differentiation protocol should be devoid of exogenous material inducing reprogramming factors, because their expression would negatively impact the differentiation process (6). Thus, vectors for reprogramming factors that do not integrate in the host cell DNA and that can be rapidly eliminated from hiPSCs are preferred. Among these vectors, mRNA has shown a low aneuploidy rate and a short life span (~48 h) in reprogrammed hiPSCs (6).

Luni et al. (19) showed that freshly derived hiPSCs generated by microfluidic reprogramming are competent to be differentiated into all three germ layers 2 days after the last mRNA transfection with reprogramming factors. Moreover, more mature phenotypes could be also achieved within an integrated reprogramming-differentiation process lasting 30 days overall. In particular, the authors obtained cardiomyocytes displaying sarcomeric organization and contraction activity and hepatocytes able to secrete albumin and store glycogen.

Lin et al. (28), whose reprogramming process within nonmicrofluidic alginate tubes was discussed above, also performed integrated differentiation into the same process. Within the same

microtubes where reprogramming occurred, they expanded the hiPSCs to fill their volumes, and, 30 days after fibroblast seeding, induced the formation of dopaminergic progenitor cells. By day 41, they obtained 90% of $LMX1A^+/FOXA2^+$ cells.

5. TECHNOLOGICAL DIRECTIONS

We now turn our attention to the technological directions affecting a wider application of microfluidics in the field of hPSCs. We discuss GMP compliance for the microfluidic process, together with the critical role of automation and high throughput. We analyze the role of PDMS as the main material currently used for microfluidic systems. Last, we discuss the role of microfluidics in the field of hPSC microencapsulation, at the crossroads of hydrogel and bioprinting technologies.

5.1. Toward GMP Compliance in hiPSC Derivation

Implementation of GMP-compliant protocols for the generation of hiPSC lines is crucial to increase the application safety as well as to fulfill the legal requirements for clinical trials approval. The development of a large-scale process for deriving GMP-compliant hiPSCs has been previously described (52). Here, we highlight the competitive advantages that microfluidics offers with respect to large-scale reprogramming systems, apart from the trivial cost-effectiveness due to scale reduction.

To develop our protocols toward the production of a clinical-grade patient-specific hiPSC, somatic cells can be isolated from cells available in the clinical practice, such as peripheral blood-derived cells. For instance, $CD34^+$ peripheral blood hematopoietic stem cells (PBHSCs) can be isolated from blood donations using currently available complete closed and automated cell manufacturing platforms, such as CliniMACS® Plus (Miltenyi Biotec Inc.). In general, cells need to be expanded to reach a certain numerosity that ensures efficient reprogramming in the conventional GMP culture system. However, on the other hand, the low number of $CD34^+$ PBHSCs isolated can be directly integrated within a microfluidic platform with all benefits of working in a close and automatable system. Microfluidics requires a low number of cells, and this will avoid intermediate cell manipulation and expansion before the reprogramming. Additionally, the use of low-passage cells will avoid prolonged expansion and mitigate the risk of induced senescence. Compared to skin fibroblasts, PBHSCs are also less prone to UV-induced mutagenesis.

The recent development of mRNA technology for clinical application (including mRNA-based vaccines) (53) could be a strong booster in the coming years for generating clinical-grade hiPSC products. We already demonstrated that reprogramming can be achieved under clinical-compliant media in microfluidics with high efficiency (24). Once again, the microfluidic setup can provide a competitive edge, compared with conventional technology, for both mRNA delivery and reprogramming.

Last, for achieving high throughput, microfluidics needs to be paralleled by methods of microchannel automated perfusion. Toward this aim, Luni et al. (19) applied the principles of microfluidic large-scale integration (54), with a system of pneumatic valves embedded inside a multilayer microfluidic chip for medium flow distribution. Different methods of microfluidic pumping have been developed (55) that may also be implemented by microfluidic integration with current robotic liquid handlers.

5.2. Role of PDMS in Microfluidics

Current microfluidic systems for hPSC culture are made of PDMS, whose use for rapid prototyping was developed in 1998 (56). PDMS has several features that make it the preferential material

in these applications (57). As mentioned above, PDMS is easy to mold and to attach to different substrates such as glass, PDMS itself, or polystyrene, by oxygen plasma treatment. It is transparent with good optical properties. It is deformable, which makes it possible to integrate built-in valves and pumps (54). PDMS is used also in medical implants because of its biocompatibility. Toxic uncrosslinked oligomers present after polymerization are typically extracted by solvents, such as 1-propanol, before use (58). A very important feature of PDMS, specific for its application in cell culture, is its permeability to gases. Oxygen and carbon dioxide diffusivities in PDMS are similar to those in water: 34×10^{-6} cm^2/s for oxygen and 22×10^{-6} cm^2/s for carbon dioxide (59). Thus, for microfluidic devices, whose typical PDMS height is a few millimeters, the gas exchange rates are similar to those in standard wells, where the medium is a few millimeters high. PDMS absorption of small molecules has been reported previously as a limitation for some of its applications (60). However, this is not an issue for microfluidic applications to hPSC, given the length scale of their derivation and expansion. During these month-long processes, the microfluidic walls of cell culture chambers are conditioned by medium and cell-secreted molecules, to the point that absorption is not playing a role in the long term.

Overall, considering PDMS performance reported in the bulk of literature already published and the importance of comparability between studies, PDMS is currently not a controversial subject in this field. On the other hand, other microfluidic applications may require upgraded biomaterials (57).

5.3. Microfluidics for hPSC Microencapsulation

Microfluidics is instrumental for another emerging technology in the field of in vitro models derived from hPSCs: microencapsulation. Recent reviews reported an overview of how microencapsulation is achieved by droplet microfluidics and a range of biomedical applications (61, 62). Here, we describe the first applications of this technology to confine hPSCs for expansion and differentiation. Xu et al. (63) used microfluidic technology to encapsulate hiPSCs within ~300-μm-diameter hydrogel spheroids. Their rational for encapsulation was to mimic a blastomere-like environment for a 4-day expansion, with zona pellucida replaced by alginate walls. They found that hydrogel spheroids promoted higher expression of pluripotency markers after 4-day expansion and higher propensity to produce cardiac beating spheroids when hiPSC aggregates were differentiated afterward. Lipke and colleagues (64) performed cardiac differentiation from hPSCs within microfluidic-based spheroids. The authors had previously optimized a microfluidic system for production of ~1-mm-diameter hydrogel particles with high throughput (65). In this study, they used a PEG-fibrinogen matrix, currently undergoing clinical trials (66), and engineered cardiac microsphere tissues that could be cultured for more than three years with remarkable functionality (64). The hydrogel gave initial support for aggregate formation, and then cells progressed with strong endogenous collagen deposition and ECM remodeling.

Overall, microencapsulation confines the cellular environment and locally controls the size and extent of cell–cell and cell–ECM interactions. Microencapsulated cells and tissues hold promise for applications such as large-scale cell expansion in bioreactors and in vivo injection, with cells protected from high-shear conditions by the biomaterial (7). Another field of application with high potential is the integration of microencapsulation within droplet-based bioprinting, to produce constructs with controlled topology (67).

6. CONCLUSIONS AND PERSPECTIVES

Microfluidic technology has shown the potential to fully match the requirements for handling the reprogramming of human somatic cells into hiPSCs and their subsequent differentiation into

desired functional phenotypes. However, most of the time, this has been based on the idea of transferring or adapting a preexisting protocol that has been shown to be successful in a conventional cell culture system. It would be interesting in the future to design reprogramming trajectory and differentiation patterns by an integrative approach in which microfluidic design captures the spatial-temporal environmental evolution underpinning epigenetic remodeling and the morphogenetic process. This approach would need to include fundamental aspects ranging from capturing the cellular diversity to addressing the multitude of biochemical and biophysical signals. There is emerging evidence that cellular population dynamics is contributing to both reprogramming and differentiation processes. Microfluidics could offer an unprecedented opportunity to dissect cross talk between subpopulations and population dynamics in a context-dependent manner, that is, mediated by cell-secreted factors. On the other hand, intricate dynamics between multiscale force fields and biochemical factors acting upon heterogeneous cell populations impose evolving physical processes and allow developing embryos to robustly self-organize into functional tissues. This process can be to some extent controlled by an integrative approach considering the simultaneous modulation of physical and biochemical signals within a microfluidic platform. From a more technical point of view, microfluidic development could enable the generation of methods for reprogramming and differentiation that fully match requirements for clinical applications. To accomplish this goal, automatization and the possibility of operating in a closed environment are fundamental aspects to ensure compliance with regulatory frameworks. These clinical-compliant aspects will include trackability, monitoring, intermediate quality control, and intervention.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The writing of this review was supported by the University of Bologna and the University of Padova.

LITERATURE CITED

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, et al. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282(5391):1145–47
2. Stevens KR, Murry CE. 2018. Human pluripotent stem cell-derived engineered tissues: clinical considerations. *Cell Stem Cell* 22(3):294–97
3. Luni C, Serena E, Elvassore N. 2014. Human-on-chip for therapy development and fundamental science. *Curr. Opin. Biotechnol.* 25:45–50
4. Shi Y, Inoue H, Wu JC, Yamanaka S. 2017. Induced pluripotent stem cell technology: a decade of progress. *Nat. Rev. Drug Discov.* 16(2):115–30
5. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, et al. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131(5):861–72
6. Schlaeger TM, Daheron L, Brickler TR, Entwisle S, Chan K, et al. 2015. A comparison of non-integrating reprogramming methods. *Nat. Biotechnol.* 33(1):58–63
7. Madl CM, Heilshorn SC, Blau HM. 2018. Bioengineering strategies to accelerate stem cell therapeutics. *Nature* 557(7705):335–42
8. Zhu M, Zernicka-Goetz M. 2020. Principles of self-organization of the mammalian embryo. *Cell* 183(6):1467–78
9. Whitesides GM. 2006. The origins and the future of microfluidics. *Nature* 442(7101):368–73
10. Gagliano O, Elvassore N, Luni C. 2016. Microfluidic technology enhances the potential of human pluripotent stem cells. *Biochem. Biophys. Res. Commun.* 473(3):683–87

11. Przybyla LM, Voldman J. 2012. Attenuation of extrinsic signaling reveals the importance of matrix remodeling on maintenance of embryonic stem cell self-renewal. *PNAS* 109(3):835–40
12. Giulitti S, Magrofuoco E, Prevedello L, Elvassore N. 2013. Optimal periodic perfusion strategy for robust long-term microfluidic cell culture. *Lab Chip* 13(22):4430–41
13. Squires TM, Quake SR. 2005. Microfluidics: fluid physics at the nanoliter scale. *Rev. Mod. Phys.* 77(3):977–1026
14. Loh Y-H, Agarwal S, Park I-H, Urbach A, Huo H, et al. 2009. Generation of induced pluripotent stem cells from human blood. *Blood* 113(22):5476–79
15. Zhou T, Benda C, Duzinger S, Huang Y, Li X, et al. 2011. Generation of induced pluripotent stem cells from urine. *J. Am. Soc. Nephrol.* 22(7):1221–28
16. Brouwer M, Zhou H, Kasri NN. 2016. Choices for induction of pluripotency: recent developments in human induced pluripotent stem cell reprogramming strategies. *Stem Cell Rev. Rep.* 12(1):54–72
17. Takahashi K, Yamanaka S. 2016. A decade of transcription factor-mediated reprogramming to pluripotency. *Nat. Rev. Mol. Cell Biol.* 17:183–93
18. Stewart MP, Sharei A, Ding X, Sahay G, Langer R, Jensen KF. 2016. In vitro and ex vivo strategies for intracellular delivery. *Nature* 538(7624):183–92
19. Luni C, Giulitti S, Serena E, Ferrari L, Zambon A, et al. 2016. High-efficiency cellular reprogramming with microfluidics. *Nat. Methods* 13(5):446–52
20. Skelley AM, Kirak O, Suh H, Jaenisch R, Voldman J. 2009. Microfluidic control of cell pairing and fusion. *Nat. Methods* 6(2):147–52
21. Okanojo M, Okeyo KO, Hanzawa H, Kurosawa O, Oana H, et al. 2019. Nuclear transplantation between allogeneic cells through topological reconnection of plasma membrane in a microfluidic system. *Biomicrofluidics* 13(3):034115
22. Sharei A, Zoldan J, Adamo A, Sim WY, Cho N, et al. 2013. A vector-free microfluidic platform for intracellular delivery. *PNAS* 110(6):2082–87
23. Uchugonova A, Breunig HG, Batista A, König K. 2016. Optical reprogramming of human cells in an ultrashort femtosecond laser microfluidic transfection platform. *J. Biophoton.* 9(9):942–47
24. Gagliano O, Luni C, Qin W, Bertin E, Galvanin S, et al. 2019. Microfluidic reprogramming to pluripotency of human somatic cells. *Nat. Protoc.* 14:722–37
25. Hackett JA, Surani MA. 2014. Regulatory principles of pluripotency: from the ground state up. *Cell Stem Cell* 15(4):416–30
26. Osafune K, Caron L, Borowiak M, Martinez RJ, Fitz-Gerald CS, et al. 2008. Marked differences in differentiation propensity among human embryonic stem cell lines. *Nat. Biotechnol.* 26(3):313–15
27. Giulitti S, Pellegrini M, Zorzan I, Martini P, Gagliano O, et al. 2019. Direct generation of human naive induced pluripotent stem cells from somatic cells in microfluidics. *Nat. Cell Biol.* 21(2):275–86
28. Lin H, Li Q, Du Q, Wang O, Wang Z, et al. 2019. Integrated generation of induced pluripotent stem cells in a low-cost device. *Biomaterials* 189:23–36
29. Warren L, Manos PD, Ahfeldt T, Loh Y-H, Li H, et al. 2010. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 7(5):618–30
30. Manfrin A, Tabata Y, Paquet ER, Vuaridel AR, Rivest FR, et al. 2019. Engineered signaling centers for the spatially controlled patterning of human pluripotent stem cells. *Nat. Methods* 16(7):640–48
31. Zheng Y, Xue X, Shao Y, Wang S, Esfahani SN, et al. 2019. Controlled modelling of human epiblast and amnion development using stem cells. *Nature* 573(7774):421–25
32. Rifes P, Isaksson M, Rathore GS, Aldrin-Kirk P, Møller OK, et al. 2020. Modeling neural tube development by differentiation of human embryonic stem cells in a microfluidic WNT gradient. *Nat Biotechnol.* 38(11):1265–73
33. Giobbe GG, Michielin F, Luni C, Giulitti S, Martewicz S, et al. 2015. Functional differentiation of human pluripotent stem cells on a chip. *Nat. Methods* 12(7):637–40
34. Naumovska E, Aalderink G, Wong Valencia C, Kosim K, Nicolas A, et al. 2020. Direct on-chip differentiation of intestinal tubules from induced pluripotent stem cells. *Int. J. Mol. Sci.* 21(14):4964

35. Hirano K, Konagaya S, Turner A, Noda Y, Kitamura S, et al. 2017. Closed-channel culture system for efficient and reproducible differentiation of human pluripotent stem cells into islet cells. *Biochem. Biophys. Res. Commun.* 487(2):344–50
36. Tao T, Wang Y, Chen W, Li Z, Su W, et al. 2019. Engineering human islet organoids from iPSCs using an organ-on-chip platform. *Lab Chip* 19(6):948–58
37. Tonon F, Giobbe GG, Zambon A, Luni C, Gagliano O, et al. 2019. In vitro metabolic zonation through oxygen gradient on a chip. *Sci. Rep.* 9(1):13557
38. Michielin F, Giobbe GG, Luni C, Hu Q, Maroni I, et al. 2020. The microfluidic environment reveals a hidden role of self-organizing extracellular matrix in hepatic commitment and organoid formation of hiPSCs. *Cell Rep.* 33(9):108453
39. Tolomeo AM, Laterza C, Grespan E, Michielin F, Canals I, et al. 2021. *NGN2* mmRNA-based transcriptional programming in microfluidic guides hiPSCs toward neural fate with multiple identities. *Front. Cell. Neurosci.* 15:602888
40. Selmin G, Gagliano O, De Coppi P, Serena E, Urciuolo A, Elvassore N. 2021. MYOD modified mRNA drives direct on-chip programming of human pluripotent stem cells into skeletal myocytes. *Biochem. Biophys. Res. Commun.* 560:139–45
41. de Souza N. 2018. Organoids. *Nat. Methods* 15:23
42. Shahbazi MN, Zernicka-Goetz M. 2018. Deconstructing and reconstructing the mouse and human early embryo. *Nat. Cell Biol.* 20(8):878–87
43. Warmflash A, Sorre B, Etoc F, Siggia ED, Brivanlou AH. 2014. A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. *Nat. Methods* 11(8):847–54
44. Bonnans C, Chou J, Werb Z. 2014. Remodelling the extracellular matrix in development and disease. *Nat. Rev. Mol. Cell Biol.* 15(12):786–801
45. Kim DH, Provenzano PP, Smith CL, Levchenko A. 2012. Matrix nanotopography as a regulator of cell function. *J. Cell Biol.* 197(3):351–60
46. Hu Q, Luni C, Elvassore N. 2018. Microfluidics for secretome analysis under enhanced endogenous signaling. *Biochem. Biophys. Res. Commun.* 497(2):480–84
47. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. 2002. *Molecular Biology of the Cell*. New York: Garland Science. 4th ed.
48. Jeon NL, Dertinger SKW, Chiu DT, Choi IS, Stroock AD, Whitesides GM. 2000. Generation of solution and surface gradients using microfluidic systems. *Langmuir* 16(22):8311–16
49. Flitsch LJ, Laupman KE, Brüstle O. 2020. Transcription factor-based fate specification and forward programming for neural regeneration. *Front. Cell. Neurosci.* 14:121
50. Shimojo H, Ohtsuka T, Kageyama R. 2011. Dynamic expression of Notch signaling genes in neural stem/progenitor cells. *Front. Neurosci.* 5:78
51. Laurent LC, Ulitsky I, Slavin I, Tran H, Schork A, et al. 2011. Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture. *Cell Stem Cell* 8(1):106–18
52. Ahmadian Baghbaderani B, Tian X, Neo BH, Burkall A, Dimezzo T, et al. 2015. cGMP-manufactured human induced pluripotent stem cells are available for pre-clinical and clinical applications. *Stem Cell Rep.* 5:647–59
53. Sahin U, Karikó K, Türeci Ö. 2015. mRNA-based therapeutics—developing a new class of drugs. *Nat. Rev. Drug Discov.* 13:759–80
54. Melin J, Quake SR. 2007. Microfluidic large-scale integration: the evolution of design rules for biological automation. *Annu. Rev. Biophys. Biomol. Struct.* 36:213–31
55. Byun CK, Abi-Samra K, Cho Y-K, Takayama S. 2014. Pumps for microfluidic cell culture. *Electrophoresis* 35(2–3):245–57
56. Duffy DC, McDonald JC, Schueller OJA, Whitesides GM. 1998. Rapid prototyping of microfluidic systems in poly(dimethylsiloxane). *Anal. Chem.* 70:4974–84
57. Sackmann E, Fulton A, Beebe D. 2014. The present and future role of microfluidics in biomedical research. *Nature* 507:181–89
58. Lee JN, Park C, Whitesides GM. 2003. Solvent compatibility of poly(dimethylsiloxane)-based microfluidic devices. *Anal. Chem.* 75:6544–54

59. Merkel TC, Bondar VI, Nagai K, Freeman BD, Pinnau I. 2000. Gas sorption, diffusion, and permeation in poly(dimethylsiloxane). *J. Polym. Sci. B Polym. Phys.* 38:415–34
60. Toepkea MW, Beebe DJ. 2006. PDMS absorption of small molecules and consequences in microfluidic applications. *Lab Chip* 6:1484–86
61. Li W, Zhang L, Ge X, Xu B, Zhang W, et al. 2018. Microfluidic fabrication of microparticles for biomedical applications. *Chem. Soc. Rev.* 47(15):5646–83
62. Correia CR, Nadine S, Mano JF. 2020. Cell encapsulation systems toward modular tissue regeneration: from immunoisolation to multifunctional devices. *Adv. Funct. Mater.* 30(26):1908061
63. Xu J, Shamul JG, Staten NA, White AM, Jiang B, He X. 2021. Bioinspired 3D culture in nanoliter hyaluronic acid-rich core-shell hydrogel microcapsules isolates highly pluripotent human iPSCs. *Small* 17(33):e2102219
64. Finklea FB, Tian Y, Kerscher P, Seeto WJ, Ellis ME, Lipke EA. 2021. Engineered cardiac tissue microsphere production through direct differentiation of hydrogel-encapsulated human pluripotent stem cells. *Biomaterials* 274:120818
65. Seeto WJ, Tian Y, Pradhan S, Kerscher P, Lipke EA. 2019. Rapid production of cell-laden microspheres using a flexible microfluidic encapsulation platform. *Small* 15(47):1902058
66. Trattnig S, Ohel K, Mlynarik V, Juras V, Zbyn S, Korner A. 2015. Morphological and compositional monitoring of a new cell-free cartilage repair hydrogel technology – GelrinC by MR using semi-quantitative MOCART scoring and quantitative T2 index and new zonal T2 index calculation. *Osteoarthr. Cartil.* 23(12):2224–32
67. Derakhshanfar S, Mbeleck R, Xu K, Zhang X, Zhong W, Xing M. 2018. 3D bioprinting for biomedical devices and tissue engineering: a review of recent trends and advances. *Bioact. Mater.* 3(2):144–56