

# Biotechnological potential of stem cells

## Abstract

Stem cells hold the potential for manifold applications in biotechnology-based next-generation therapeutics. Scientists are trying to formulate better and more personalized treatment modalities against some seemingly irremediable diseases, by harnessing body's own stem cells and stem cell niche. Advancement of fundamental research techniques like tissue biomechanics along with continuous emergence of cutting-edge technologies like 3-D printing, microfluidic-platforms etc. lead to a flood of stem cell-based applications in clinical practice. The pros and cons of stem cell therapy in routine healthcare practices must be critically appreciated in order to fetch its benefits to the mankind. This review is meant for discussing some recent breakthroughs and future aspects of stem cell biology.

**Keywords:** biotechnology, stem cells, cell-potency, therapeutic cloning, tissue engineering, scaffold, scnt, vsels, oct4, sox2, cmyc and klf4 mesc, prolotherapy, niddm, pde5 inhibitors, sci, vegf-a

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**Abbreviations:** ESCs, embryonic stem cells; FSCs, fetal stem cells; ASCs, adult stem cells; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; PGCs, primordial germ cells; iPSCs, induced pluripotent stem cells

## Introduction

Biotechnology deals with developing strategies using biological systems, living organisms or derivatives aimed to make or modify products or processes for specific use and address a broad range of issues in the fields of agriculture, industry, environment and medicine. Though the term 'Biotechnology' is not more than a century old until a Hungarian engineer Karl Ereky coined it in 1919, the concept of biotechnology had its first footstep right since the discovery of fermentation around 7000 BC.<sup>1</sup> The subject has a bygone soul with a dynamic nature and promising outlook towards future. Biochemistry, cell biology, pharmacology, immunology, genomics, proteomics,

structural biology etc. are relentlessly contributing into biotechnology. Cell-based approaches of biotechnology, particularly focussing on mammalian or human cells, came up in the late 20<sup>th</sup> century with the advent of procedures for artificial insemination and reproductive cloning.<sup>2</sup> In this context, the isolation of inner cell mass from human blastocyst and their characterization as stem cells by the scientists of University of Wisconsin in 1998 set the cornerstone of stem-cell research.<sup>3</sup> Table 1 summarizes the chronological progresses in stem cell-research. In this age of Biology, scientists throughout the globe are looking for alternative therapeutic measures using the inherent potential of the stem cells, holding great promise for the treatment of debilitating diseases. Stem cells of different origin and level of potency are being investigated for tissue regeneration, treatment of bone defect, drug testing, gene therapy and cell based therapy for muscle damage, spinal cord injury, cancer therapy etc.<sup>4</sup> This review concentrates on types of stem cells and their multidisciplinary applications, mainly as a tool for biotechnological advances in modern therapeutics.

**Table 1** Chronological development of stem cell research.<sup>2</sup>

Year	Major discoveries
1959	First animal made by IVF
1968	First human egg fertilized <i>in vitro</i>
1970	Embryonal carcinoma cells were injected into mouse blastocysts to make chimeric mice. Cultured SC cells were explored as models of embryonic development in mice
1981	First mouse embryonic stem (ES) cells were isolated and grown in culture
1988	First cord blood transplant
1989	A clonal line of human embryonal carcinoma cells was derived that yields tissues from all three primary germ layers
1994	Human embryonic stem (ES)-like cells were generated
1998	University of Wisconsin-Madison and Johns Hopkins University isolate the first human embryonic stem cells
2001	Human ES cells successfully developed into blood cells
2003	Dolly dies after developing progressive lung disease
2007	Development of first iPSCs created from human cells

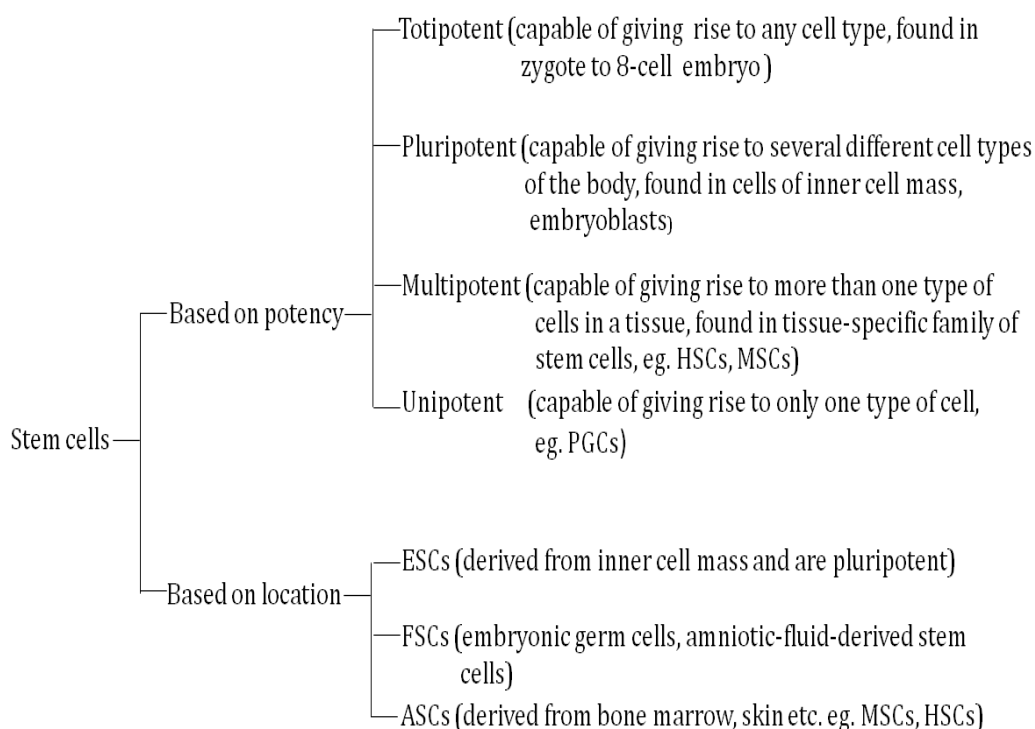
IVF, *in vitro* fertilisation; SC, stem cells; ES, embryonic stem cells; iPSCs, induced pluripotent stem cells

## Stem cells

Stem cells are defined as cells with infinite proliferative capacity and property of self-renewal and they can differentiate in diverse kinds of cells, tissues, and organs of the body.<sup>2</sup> Since the advent of microscopy followed by discovery of cells, scientists showed curiosity in understanding the cellular biochemistry, cell division machinery and lineage-determination to construct tissues and higher structures. In 1998, Thompson et al.,<sup>3</sup> isolated inner mass of cells from a human blastocyst and found their stem cell properties.<sup>3</sup> Practically each of more than 200 different types of differentiated adult cells can be traced back to its origin from a single totipotent zygote. Adult body also retains a big pool of stem cells. Blood, intestinal, epithelial or skin cells are shed off and replaced with new cells by a highly active

reserve of stem cells.<sup>2</sup>

Stem cells are categorized based on their potency and location (Figure 1).<sup>2</sup> Cell potency specifies the differentiation potential of stem cells into different cell types. Stem cells must have to maintain the chastity of their genome. For the sake of it, non-random chromosome segregation, during cytokinesis of asymmetric cell division of stem cells preserves the original template genome to the daughter stem cell and the replicated copy passes on to the daughter cell destined for differentiation.<sup>5,6</sup> A unique property of cross-differentiation of adult stem cells in certain microenvironments is known as its plasticity.<sup>7</sup> Different types of stem cells have been discovered (e.g. embryonic, fetal and adult stem cells) from different sources having different properties.<sup>8</sup>



**Figure 1** Types of stem cells.

## Stem cell niche

Niche is referred to as the in-situ microenvironment that interacts with the stem cells to regulate the cell-fate.<sup>9</sup> Several factors like cell-cell interactions between stem cells and neighbouring differentiated cells, extracellular matrix components, PO<sub>2</sub>, growth factors, cytokines, pH, ionic concentrations and metabolites are the essential components in determining a niche.<sup>10</sup> The ability of a niche to harbour stem cells is also responsible for recruitment of stem cells during development, a process referred to as “homing”.<sup>11</sup> An age-compromised niche does not allow very-small embryonic-like stem cells (VSELs) to maintain tissue homeostasis, like in pancreatic cells and could be a plausible explanation for type-II diabetes mellitus and cancer.<sup>12</sup>

## Multidisciplinary applications of stem cells

### Therapeutic cloning

Nuclear cloning involves introduction of a donor cell nucleus into an enucleated oocyte to produce an embryo that is genetically identical to the donor.<sup>13</sup> Tremendous interest in the field of nuclear

cloning developed since the birth of first mammalian clone, Dolly in 1997.<sup>14</sup> There are mainly two types of nuclear cloning processes: reproductive cloning and therapeutic cloning. Reproductive cloning is used to generate an embryo with genetic make-up identical to its cell source, which can be implanted into the uterus of a female to give birth to the clone of the donor. On the other hand, therapeutic cloning is aimed to generate early-stage embryos that are explanted in culture to produce ESC lines, genetically identical with its source. These autologous stem cells have the potential to differentiate into almost any adult cell-type and thus would be useful in tissue and organ replacement applications.<sup>15</sup> They can be used in the treatment of diseases like end-stage kidney disease, neurodegenerative diseases, and diabetes, for which limited availability of immune-compatible tissue transplants is a big challenge. Therefore, therapeutic cloning, alternatively called as somatic cell nuclear transfer (SCNT) provides an alternative source of transplantable cells. Use of transplantable tissues and organs, derived from SCNT can bypass the allogenic graft-rejection response.<sup>16</sup> Patient-specific pluripotent cells can be obtained from SCNT constructs, by replacing the oocyte genetic material with a somatic nucleus.<sup>17</sup>

One of the major ground-breaking discoveries in stem cell biology in recent times has been the development of patient-specific pluripotent cells or iPSCs through over expression of a set of four transcription factors (OCT4, SOX2, cMYC and KLF4) in somatic cells. In the last few years it has become evident that pluripotent stem cells can be differentiated into primordial germ cells (PGCs). Putative PGCs were derived *in vitro* from mouse embryonic stem cells (mESC) and iPSCs.<sup>18–20</sup> Recently, human iPSCs (hiPSC) from azoospermic and normospermic males were transplanted into sterile mouse testes and showed partial colonization of the testicular niche with signs of early stage spermatogenesis.<sup>21</sup> Hayashi et al.,<sup>22</sup> have recently shown that transplanting *in vitro* produced PGC-like cells into the mouse testes could be used to obtain functional mouse sperm, capable of producing healthy and fertile pups.<sup>22</sup> It has been recently proposed that bone marrow-derived stem cells mainly contribute to the stromal cell compartment of the endometrium.<sup>23–26</sup> Further, it has been reported that bone marrow-derived stem cells improve endometrial regeneration in murine model of Asherman's syndrome.<sup>27</sup>

### Regenerative medicine

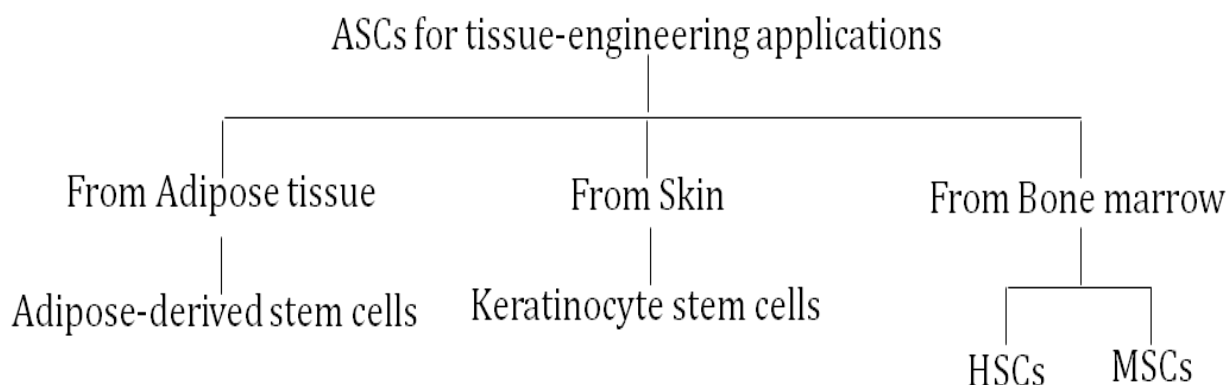
The properties of embryonic and adult stem cells to either self-renew or differentiate into multiple cell lineages make them an attractive source for cell therapies, tissue engineering and as model systems for drug screening.<sup>28</sup> Vertebrates exhibit three types of regeneration: 1. 'Epimorphic' through blastema formation, 2. 'Compensatory growth' and 3. 'Tissue regeneration'.<sup>29</sup> Regenerative medicine aims in engineering damaged tissues to replenish the normal function using body's own process of healing.<sup>30</sup> Stem cells are easily manipulable and are a promising biomedical tool in the emerging field of regenerative medicine.<sup>31</sup> It includes the injection of stem cells or progenitor cells (cell therapy), induction of regeneration by biologically active molecules (immunomodulation therapy, prolotherapy) and the transplantation of *in vitro* grown tissues or organ (tissue engineering). The cell-based approach has grown largely from the successes in the use of HSCs for the cure of blood diseases.<sup>32</sup> The potential of adult stem cells to participate in the formation of various organ systems when introduced in early embryos, the feasibility of transforming adult cells into pluripotent stem cells, and the isolation and characterization of adult multipotent stem cells from virtually every tissue of the body made scientists feel enthusiastic.<sup>33–35</sup> Use of ESCs and reprogrammed cells holds great promise in regeneration of pancreatic  $\beta$ -cells for the treatment of NIDDM.<sup>36</sup> Some stem cells

other than MSCs are being investigated for their curative values. As for example, a preclinical study involving patients was carried out with intracavernosal injections of umbilical cord blood stem cells in seven patients with diabetic-erectile dysfunction: patients reported an improvement in penile erection and an increase in penile rigidity, especially when in combination with PDE5 inhibitors.<sup>37</sup>

### Tissue engineering

The term was coined at key workshops held at the Granlibakken Resort, Lake Tahoe, California, in February 1988 and UCLA symposium in 1992.<sup>38</sup> "Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function".<sup>39</sup> Stem cell-based tissue engineering focuses on building tissues or even organs on 3-D scaffolds made up of biocompatible materials that mimic various structures making the tissue (cartilage, bone, tendon etc.) and provide a hospitable 'niche'. The concept of tissue engineering is based on assembling three essential components: the cells, signals (chemically by growth factors or physically by bioreactor) and the scaffold that acts as a template for tissue formation by allowing cells to adhere, migrate and produce tissue. This combination is referred to as tissue engineering-triad.<sup>40</sup> Tissue engineering can be broadly categorized into open and closed systems.<sup>41</sup> In open system, cells are immobilized in a porous, 3-D matrix, whereas closed system employs immobilized cells in semipermeable membrane that helps overcome potential immunological issues associated with implanted cells. Current approaches to tissue engineering are divided into substitutive, histioconductive, and histioinductive.<sup>42</sup> Substitutive approaches are meant for whole organ replacement; histioconductive approaches involve the replacement of damaged tissue with ex-vivo constructs, whereas histioinductive approaches are based on self-repair which involve gene therapy using DNA delivery via plasmid vectors or growth factors.

The shortage of transplantable organs and histocompatibility issues can be overcome with immortalization by genetic manipulation of ASCs. Immortalized human hepatocyte stem cells grafted into rats with acute liver failure could keep them living.<sup>43</sup> Many types of ASCs (Figure 2) have been reported over several decades. However, stem cells that would be useful in tissue engineering need to be: 1) Easily obtained in large numbers; 2) Safe to implant and 3) Able to differentiate into the cells needed.<sup>44</sup>



**Figure 2** Types of adult stem cells (asc) used in tissue engineering.

Dermal fibroblasts obtained from neonatal foreskin, are expanded *in vitro*, seeded onto a scaffold of polylactic or polyglycolic acid to generate a layer of dermis.<sup>45</sup> A bilaminate construct is produced by laminating the dermal construct with multiple layers of keratinocytes. At present, liver transplantation is the most commonly used treatment modality for end-stage liver failure. The scarcity of donor livers can be resolved by tissue engineering. Highly porous biodegradable discs can be used to deliver hepatocytes. This approach is constrained by the low proliferative ability of hepatocytes and the suboptimal cell survival due to poor blood-supply for nutrient provisions and removal of metabolic waste.<sup>46</sup> Various types of stem cells, such as HSCs and MSCs, have been demonstrated to differentiate into hepatocytes under the suitable condition, and may be used as an unlimited cell source for therapy.<sup>47</sup>

Potential role of ESCs for treating brain diseases is under active investigation. The first ever CNS stem cell-derived functional synapse and neuronal network formation on a 3-dimensional collagen gel has been recently demonstrated.<sup>48</sup> MSCs are ideal for replacing the dead neural cells because of their high proliferative ability, easy acquisition, and tolerance to genetic modifications.<sup>49</sup> The presence of endogenous stem cells in the mammalian spinal cord, suggests an inherent capacity for regeneration able to remyelinate spinal cord axon. Traumatic spinal cord injury (SCI) can lead to severe neurological damage.<sup>50</sup> Even though endogenous stem cells are present, recovery from this injury is difficult. A clinical study on five patients with acute SCI, using bone-marrow stem cells and granulocyte-macrophage colony stimulating factor confirmed a significant improvement of sensory and motor functions.<sup>51</sup> ESCs have been shown to give rise to functional vascular tissue. Three different strategies have been employed to induce vascular differentiation of ESCs: 1) Embryoid body (EB) formation; 2) Co-culture with fibroblast feeder layers or target cells; and 3) 2D monolayer culture of ESCs in defined chemical conditions combined with differentiation stimuli.<sup>52</sup> Various methods such as addition of vascular endothelial growth factor-A (VEGF-A) or bone morphogenetic protein-4 (BMP4) to the culture media and magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) are employed to improve the efficiency of EB protocols in order to promote vascular cell differentiation.<sup>53-55</sup>

MSCs are currently the most prolific cell source that could be potentially used for the treatment of human disease and numerous clinical trials are also going on. MSCs may be isolated from human bone-marrow (BM).<sup>44</sup> Cells of other tissues could be induced to generate bone by the transplantation of de-cellularized bone to ectopic sites. Friedenstein identified adherent 'fibroblast-like' cells in those tissues arising from BM were capable of osteogenesis *in vivo*.<sup>56</sup> By the late 1990s, umbilical cord blood, a valuable source of HSCs, was found to also contain multipotential cells similar to those found in the BM.<sup>57</sup> Recently, MSCs with similar characteristics to BM-derived MSCs have been isolated from other tissue sources, including trabecular bone, periosteum, synovial membrane, skeletal muscle, skin, peripheral blood, deciduous teeth and periodontal ligament.<sup>44</sup>

**Scaffolds used in tissue engineering:** The phrase 'scaffold' in tissue regeneration is used for biocompatible templates that can provide support to the cells by guiding their proliferation and differentiation during repair or restoration of physiological features of injured tissues. Ideally, scaffolds provide cells with a suitable growth environment, facilitated transport of oxygen and nutrients, drainage of waste products, mechanical integrity and suitable degradation. The scaffold

compartmentalizes the cells into close proximity and thus facilitates tissue-formation. Despite the ability of stem cells to differentiate into cells with desired phenotypic and morphological properties, there have been very few scaffold-based tissue-engineering studies that use ESCs, by differentiating these cells *in vitro*, selecting desired cell types and seeding these into scaffolds.<sup>52</sup>

Some key considerations are important while designing or determining the suitability of the scaffold for use in tissue engineering: 1) Biocompatibility: suitable for cell adherence, migration and elicits minimal immune response after being implanted; 2) Biodegradability: provides a temporary bed for the cells, allows the cells to produce their own extracellular matrix by getting degraded and replaced over time; 3) Mechanical properties: should be mechanically consistent with the anatomical position of implantation, sturdy to be handled during surgical procedures and optically porous for cell infiltration and neovascularisation; 4) Architecture: interconnected pore-system for cell invasion, vascularization, diffusion of waste products out of scaffold with optimal pore-size and ligand-groups for cellular attachment (eg. Arg-Gly-Asp for collagen); and 5) Manufacturing technology: clinically viable, cost-effective, off-shelf availability, large-scale productivity.<sup>40</sup>

Tissue-engineering scaffolds are comprised of either synthetic or natural materials, or a composite of the two. Scaffolds are commonly made of synthetic materials such as hydroxyapatite, calcium carbonate, PGA (poly glycolic acid), PPF (poly propylene fumarate) and natural materials such as collagen, Matrigel or alginate. Natural materials show better biocompatibility while synthetic materials provide better control of various properties such as degradation rate, biomechanics, and structure.<sup>58</sup>

Xenogenic materials are used as replacement blood vessels for complex cardiovascular lesions. As these materials lack growth potential, they may put the recipients at risk for complications such as stenosis and thromboembolisation.<sup>59</sup> Tissue-engineered vascular grafts have been constructed using autologous cells and biodegradable scaffolds and have been tested in dog and lamb models.<sup>60</sup> The design of robust scaffolds and ECM that mimics a native physiological milieu and a capillary network in a 3-dimensional (3-D) structure is equally important. In collaboration with Draper laboratories, Shieh et al.,<sup>61</sup> have developed a computational model of circulating vasculature.<sup>61</sup> hESC-derived endothelial progenitors were seeded onto highly porous PGA biodegradable polymer scaffolds to create blood vessels that got fused with the host vasculature when implanted into immunodeficient mice. These endothelial progenitor cells were also able to support the formation of vascularized skeletal muscle.<sup>62</sup> After 35 days of transplantation of osteoblast-like cells derived from hESCs into an animal model by using a poly (D, L-lactide) scaffold, regions of mineralized tissue could be identified within the scaffold by Von Kossa staining and expression of human osteocalcin.<sup>63</sup> For cardiac tissue-engineering, synthetic materials were used in the form of injectable hydrogels and surfaces that can be treated to get detached cardiomyocyte layers.<sup>64,65</sup>

The high porosity of collagen-GAG scaffold promotes improved cell infiltration and vascularization. It also has a drawback of having poor mechanical properties. The hydroxyapatite scaffold has better mechanical properties but poorer capacity for cell infiltration and vascularization. By producing a composite collagen-HA scaffold, it is possible to overcome the problems with both materials while retaining their positive attributes.<sup>40</sup> Some unique sources of biomaterial for

building scaffolds are also being experimented. For example, the silk scaffolds seeded with human bone marrow stem cells and differentiated over time in chondrogenic culture in the presence of transforming growth factor- $\beta$  3 have been used to generate meniscus-like tissue *in vitro*.<sup>66</sup> Chemically and thermally stable carbon nanotube (CNT)-based single-walled or multi-walled 3-dimensional architectures are also under investigation for their supposedly better potential as scaffolds especially in skeletal, vascular and neural defects.<sup>67</sup>

### Three dimensional (3-D) bioprinting

Bioprinting is defined as the use of printing technology to deposit living cells, extracellular matrix (ECM) components, biochemical factors, proteins, drugs, and biomaterials on a receiving solid or gel substrate or liquid reservoir.<sup>68</sup> Handling with picoliter to nanoliter droplets has been a challenge for various applications including biochemical surface patterning, tissue engineering, and direct placement of cells and biomaterials for wound dressing etc.<sup>69-71</sup> In this context, emerging technologies of ejecting droplets via an actuator can address the issue of precise manipulation and deposition. Several bioprinting methods have been developed utilizing acoustics, piezoelectricity, and lasers to deposit living cells onto receiving substrates.<sup>68</sup> Among the numerous advantages of bioprinting are: 1) simplicity of use; 2) generation of geometrically well-defined scaffolds in a rapid and inexpensive manner using polymers or ceramics and other stimulating factors providing support and induction for seeded cells; 3) high-throughput generation of replicas with spatio-temporally well-controlled complex constructs; and last but not the least 4) facilitation of 3-D complexity by multilayer printing.<sup>68,72,73</sup> Stem cells such as ESCs, human bone marrow stem cells, and adipose-derived stem cells have been directly bioprinted onto substrates providing microenvironment with controlled gradients of immobilized macromolecules, engineered to direct stem cell fate.<sup>74-76</sup>

Laser-induced forward transfer (LIFT) was used to print human mesenchymal stem cells (hMSCs) to produce grafts, able to differentiate towards cartilage and bone. In another experiment, human adipose-derived stem cells and endothelial colony-forming cells, printed using LIFT showed vascular-like network formation by direct cell-cell interaction in VEGF-free medium.<sup>74,77</sup> LIFT was also used to print hMSCs and human umbilical vein endothelial cells onto a polyester urethane urea cardiac patch and significant functional progress of infarcted heart was reported after transplantation of a bioprinted patch.<sup>78</sup>

### An industrial venture towards future clinics

Extensive use of ESCs and ASCs for therapeutic applications requires reproducible production of large numbers of well-characterized cells under well-controlled conditions in bioreactors. Various types of tissue-specific stem cells have been successfully cultured as aggregates in stirred reactors using defined serum-free media. Scientists have employed bioreactors for embryonic stem cell (ESC) expansion and/or differentiation.<sup>79</sup> Expansion of undifferentiated mouse ESCs (mESCs) is carried out as bioreactor culture because feeder layers and conditioned medium are not required if leukemia inhibitory factor (LIF) is provided. Expansion of undifferentiated human ESCs (hESCs) is more arduous than for mESCs, and such expansion has not yet been reported in stirred

cultures. As LIF does not support the expansion of undifferentiated hESCs, feeder layers or conditioned medium are required. Feeder-free hESC expansion in defined, serum-free media on surfaces coated with laminin or fibronectin are employed to improve the conditions for hESC expansion in stirred reactors.<sup>80,81</sup> HSCs were among the first stem cells to be cultured in stirred or suspended cultures in bioreactors. Most bioreactor studies with MSCs have employed perfusion of cells embedded within a 3-dimensional (3-D) scaffold. Optimal  $PO_2$ , stirring, perfusion with fresh media are some of the crucial factors determining habitable bioreactor conditions for stem-cell culture.<sup>79</sup>

Microfluidics deals with the behaviour, precise control and manipulation of fluids that are geometrically constrained to a small, typically sub-millimeter scale. Microfluidic systems can also be used to investigate the effect of growth factor or chemical environments on stem cell differentiation in a high-throughput manner. For example, a microfluidic device was developed to fix a concentration gradient of growth factors for optimizing the proliferation and differentiation of stem cells. The microfluidics-based platform enabled rapid optimization of media compositions by exposing the cells to a continuous gradient of various growth factors within the microfluidic environment to induce proliferation and differentiation in a graded and proportional manner, depending on growth factor concentration.<sup>82</sup> In another study, micro-bioreactor arrays (MBAs) system composed of a microfluidic platform and an array of micro-bioreactors was devised to investigate the effect of culture microenvironments on hESCs differentiation both in 2-D and 3-D culture conditions.<sup>83,84</sup> Medium perfusion promoted the viability of encapsulated hESCs within hydrogels (67% viability in perfused culture compared to 55% in static culture). Using this system, it became possible to induce the vascular differentiation of hESCs through the addition of vascular growth factor (hVEGF) to the culture media.<sup>84</sup>

### Conclusion

The current state of the art stem cell-based technologies are directing towards their widespread futuristic uses in the fields of basic biology, medicine and engineering. Stem cells hold great promises for cell therapy, drug development and tissue engineering. At present more trials are needed to explore the utility of stem cells in regenerative medicine. MSCs have shown great promise in several animal studies and clinical trials. ESCs also have a great potential but their use is limited by ethical considerations. The use of amniotic fluid cells, umbilical cord cells, adipose-derived stem cells and iPSCs could be a good alternative. Current laboratory and animal trials are studying the possibility of introducing stem cell therapy into clinical practice for tissue-regeneration and transplantation in muscular dystrophy, intervertebral disc degeneration, cerebral and cardiac infarcts. Studies are showing positive results that enable augmentation of the body's own regenerative potential under controlled conditions. We have been trying to introduce the importance of designing scaffolds from different sources in concert with bioprinting techniques, empowering us with great tool to construct functional and 3-dimensional intricate replicas of human tissue. As plenty of cells are required for clinical trials and industries, variety of bioreactor systems have been developed and are used in controlled and reproducible bioprocesses for large-scale expansion and differentiation of stem cells. Present trends are encouraging and it is well expected that there will be many stem cell based biotechnological breakthroughs in the coming years.

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## Conflict of interest

The author declares no conflict of interest.

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