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Applications of Microfluidics in Stem Cell Biology

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Abstract Stem cell research can significantly benefit from recent advances of microfluidics technology. In a rationally designed microfluidic device, analyses of stem cells can be done in a much deeper and wider way than in a conventional tissue culture dish. Miniaturization makes analyses operated in a high-throughput fashion, while controls of fluids help to reconstruct the physiological environments. Through integration with present characterization tools like fluorescent microscope, microfluidics offers a systematic way to study the decision-making process of stem cells, which has attractive medical applications. In this paper, recent progress of microfluidic devices on stem cell research are discussed. The purpose of this review is to highlight some key features of microfluidics for stem cell biologists, as well as provide physicists/engineers an overview of how microfluidics has been and could be used for stem cell research.

Keywords Microfluidics · Stem cells · Cancer

1 Introduction

Microfluidics provides a reproducible and controllable way to reconstruct various important factors of in vivo environments, which is challenging to achieve via the conventional plastic tissue culture dish [1–3]. The naturally ecological niches of cells are complex, varying in both space and time. For example, cells experience a transient high level of drugs after a certain period of time since patients take chemotherapy. Even at the same time point, cells living at different distances to blood vessels experience different concentrations of drugs, growth factors, cytokines, etc. The biochemical and mechanical interactions between cells and extracellular matrix or between a sub-population of cells and their neighborhood cells also change dynamically. Further complexity reveals at the internal organization of tissues. Cells in tissues are neither well mixed nor homogeneous, but rather organized in a systematic way that maintains the normal functions of tissues. It is the complex biochemical, physical and mechanical structures of microenvironment that ultimately influences cellular decisions like the differentiation of stem cells.

Stem cells are defined as having the capacity to both self-renew and give rise to descendants which can commit themselves in a number of distinct directions [4]. In general, there are two types of stem cells. One is embryonic stem cell (ESC), which is pluripotent, that can direct its descendants into nearly all of the differentiation lineages in the body [5]; the other is adult stem cell (mesenchymal, hematopoietic, etc), which is multipotent that only differentiate to a limited number of cell types [6].

ESCs are isolated from the inner cell mass of mammalian blastocysts and can be propagated indefinitely while maintaining pluripotency under appropriate culture conditions [7]. Such properties of ESCs make them very attractive for therapeutic applications [8]. ESCs are promising donor sources for cell transplantation therapies for diseases such as juvenile diabetes, Parkinson's disease, and heart failure [9]. To circumvent the ethical controversies regarding with getting human ESCs from fertilized egg, direct generation of pluripotent

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cells from the patients' own cells was developed. Induced pluripotent stem (iPS) cells can be generated from mouse embryonic fibroblasts and adult mouse tail-tip fibroblasts by the retrovirus-mediated transfection of four transcription factors, namely Oct3/4, Sox2, c-Myc, and Klf4 [10, 11]. The success of human iPS provides a rich source for understanding the role of pluripotent stem cell in disease, drug screening, and possibly replacing human ESCs in medical applications.

Adult stem cells, although cannot reconstitute a whole organism, play crucial roles in replenishing adult tissues [12, 13]. Adult stem cells are able to make a decision to either differentiate to specific cell lineage or self renew, as a response to environmental cues. The slow growth rate of adult stem cells keeps the genomic integrity from replication errors. Once a daughter cell is committed to a lineage, it undergoes exponential expansions in a process of creating a population of cells that are called transit amplifying cells. The process finishes with hundreds of functional differentiated cells after the transit amplifying cells exhaust their proliferative potential and go through the last stage of differentiation [14]. To direct the differentiation with precise control, it is important to analyze stem cell microenvironment and elucidate the key components that regulate the differentiation commitments [6].

In this review, we first introduce the microfluidics, then illustrate the recent advances of the study of stem cells in microfluidics, with a focus on stem cell differentiation, and finally discuss its future directions.

2 Microfluidics

Microfluidics is the science and technology of systems that process or manipulate small (10^{-9} to 10^{-18} L) amounts of fluids [1]. To fabricate a microfluidic device, the first thing to do is to pattern the tens to hundreds of micrometer fluidic channels on a substrate. A typical patterning process includes the following steps (Fig. 1): (1) Prepare the substrate. Silicon with different types of doping is most commonly used substrate [15, 16]. But other types of substrate can also easily be made on silicon wafers, like silicon dioxide, silicon nitride, depending on the applications. (2) Spin coat the photoresist. There are a library of photoresists available on the market, with a great variety of thickness, resolution, working wavelength, tolerance of etchant, etc. (3)

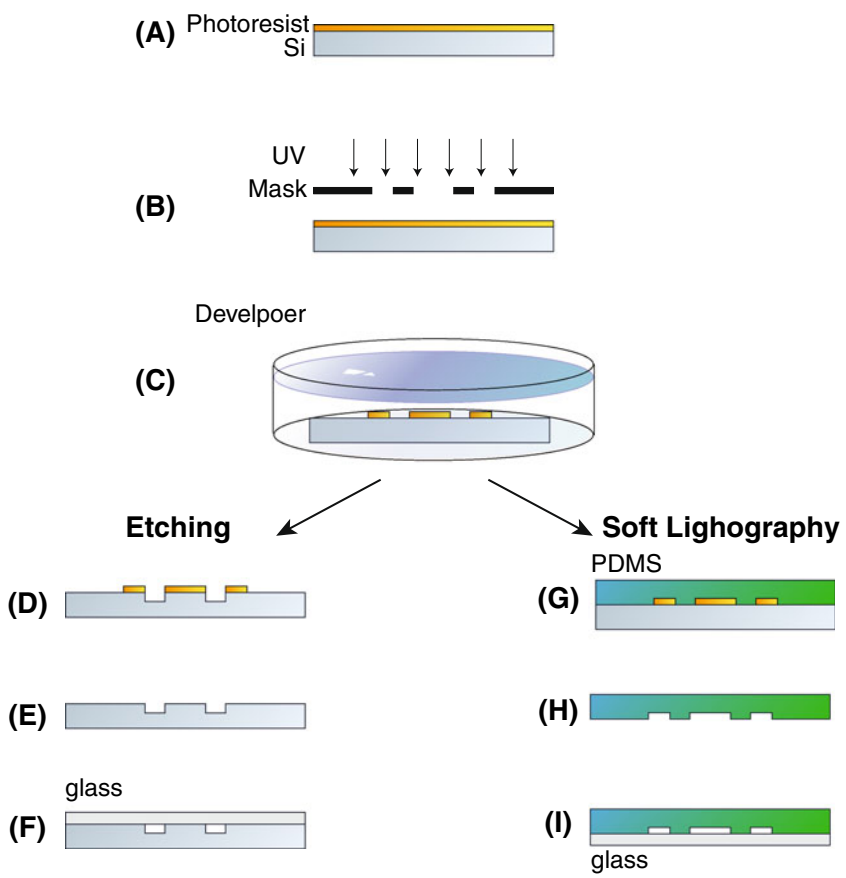

Fig. 1 Two typical approaches to fabricate one layer microfluidic devices: etching and soft lithography. Both approaches share the same processes from *A*–*C* to transfer pattern from mask to photoresist (we use negative photoresist as an example in this figure): *A* spin coating, *B* photolithography, and *C* developing. With a patterned layer of photoresist on the silicon substrate, one can choose either following etching steps *D*–*E*: *D* etching, *E* stripping photoresist, *F* binding to glass slide; or soft lithography steps: *G*–*I*: *G* casting PDMS against the substrate, *H* peeling PDMS off the substrate, *I* binding to glass slide

Photolithography. This process transfers micrometer-sized features on the photo-mask to photoresists coated on top of the substrate, utilizing the photochemical reaction in the exposed region of photoresists. There are two kinds of photoresist. For positive photoresist, the exposed region is removed by developer, while for negative photoresist, the unexposed region is removed by developer. (4) Etch the substrate. This step transfers the pattern on the photoresist to the substrate via either plasma etching or wet etching using chemical solutions such as HF, KOH, etc. (5) Steps 1–4 can be repeated to fabricate another layer of features, which ends up with a 3-dimensional (3D) hierarchy structure with different heights or chemical compositions. Alternatively, micromilling can be used to direct write the pattern on a wide range of hard materials [17].

The other commonly used patterning technique is soft lithography [18, 19]. Instead of etching the silicon, soft lithography uses polydimethylsiloxane (PDMS) to cast against the photoresist (usually SU-8) mold. In some applications involving high aspect ratio features, patterned silicon wafers are used as the mold, since photoresists tend to bend when aspect ratio is high. Compared to silicon, PDMS is soft, transparent, permeable to gasses, biocompatible, which makes it very attractive for biological applications [20–22]. The patterned PDMS can be directly used as a substrate for cell culture or be used as a stencil to pattern the inoculation of cells onto other substrates.

With a patterned substrate, the next step is to seal the substrate so that the fluidic channel can sustain a desired amount of pressure. After establishing macro-to-micro adapters, the chip is ready to run. In the applications discussed in this review, microfluidic devices are usually combined with different optical characterization techniques such as fluorescent microscopy.

There are basically two distinct design philosophies behind microfluidic devices. One is trying to control the reaction inside device via the law of fluidics. Our group pioneered this approach by demonstrating rapid mixing in a hydrodynamic focusing device [23] and further added additional passive controls by creating regular structures on silicon wafers [24–27]. The other approach of the control is to make on-chip active components—valves [28–30], mixers [31–33], and pumps [34, 35]. By rational integration, a microfluidic device resembles a “lab on a chip”.

3 Stem Cells in Microfluidics

The fate of stem cells is highly regulated by microenvironment [36]. Microenvironment promotes stem cell maintenance and controls the differentiation of stem cells to achieve homeostasis. For example, at the bottom of finger-like projections (villi) that protrude into the lumen of the small intestine, there are small cavities called crypts [37]. The stem cells living in the crypts produce progeny that have committed themselves to differentiate and migrate to the tip within 5 or 6 days. The newly arrived cells replace the old cells that have been damaged by corrosive materials in the lumen. The microenvironment in the crypts not only protects the stem cells being damaged by corrosive materials but also precisely controls the timing of the differentiation.

To direct the stem cell differentiation, it is crucial to know the role of various biochemical cues (e.g., growth factors, glucose, oxygen, etc, [38–40]) in the decision-making process of stem cells. Microfluidic devices have been used to study microenvironment mainly from two aspects: screening a wide range of conditions in a high-throughput fashion, and reconstructing the physiological environment like heterogeneous and 3D growth conditions.

3.1 High-Throughput Screening

Conventional tissue culture dishes have been used to test different culture conditions for their ability to drive stem cell growth and differentiation. This approach, although provided insights before, has several limitations. For example, it requires significant amounts of stem cells, which reports average response of the population and could miss the intrinsic heterogeneity; it is very difficult to precisely control the cell number, which could add artificial uncertainty to the results; it is also expensive and labor-intensive to maintain hundreds of dishes over long time; etc. Microfluidics offers a revolutionary way to perform high-throughput screening with many advantages like much lower amounts of starting cells, precise control of inoculation number and dynamic adjustment of culture conditions, etc. [41–44].

A fully automated cell culture screening system based on a microfluidic chip has been used to create arbitrary culture media formulations in 96 independent culture chambers [41]. This highly integrated device was made from multilayer PDMS soft lithography. The device was able to change the condition of each culture chamber separately by using combinations of valves. As a proof of principles, the researchers inoculated the human primary mesenchymal stem cells (hMSCs) to each chamber with a feedback control to achieve the desired inoculation number and monitored the effect of osteogenic stimulation on differentiation and proliferation of hMSCs over weeks [41]. The precise control of cell seeding density, composition of culture medium,

and feeding schedule make it possible to quantitatively measure the cellular responses to external stimulus [45]. The gene and protein expressions change after a stem cell receives cues to initiate differentiation [46], so that the whole differentiation process can be studied by combing high-throughput microfluidic culture platforms and fluorescent reporters engineered to stem cells.

More recently, a microfluidic device featuring 1,600 cell culture chambers, each with a volume of 4.1 nL, and integrated microvalves for precise control and exchange of medium has been made [42]. One main advancement of this device is the introduction of "isoosmotic bath" to keep the nanoliter medium from dehydration. The device has been used to perform analysis on single hematopoietic stem cell (HSC) proliferation [42]. Such device is particularly attractive for the analysis of rare cell types or minority subpopulations and should allow for the investigation of autocrine signaling by single cells in isolated chambers [42].

3.2 Reconstructing the Physiological Environment of Stem Cells

In addition to the high-throughput advantage, microfluidics has the ability to reconstruct the complex physiological environment. By controlling the fluidic properties like convection, diffusion, and reaction, microfluidics can tune the microenvironment around stem cells in a variety of ways, more than setting a concentration of chemicals (Fig. 2). By combing with different structures on the substrate, microfluidics can also tune the composition of the local population and cell–cell interactions (Fig. 2). Microfluidics provides a handy platform to probe various important biological processes like differentiation and evolution in more biological relevant conditions than conventional tissue culture dishes.

3.2.1 Differentiation

As the first step to move beyond the homogeneous environment in conventional tissue culture dishes, chemical gradients have be established in microfluidic devices by either laminar flow [47–49] or controlled diffusion [50, 51]. A gradient-generating microfluidic platform has helped optimize proliferation and differentiation of neural stem cells (NSCs) in culture [52]. NSCs from the developing cerebral cortex were cultured for more than 1 week in the microfluidic device while constantly exposed to a continuous gradient of a growth factor mixture containing epidermal growth factor, fibroblast growth factor 2, and platelet-derived growth factor [52].

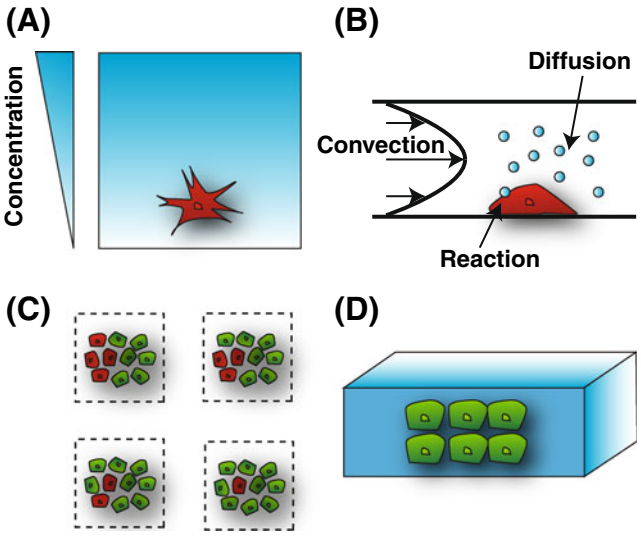

Fig. 2 Illustrations of some key ideas of how microfluidics is used to analyze stem cells: **a** a concentration gradient of chemicals (e.g., growth factors, drugs) can be established in microfluidic devices by combining convection and diffusion, depending on the desired gradient profile. This is to mimic the heterogeneous environment in natural stem cell niches. **b** By changing the geometry of devices or driving pressures, the flow and diffusion profile can be fine tuned. This can be used to investigate the role of different signaling pathways on stem cell differentiation in a dynamic fasion. **c** The substrate of microfluidic devices can be patterned to study the effect of cell–cell interaction within same or different types of cells (e.g., if stem cell differentiation can be stimulated or suppressed by the presence of other cells nearby and how). **d** 3D microfluidic devices can be made from extracellular matrix, where stem cells are cultured in the more in vivo like environment

Not only growth factors but also oxygen gradient can also be established in microfluidic devices. Researchers have used diffusion to localize oxygen delivery without exposing cells to mechanical stresses inside microfluidic culture chambers [53] and, thus, are able to modulate the intracellular reactive oxygen species. Even a stable temperature gradient can be established in microfluidic devices. When embryo are put inside a microfluidic device with steep temperature gradient, it can adapt to the effects of temperature step by having different developing rate at anterior and posterior halves [54]. Therefore, a gradient microfluidic device serves as an ideal platform to probe how stem cells compensate for the fluctuating environment, which reveals the internal dynamics of biological networks [55, 56].

A further step beyond gradients is to fine tune how cells interact with chemicals and how cells interact with other cells. Autocrine and paracrine signaling are widespread both in vivo and in vitro and are particularly important in ESC pluripotency and lineage commitment. It is challenging to evaluate if specific autocrine

signaling is sufficient, or other soluble ligands are also involved in fate specification. A microfluidic device has fine-tuned transport phenomena at cellular resolution to downregulate overall diffusible signaling through the physical removal of cell-secreted ligands [57]. In this microfluidic device, researchers demonstrate that autocrine signaling drives neuroectodermal commitment of mESCs through both fibroblast growth factor-4 (FGF4)-dependent and -independent pathways [57]. The differentiation process is also affected by cell–cell communications such as Notch signaling pathway [58]. Compared with conventional tissue culture dishes, it is much easier to control the composition and size of population in microfluidic device via patterning surfaces by microfabrication and tuning fluidic patterns. Different cell types like fibroblasts, mESC and myeloma cells have been paired in a microfluidic trap [59]. Microfabricated PDMS stencils have been used to make mouse embryonic stem cell aggregates of specific sizes ranging from 100 to 500 μm in diameter, which allows systematic investigation on the effect of initial aggregate size on differentiation [60].

Finally, 3D cell culture can be implemented in microfluidic devices, using all the advantages of microfluidics. Compared with 2D, cellular responses can be significantly altered in 3D microenvironment [61]. 3D culture has more biological or clinical relevance since it mimics physiological conditions in vivo [62, 63]. As early as 2003, a device with 3D microfluidics structure composed with two stacked layers of PDMS was made for mammalian cell culture, and the test with Hepatocarcinoma liver cells showed very promising results [64]. In the high-resolution 3D cellular structures within a photo-polymerizable hydrogel, >20,000 cell clusters of precise size and shape are used to demonstrate that microscale tissue organization regulates bovine articular chondrocyte biosynthesis [65]. Such platform can be extended to study tissue architecture in multicellular processes like embryogenesis and regeneration. In a microfluidic channel-based system, 3D cell culture is achieved by supporting with adequate 3D cell–cell and cell–matrix interactions [66]. Bone marrow mesenchymal stem cells maintain the potency of differentiation up to 1 week in the 3D microfluidic culturing device [66]. A natural cross-linking process has been used to fabricate microfluidic devices from gelatin [67]. The morphology of cells significantly differs when cells are cultured on cross-linked gelatin microchannels instead of rigid tissue culture plastic. In a different type of microfluidic 3D culturing device, the selected biocompatible self-assembling peptide hydrogel, PuraMatrix, is hydrodynamically focused in the middle of main channel of a microfluidic device, and at the same time, the cells are 3-dimensionally immobilized and encapsulated without any additional surface treatment [68]. Instead of using gel, microfluidic device integrated with inter-cellular polymeric linker and microfabricated pillar arrays has also been developed to realize 3D cell culture, where cells displayed 3D cellular morphology, cellular functions, and differentiation capability [69].

3.2.2 Evolution

By reconstructing physiological environment, microfluidic devices can be used not only as an assay to evaluate differentiation but also as an evolution reactor to study the somatic evolution of stem cells. Here, we use the evolution of cancer stem cell as an example. Cancer stem cell model has been proposed to explain the tumor heterogeneity by hypothesizing that rare cancer stem cells are the seeds of tumor, and different differentiation pathways give rise to the final heterogeneity [70–73]. This is fundamentally different from conventional clonal expansion model, where heterogeneity comes from a series of genetic alternations acquired in the clonal expansion of initially altered cells. There is no master seeding cell in the clonal expansion model. The hierarchy structure in the cancer stem cell model put the tumorigenesis as an uncontrolled version of tissue formation from normal adult stem cells.

Identifying the evolution of cancer stem cells is of great importance to chemotherapy [74]. The majority of present chemotherapeutic drugs target on the abnormal proliferating cells, which are supposed to be cancer cells, since most differentiated cells like epithelia cells are post-mitotic. If the tumor is formed by clonal expansion, such drugs work in the right direction to kill the active cells. Clinically, this can be measured by the reduction of tumor size. However, if tumor is a result of differentiation from a rare population of cancer stem cells, the strategy to kill active cells will not work, because the active cells got killed are irrelevant. Cancer stem cells grow very slowly so that they can easily escape from these drugs. What's worse is that cancer stem cells are capable to regenerate the whole tumor, like stem cells in the liver can regenerate the whole liver [74]. Therefore, the chemotherapeutic drugs should target on cancer stem cells, if existing, instead of rapid proliferating cells.

One example of cancer due to stem cell damage is chronic myeloid leukemia (CML), a HSC disorder [75, 76]. Understanding the mechanisms that govern chemotherapy resistance of CML require analyses at the single stem cell level. It is difficult to study single CML stem cell over time using conventional tissue culture dishes, because hematopoietic cells are largely

non-adherent. By fabricating novel microfluidic single cell arrays, researchers are able to perform functional interrogation of hundreds of non-adherent single cells in parallel [77]. As a first test, researchers have revealed the differences in the responses of normal and CML stem cell to the tyrosine kinase inhibitor, dasatinib, a drug approved for the treatment of CML [77].

In addition, to be an analysis platform, microfluidics could be a promising platform to investigate the evolution dynamics of cancer stem cells, by integrating the power of precisely handling cells and reconstructing physiological environment. It has been demonstrated that evolution of bacterial antibiotic resistance is much faster in a gradient environment than homogeneous culture [27, 78]. We believe that this same principle can be extended to the evolution of chemotherapy resistance in cancer [79, 80]. One possible microfluidic device could mimic the structure of small intestine by combining a main channel (lumen) and several side projections (villi). Cancer stem cells are put at the bottom of the projections (crypts) and are allowed to form a tumor. By flowing drugs in the main channel, one can watch how tumor shrinks and how cancer stem cells acquire resistance and differentiate to regenerate the whole tumor again if it happens. The other possible device could mimic the complex vascular structures of solid tumor like breast cancer. We know that the vascular network generates complex spatial gradients of chemicals. Therefore, by placing cancer stem cells at different places, we can gain insights about what compositions of microenvironment promote or suppress the evolution. Beside providing new knowledge of how the evolution of drug resistance happen in tumor, such device can also be a new platform for drug testing in the pharmaceutic industry.

3.3 Isolating Stem Cells in Microfluidic Devices

Both applications discussed above—high-throughput screening or reconstructing physiological environment—require a high-quality stem cells to start with. This leads to a major challenge in stem cell research—how to isolate stem cells from other differentiated cells?

Cell sorting is well developed in hematology [81]. The components of blood can be fractionated according to buoyant density [82], electric charge [83], immunologic labeling [84], etc. If the label is attached with magnetic particles or fluorophores, then magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) can be applied. Various microfluidics versions of MACS and FACS have been actively developed to achieve lower cost, reduced reagent usage, and rapid analysis time [85–88]. A highly efficient microfluidic fluorescence-activated droplet sorter has been developed to combine many advantages of microtiter-plate screening and conventional FACS [89]. A more efficient (high sorting rates) microfluidics sorter utilizes a surface acoustic wave cell-sorting scheme [90]. Since it is difficult to get consensus on biochemical makers to label, label-free sorters are developed. Various physical biomarkers have been explored to identify cells of interest, including cell size [24, 91], shape [92], density [93], deformability [94, 95], electrical polarizability [96, 97], magnetic susceptibility [98], optical properties [99], etc. The scale of microfluidics provides an interface to manipulate single cells and apply separation forces. There are plenty of microfluidic-based cell sorters. We would suggest readers to refer to more specific reviews on microfluidic cell separation techniques [100–103]. Here, we would like to focus on applications of microfluidic-based cell sorters in stem cell research.

We choose cancer stem cell as one example to illustrate the use of microfluidics in sorting rare stem cells. The evidences for the existence of cancer stem cells have been provided in the context of acute myelogenous leukemia [104, 105], breast [106], brain [107], and pancreatic [108] tumors. However, it remains difficult to isolate cancer stem cells in the primary tumor since cancer stem cells are embedded in a large number of differentiated cells. Recently, researchers found that circulating tumor cells (CTC) of metastatic breast cancer patients frequently over-expresses stem cell and epithelial–mesenchymal transition markers [109, 110]. Therefore, development in sampling CTCs from peripheral blood is highly demanded. As discussed in the last paragraph, numerous intrinsic properties could be used to sort CTCs. A microfluidic device has been developed to efficiently and reproducibly isolate circulating tumor cells from the blood of patients with common epithelial tumors [111]. This CTC chip consists of an array of microposts that are made chemically functional with anti-epithelial cell adhesion molecule (EpCAM) antibodies. Anti-EpCAM provides the specificity for CTC capture from unfractionated blood, because EpCAM is frequently over-expressed by carcinomas of lung, colorectal, breast, prostate, head and neck, and hepatic origin, and is absent from haematologic cells. The efficiency of stem cell capture is affected by duration and robustness of cell–micropost contact, which can be controlled by flow velocity and shear force in microfluidics. To maintain capture efficiency, the antibody-based devices must be operated slowly (~mL/hr). Also, viable cell recover is difficult because cells are strongly bound to the surface once they attach. Could label-free sorters be developed to sort CTCs?

Since CTCs (diameters 15–30 μm) are on average larger than other cells in blood (2–15 μm) [112], it is possible to utilize a size-based separation technique. Researchers have developed a microfluidic device for ~mL/min flow rate, continuous-flow capture of viable CTCs from blood using deterministic lateral displacement arrays [113]. A further development would be made to enrich the subpopulations of cancer stem cells from other types of cells like metastatic precursor cells in circulating tumor cells. Also, the cancer stem cells after sorting must be able to be recovered for down-stream applications, which means the unwanted damages of the sorter to cells must be eliminated or minimized. Microfluidic sorters can also be extended to isolate other types of stem cells, depending upon the knowledge of biomarkers for developmental maturity for that type of stem cells. Nevertheless, it is promising for microfluidic sorters to be applied to stem cell-based regenerative medicine. Such technology could be used by cancer clinicians as a clinically useful point-of-care diagnostic and a prognostic tool.

4 Outlook

In conclusion, stem cell research has already started to take advantages provided by microfluidics. This paper reviews recent advancements and shows that microfluidics is attractive in many aspects like high-throughput screening, reconstructing physiological environment, and isolating rare stem cells. However, in our opinion, following issues need to be solved before microfluidics can be widely applied to the resolution of biological problems rather than simply to the proof of concepts. The first issue is to make microfluidic devices easy to use by stem cell biologists. Two approaches will help solve this issue. One is to commercialize the microfluidic technology developed in academic labs. The other is to train biologists who don't have experience in fluid physics or microfabrication. The authors of this review and their colleagues indeed make a great effort in training biologists by organizing annual microfluidics courses. The second issue is the materials to make microfluidic devices for stem cell research. Presently, PDMS is the common choice for microfluidic devices. But it does not necessarily mean that it is suitable for stem cells, which are very sensitive to the microenvironment. For example, dehydration can cause bubbles in the devices; absorption of molecules in PDMS can change the local chemical concentration; etc. Such factors could reduce the reproducibility of experiments. Searching for a more biocompatible material for stem cell research should keep continuing. The third issue is the integration of microanalysis systems. Most existing microfluidic devices, as discussed in this review, implement only one or two functions. Highly integrated systems are needed to address a specific question in stem cell biology from different aspects, which also minimizes the uncertainty introduced in sample transferring. Nevertheless, considering the advantages of microfluidics that are too compelling to let pass, we believe microfluidics will become a powerful tool for both fundamental understanding and medical applications of stem cells in the near future.

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