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# Scaffold-free 3D culture systems for stem cell-based tissue regeneration

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## ABSTRACT

Recent advances in scaffold-free three-dimensional (3D) culture methods have significantly enhanced the potential of stem cell-based therapies in regenerative medicine. This cutting-edge technology circumvents the use of exogenous biomaterial and prevents its associated complications. The 3D culture system preserves crucial intercellular interactions and extracellular matrix support, closely mimicking natural biological niches. Therefore, stem cells cultured in 3D formats exhibit distinct characteristics, showcasing their capabilities in promoting angiogenesis and immunomodulation. This review aims to elucidate foundational technologies and recent breakthroughs in 3D scaffold-free stem cell engineering, offering comprehensive guidance for researchers to advance this technology across various clinical applications. We first introduce the various sources of stem cells and provide a comparative analysis of two-dimensional (2D) and 3D culture systems. Given the advantages of 3D culture systems, we delve into the specific fabrication and harvesting techniques for cell sheets and spheroids. Furthermore, we explore their applications in pre-clinical studies, particularly in large animal models and clinical trials. We also discuss multidisciplinary strategies to overcome existing limitations such as insufficient efficacy, hostile microenvironments, and the need for scalability and standardization of stem cell-based products.



## I. INTRODUCTION

Employing stem cells with self-renewal and regenerative capabilities to rescue dysfunctional tissues and organs offers new treatment modalities for currently incurable diseases. Conventional regenerative therapy involving cell suspensions has encountered challenges of stem cell survival and limited therapeutic effect after transplantation. The biomaterials and trypsin used in the culture process may compromise the cell viability and functionality, leading to reduced therapeutic efficacy in the targeted sites. Subsequently, the three-dimensional (3D) cultivation technology has gained considerable attention to mimic the cell–cell and cell–extracellular matrix (ECM) interactions in the biological niche, displaying promising results in many disease conditions.[1] This strategy frequently involves biocompatible and sometimes biodegradable scaffolds as carriers for stem cells; however, the use of exogenous biomaterials in the cell-scaffold composite platforms often raise concerns of untoward immune responses and biosafety issues about the degradation of scaffolds.[2,3] Hence, scaffold-free culture systems have been investigated to mimic the *in vivo* physiological microenvironment of stem cells. The 3D structures can enhance the survival, the stemness and the differentiation abilities of stem cells.[4–7] Additionally, the structural integrity of 3D cell format, such as sheets and spheroids, can also facilitate manipulation, stacking, and transplantation into lesions in various anatomical sites.[8–12]

To date, multidisciplinary technologies have been integrated to fabricate cell sheets and spheroids. By applying specific factors or gene modifications to stem cells, their therapeutic effects can be synergistically enhanced. Collaboration between experts in academia and industry has driven the development of stem cell-based products, including the introduction of automated manufacturing systems for large-scale production of clinical-grade tissues.[13] This review aims to elaborate on the establishment of scaffold-free 3D culture systems of stem cells and their applications in tissue regeneration. We will first discuss the engineering methods of cell sheets and spheroids, and then introduce their current employment in biomedical research and clinical use.

Finally, we will highlight the current challenges and future perspectives of scaffold-free 3D culture technologies for stem cell research and clinical application.

## II. THE SOURCES OF STEM CELLS

The selection of stem cells for tissue engineering is guided by their characteristics and intended clinical application. Generally, stem cells can be categorized into pluripotent and adult stem cells. Embryonic stem cells (ESCs) exhibit pluripotency, capable of differentiating into various cell types. However, their clinical use is limited due to concerns about high mutation rates, heterogeneous differentiation, potential immune rejection, and ethical challenges.[14] Induced pluripotent stem cells (iPSCs) can differentiate into a wide range of adult cells, including cardiomyocytes, neurons, endothelial cells, and hepatocytes, making them valuable for tissue regeneration, disease modeling, and pharmaceutical testing.[15,16] Nevertheless, iPSCs may trigger immune reactions and carry risks of genetic alterations and tumorigenesis, resulting in limited clinical use.[17]

Hematopoietic stem cells (HSCs), multipotent cells from which myeloid- and lymphoid-lineages derive, have shown therapeutic effects in some clinical trials,[18,19] such as liver rejuvenation in collaboration with local hepatic progenitor cells.[20] Neural stem cells (NSCs) can differentiate into neurons, oligodendrocytes, and astrocytes, thus supporting neurogenesis and nerve regeneration.[21] They protect both the central and peripheral nervous systems by the release of glial cell line-derived neurotrophic factor (GDNF).[22] Mesenchymal stem cells (MSCs) can be isolated from several types of mesenchymal tissues, including bone marrow, adipose tissue, dental pulps, periodontal ligaments, umbilical cord, and placenta, indicating their diverse accessibility for research.[23] Adipose-derived stem cells (ASCs) and bone marrow-derived MSCs (BM-MSCs) are among some of the most frequently employed MSCs for cell therapy. MSCs possess pro-angiogenic properties through secretion of several angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).[24] MSCs can regulate numerous complex immune responses and mobilize a varied kinds of immune cells involving T cells, B cells, and natural killer cells. These immunomodulatory effects are mediated by MSC-derived cytokines, including interleukin-10 (IL-10), prostaglandin E2 (PGE2), transforming growth factor-$\beta$ (TGF-$\beta$), indoleamine 2,3-dioxygenase (IDO), and nitric oxide.[25]

## III. CELL PHENOTYPES IN 3D CULTURE SYSTEMS

Two-dimensional (2D) cell cultures on tissue culture polystyrene surfaces (TCPS) represent an artificial and less physiological environment. Without the support of ECM and intercellular interactions, the cell morphology and characteristics can change significantly from their *in vivo* state.[26] In contrast, 3D scaffold-free cultures simulate tissue structure by secreting matrices and forming 3D cell constructs, such as cell sheets and spheroids. Studies have shown that increased stem cell–ECM interaction in 3D culture systems promotes stemness, potency, and the release of trophic factors (Table I).[27,28]

Pluripotent-based organoids have become valuable tools for small-scale drug screening, providing rapid insights into physiological relevance and potential disease-modulating therapies.[29] In the context of specific diseases, particularly those arising from monogenic mutations, iPSC-derived organoids have been effectively developed to recapitulate disease characteristics *in vitro*.[30] These iPSC-based microphysiological 3D models provide a unique opportunity to decipher sporadic cases of certain degenerative diseases, which are challenging to investigate in animal models.[31,32] Similarly, human iPSC-derived organotypic cardiac microtissues, incorporating multiple heart cell types, have contributed innovative insights into heart diseases. These long-term 3D cultures successfully recapitulated self-organization, cellular heterogeneity, and ultrastructural maturation within the microtissues.[33]

MSC sheets contain ECM proteins such as fibronectin and laminin, and cell junction proteins such as integrin $\beta$1 and connexin 43.[34] Similarly, MSC spheroids produced substantially greater amounts of ECM protein, including tenascin C, collagen VI $\alpha$3, and fibronectin.[35] Thus, cells within these scaffold-free 3D formats maintained their

**TABLE I.** A comparison of 2D and 3D stem cell culture.

| | 2D cell culture | 3D sheet culture | 3D spheroid culture |
|---|---|---|---|
| Cell morphology | Mostly spindle-shaped cells[201] | Unaligned rounded cell shape[202] | Rounded cell shape[203]<br>More homogenous in size[28] |
| ECM deposition | Limited | Enriched | Enriched |
| Cell–Cell interaction | Limited cell–cell interaction[34] | Enhanced cell–cell interaction[34,204] | Enhanced cell–cell interaction[205] |
| Cell viability | Decreased over time[203] | Enhanced viability[34] | Enhanced viability[203] |
| Proliferation and senescence | Replicative senescence occurs as passage number increase[206] | Decreased proliferation[202] | Decreased proliferation[207]<br>Decreased senescence[203] |
| Differentiation potential | Compromised[206] | Preserved[204] | Preserved[28] |
| Cytokine and growth factor expression | Reduced growth factor expression compared to 3D culture formats[208] | Maintained or increased secretion of pro-angiogenic and immunomodulatory factors[137] | Increased secretion of pro-angiogenic, immunomodulatory, and anti-fibrotic factors[209] |
| Applications | Cell expansion, basic research, and cell therapy | Tissue engineering, disease modeling[210,211] | Tissue engineering, disease modeling,[212,213] and drug screening[214,215] |

multilineage potential, exhibited good viability and collagen production, and showed higher expression of HGF, fibroblast growth factor-2 (FGF2), and insulin-like growth factor 1 (IGF-1).[36,37] Moreover, enhanced expression of stemness markers Sox-2, Oct-4, and Nanog was noted in ASCs within cell spheroids and sheets.[11,38] These cells retain the ability to differentiate, and can modulate the expression of cytokines and growth factors in response to environmental stimuli.[39,40] For example, 3D MSC spheroids promote the secretion of HGF, VEGF, FGF2, matrix metalloproteinase-2 (MMP-2), and MMP-14, compared to 2D cultures.[35,40] ERK and AKT pathways are activated through E-cadherin in MSC spheroids, leading to increased VEGF secretion.[8] Additionally, MSC spheroids exhibited increased levels of hypoxia-induced survival factors produced by spheroids, such as C-X-C motif chemokine 12 (CXCL12) and hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$).[41] The MSC spheroids have also been shown to display reduced production of pro-inflammatory chemokines.[42] Meanwhile, enhanced expression of some immunomodulatory factors, including cyclooxygenase-2 (COX-2), tumor necrosis factor-inducible gene 6 (TSG-6), prostaglandin E2 (PGE2), and TGF-$\beta$1, were noted in ASC spheroids.[43] Likewise, ASC sheets expressed significantly more C1q/TNF-related protein-3 (CTRP3) for immunomodulation, which inhibited the C-C motif ligand 2 released by macrophages and subsequently reduced the chemotaxis of unstimulated macrophages.[24,44] However, downregulation of *VEGF* has been noted upon ASC sheet formation.[24]

## IV. SCAFFOLD-FREE CELL SHEET STRATEGY

Cell sheet engineering is an emerging scaffold-free approach in tissue engineering that aims to deliver stem cells more effectively.[45] Traditionally, stem cells are administered through topical injection or intravascular infusion, which may lead to decreased cell retention at the target site and increased risk of thrombotic events.[46] Cell sheet fabrication preserves intercellular structures and mimics biological activities within niches. By avoiding enzymatic treatment during detachment, cell sheets can be harvested while maintaining the proliferation and differentiation capabilities of stem cells.[45] Cell sheets can, therefore, be conveniently applied to lesion sites, exerting therapeutic effects such as pro-angiogenesis and anti-fibrosis through paracrine mechanisms.[47] Cell sheet engineering can also be modified by performing multi-layer stacking, enabling the creation of thicker 3D structures that can recapitulate natural tissue architecture without the use of scaffolds.[48]

Over the decades, various strategies for culturing and transferring cell sheets have been developed (Fig. 1). Successfully fabricated cell sheets can be harvested using the following techniques: temperature-responsive systems,[49–51] chemically defined cultures using ascorbic acid derivatives,[11,44] photo-responsive culture surfaces,[52,53] and magnetic-responsive culture surfaces.[54] By ensuring the structural integrity of cell sheets during detachment, cell sheets have been successfully applied in the regeneration of various tissue types, including myocardium,[9,48] skin,[11,44,55,56] cartilage,[57,58] bones,[59,60] periodontal tissue,[61,62] nerves,[36,63,64] and tendon-to-bone junctions.[65,66]

### A. Temperature-responsive systems

Several synthetic polymers respond to alterations in temperature, chemical species, pH, electric fields, magnetic force, and other environmental factors. Poly(N-isopropylacrylamide) (PIPAAm) is a noncytotoxic and biocompatible synthetic polymer that changes its water solubility with respect to the temperature. It can be applied to TCPS using electron beam polymerization, creating a surface that becomes hydrophobic at 37 °C, which is suitable for cell adhesion, proliferation, and ECM secretion.[67] When the temperature drops below 32 °C, PIPAAm hydrates and renders the surface hydrophilic, allowing cell sheet detachment while maintaining its integrity. Most studies set the detachment temperature at 20 °C,[68] although some set it at 32 °C.[69] While preserving cell morphology and proliferation rates similar to those observed on TCPS, PIPAAm-coated surfaces have successfully fabricated cell sheets from multiple cell sources for application in extensive *in vitro* and *in vivo* studies.[49] The main disadvantage of this system is the prolonged low-temperature phase during detachment, which takes up to 30–60 min and may hamper cell metabolism.[70] Revisions to accelerate the detachment process include grafting PIPAAm onto porous membranes,[71] combining it with other polymers,[72] or incorporating a transfer membrane.[49] Recently, micropatterned PIPAAm surfaces have also shown potential in the fabrication of complex tissues.[73] Commercially available thermo-responsive culture dishes (TRCD) have effectively augmented the construction of cell sheets in both preclinical and clinical studies, although they are relatively expensive.[9,61,69]

Methylcellulose (MC), a water-soluble polysaccharide, forms a temperature-responsive hydrogel under suitable conditions, making it effective for harvesting cell sheets.[74] MC hydrogels are non-cytotoxic and have been extensively used in stem cell sheet fabrications *in vitro*.[75,76] At 37 °C, the gel phase with a hydrophobic surface facilitates cell adhesion and proliferation. Cooling down to the lower critical solution temperature (LCST) reverts the polymer to a solution phase, enabling the detachment of cell sheets.[51,74,77] By adjusting the proportion of MC in the gel, the optimal LCST can be achieved at approximately 32 °C, which is in close proximity to the physiological temperature compared to PIPAAm.[77] Moreover, MC hydrogels are more economical and efficient for detachment compared to PIPAAm. Although they lack mechanical strength and stability,[50] a citric acid-based cross-linking strategy has been proposed to enhance the physical properties.[78] However, the preparation process for MC hydrogels is tedious, requiring a meticulous production process.[77] Additionally, a comparative study has indicated that cell proliferation is lower when cultured on MC surfaces than on PIPAAm surfaces.[75]

### B. Chemically defined culture medium

Ascorbic acid is an essential water-soluble vitamin and antioxidant that has been demonstrated to reduce intracellular reactive oxygen species (ROS) levels and to stimulate cell proliferation and ECM production.[79] Its positive effect on stemness has been observed in ASCs,[11] periodontal ligament-derived MSCs,[80] and corneal epithelial stem cells.[81] Ascorbic acid can be used alone or in combination with other strategies for sheet engineering.[56,82] Specifically, introducing derivatives of ascorbic acid stimulates cell proliferation and ECM deposition, enabling the fabrication and harvesting of cell sheets by simple peeling.[11,82] In a comparative study, ASC sheets cultured with ascorbic acid supplementation on TRCD or TCPS exhibited comparable collagen deposition and pro-angiogenic ability in a cutaneous wound healing model.[82] Moreover, high doses of ascorbic acid can lead to cellular apoptosis through metabolic stress and genotoxic effects, which can potentially be mitigated by increasing the cell seeding density.[83] Otherwise, ascorbic acid-2-phosphate, an oxidation-resistant

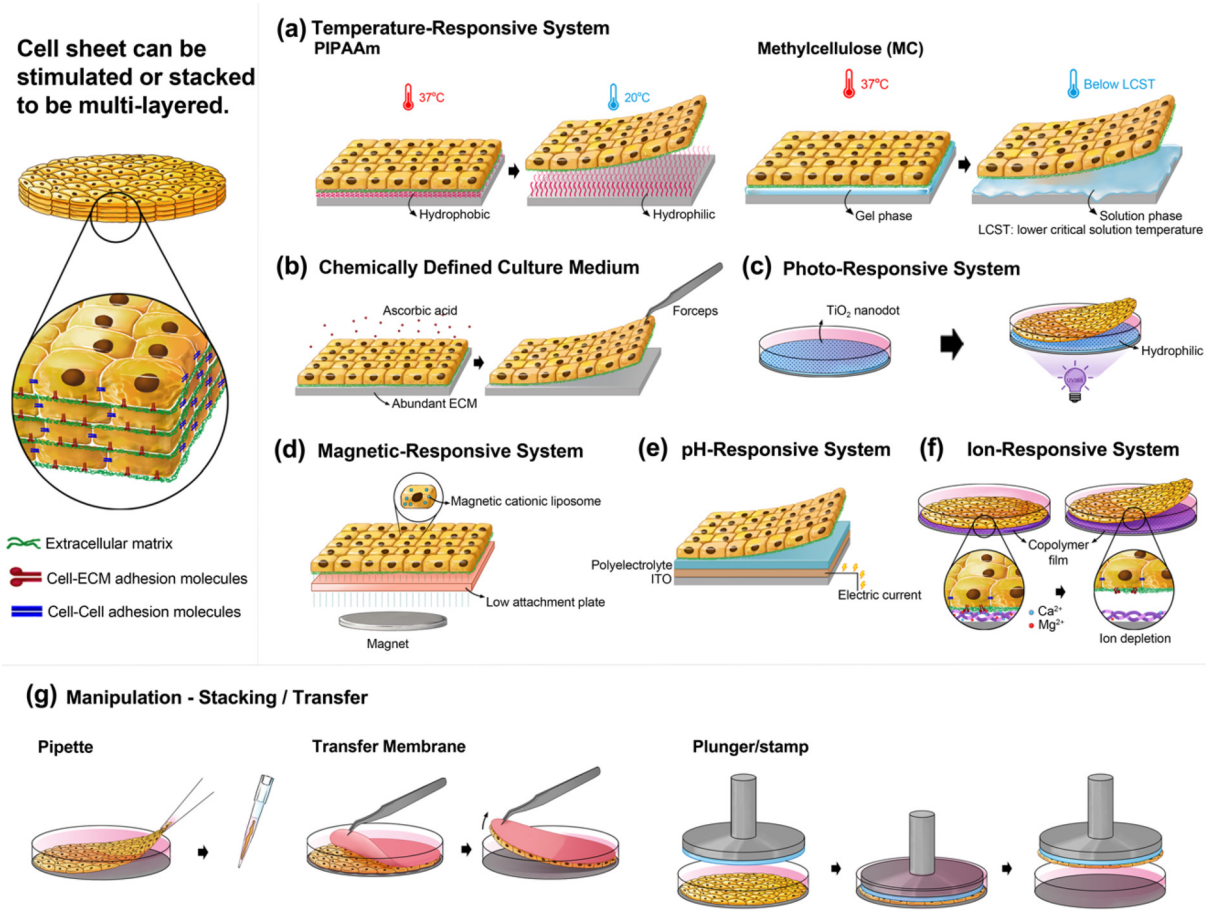


**FIG. 1.** Cell sheet fabrication and harvesting systems. (a) Temperature-responsive system: cells cultured on the N-isopropylacrylamide (PIPAAm) -coated surface at 37 °C. By lowering the temperature to 20 °C, PIPAAm became hydrophilic and the cell sheets were detached. Similarly, cells were cultured on hydrophobic methylcellulose (MC)-coated surface at 37 °C. As the temperature declined below the lower critical solution temperature (LCST), MC turned hydrophilic and the cell sheets were detached. (b) Chemically defined culture medium: ascorbic acid was supplemented in the medium for facilitating extracellular matrix (ECM) production within cell sheets. (c) Photo-responsive system: $TiO_2$ nanodot (TN) films were applied to facilitate photo-responsive cell sheet detachment. Upon exposure to 365-nm ultraviolet (UV) light, the water contact angle significantly decreased, resulting in cell detachment. (d) Magnetic-responsive system: employing the magnetite cationic liposomes (MCLs) and magnetic force, assisted by low-attachment surfaces to minimize cellular adhesion. (e) pH-responsive system: electrochemically induced pH lowering at the interface altered the protein microstructure and disrupted cell–ECM interactions for detachment. (f) Ion-responsive system: incorporating functional copolymers coating, with their hydrophobicity determined by the divinylbenzene (DVB)/4-vinylpyridine (4VP) ratio, to facilitate cell sheet detachment through changing the concentration of cations. (g) Manipulation/stacking/transfer: techniques using pipettes, membranes and plungers for layering and transferring cell sheets precisely, facilitating the construction of multi-layered tissue structures.

derivative of ascorbic acid, has been successfully applied in culturing cell sheets.[11,44] It can enhance the viability of ASCs while maintaining their stemness and differentiation capabilities.[11] This approach has been utilized in several pre-clinical studies involving large animals.[60,63,66]

### C. Photo-responsive systems

Titanium dioxide ($TiO_2$) is renowned for its biocompatibility, chemical stability, and photo-induced hydrophilicity. A $TiO_2$ nanodot film is applied to a quartz substrate to facilitate photo-responsive cell sheet detachment.[52] Upon exposure to 365-nm ultraviolet (UV) light for 20 min, the water contact angle significantly decreases, resulting in cell detachment. The total light energy absorbed is well below the safety threshold for cells. The detached cells exhibit viability and reattachment capabilities comparable to those treated with trypsin.[53] Laminin-521, a protein expressed in human pluripotent stem cells, is known to enhance cell adhesion, pluripotency, and proliferation across various cell types.[84,85] Laminin-521 immobilized on the TiO2 nanodot surface has been successfully applied to fabricate rat BM-MSC sheets consisting of four to eight layers of cells, while maintaining high viability, pluripotency, and DNA integrity.[53]

Koo *et al.* integrated the photosensitizer hematoporphyrin (Hp) into polyketone (PK) polymers, creating a ROS-producing Hp-incorporated polyketone (Hp-PK) film that served as an adhesion surface without cytotoxic effects. The process involved placing the Hp-PK film with the cell sheet facing down on the target surface. The film's opposite side was then exposed to green LED light for 20 min, activating

ROS production by Hp. This method enables the creation of multilayer cell sheets and their direct transplantation onto target tissues.[86]

### D. Magnetic-responsive systems

Ito *et al.* developed a novel method for cell sheet engineering using magnetite nanoparticles and magnetic levitation. They incorporated non-cytotoxic magnetite cationic liposomes (MCLs) into keratinocytes, which formed five-layer sheets in low-calcium media and ten-layer sheets in high-calcium media when exposed to a magnet placed beneath a low-attachment plate. This setup minimized cellular adhesion to the culture surface, aided by a hydrophilic poly (vinylidene fluoride) (PVDF) membrane-wrapped magnet.[87] Similarly, by mixing MCL-labeled ASCs with an ECM solution containing collagen and basement membrane matrices, and seeding them in an ultra-low attachment plate, they formed multilayer ASC sheets within 1–3 h using magnetic force.[88] Zhang *et al.* improved upon these methods using magnetic nanoparticles of iron (II) iron (III) oxide coated with nanoscale graphene oxide (nGO@Fe3O4) to fabricate multilayer dental pulp-derived MSC sheets. The graphene oxide coating enabled efficient binding of growth factors like TGF-$\beta$3 and BMP2 without affecting cell viability or proliferation. This approach allowed for rapid, controlled fabrication of multilayer sheets within two days and provided better esthetic integration for skin regeneration due to the natural coloration of nGO@Fe3O4, overcoming limitations of the black appearance of MCLs.[54]

### E. pH-responsive systems

Guillaume *et al.* introduced an innovative method for cell detachment using a polyelectrolyte coating combined with electrochemically induced local pH reduction and overall pH decrease in the culture medium. This technique enhanced cell adhesion and proliferation across various cell types. The multilayers of poly (allylamine hydrochloride) (PAH) and poly (styrene sulfonate) (PSS) were constructed on indium tin oxide (ITO) electrodes. The electrochemically induced water electrolysis led to a local pH drop at the interface, altering the protein microenvironment and disrupting cell–ECM interactions.[89] In this study, a monolayer sheet of human placenta-derived MSCs was produced with 4 h of culture, and detachment was achieved within 10–20 min using an electrical current. Alternatively, introducing a buffered culture medium at pH 4.0 induced a rapid overall pH change, detaching the cell sheet in just 2–3 min. The detached cell sheet retained its adipogenic and osteogenic potential, demonstrating excellent cell viability for broad applications in tissue regeneration.[89]

### F. Ion-responsive systems

Concerning the interaction between fibronectin and integrin on the cell surface, hydrophobic substrates can distort the conformation of fibronectin, thus reducing the exposure of its integrin binding region. Baek *et al.* developed an innovative method for cell sheet detachment utilizing a functional polymer coating that alters the surface hydrophobicity by modulating the concentration of cations in the medium. Consequently, the affinity between cells and the ECM can be manipulated to induce sheet detachment. The study combined nonpolar, hydrophobic divinylbenzene (DVB) and polar, hydrophilic 4-vinylpyridine (4VP) to create a composite culture surface, with its hydrophobicity determined by the DVB/4VP ratio. An optimal ratio was identified to modify the culture surface for efficient cell sheet separation. The introduction of less concentrated divalent cations, such as calcium and magnesium ions, further decreased the integrin-fibronectin affinity, enabling detachment within 100 s. Human MSC sheets produced by this technique possess adequate viability, proliferative activities with enhanced expression of adhesion proteins.[90]

### G. Physical manipulations

Pipettes are commonly employed for transferring and stacking cell sheets, wherein the sheets can be folded and transferred in the culture medium. Once transferred to another platform, the sheets can be spread out by adding drops of medium or using forceps. This method has been shown effective both *in vitro* and *in vivo*. However, pipetting poses challenges when manipulating multi-layer sheets and carries the risk of damaging the cell sheets.[91] Forceps offer intuitive operation for peeling, transferring, and stacking cell sheets.[11,82] Despite their advantages, forceps can damage the structure of mechanically fragile or thin sheets. To mitigate this issue, customized forceps tips have been developed, which may enhance the process by providing a gentler and more controlled handling method.[92]

Plungers coated with hydrogels, such as gelatin or fibrin, are well recognized tools for transferring and stacking cell sheets.[93] Hydrogels transition into a gel phase at low temperature, acting as an adhesion surface for sheet transfer. Hydrogel-coated plungers are overlaid on TRCDs at 20 °C, allowing cell sheets to be lifted after incubation.[93] Raising the temperature to 37 °C results in gelatin melting and subsequent cell sheet release. Unlike gelatin, fibrin does not dissolve at 37 °C, and sheets adhered to a fibrin coating require mechanical separation.[70] These manipulators offer convenience, stability, and feasibility for scale production, though some biomaterials (e.g., hydrogels) may remain on the cell sheets.

Various membranes assist in cell sheet transfer by relying on the adhesion of the cell sheet to the transfer membrane through capillary pressure. These transferred sheets then adhere to new surfaces via adhesion molecules, with transfer efficacy influenced by the membrane's wetness.[94] Adjusting the wetness with a drop of culture medium can optimize membrane affinity. Several types of membranes have been developed to support this purpose, including CellShifter®,[95] PVDF membrane,[96] Seprafilm®,[97] and polyglycolic acid (PGA) membranes.[98] Biodegradable materials like Seprafilm® and PGA membranes also serve as carriers to support sheet structure, aiding in transplantation.[62,99]

## V. SCAFFOLD-FREE CELL SPHEROID STRATEGY

Cell spheroids, also known as cell aggregates,[100] pellets,[101] micromasses,[101] microtissue,[102] 3D-bullets,[103] or embryoid bodies[104] if composed of ESCs, leverage cells' inherent ability to self-organize into spherical 3D structures. While cells can aggregate on 3D scaffolds and within hydrogels, challenges arise when adapting to synthetic environments.[105] Scaffold-free cell spheroids introduce a transformative approach by replicating cell features *in vivo*, mirroring the intricate architecture and functionality of native tissues without external structural support.[106] Compared to 2D structures, stem cell spheroids demonstrate enhanced cell viability, pluripotency, and beneficial properties such as angiogenic, anti-fibrotic, and immunomodulatory effects. These attributes significantly elevate the therapeutic potential of spheroids in tissue regeneration.

The essence of scaffold-free spheroid culture hinges on reducing cell adhesion to culture surfaces while promoting the aggregation of suspended cells.[107] This is achieved through surface modifications,[108] dynamic culture conditions,[109] and the application of external forces.[110] Innovative microfluidic devices, which provide microchambers for spheroid formation and channels for cell and medium delivery, enable the development of micro-organs-on-chips (Fig. 2).[111]

### A. Forced aggregation

In a pellet culture system, centrifugation induces cell aggregation at the bottom of conical tubes,[100,112] facilitating pellet compaction and differentiation. However, this approach poses the risk of developing a central necrotic core within the spheroids due to gradients in oxygen and nutrient levels.[113,114] Moreover, the extensive use of tubes is time-consuming and requires considerable space. To mitigate these challenges, high-throughput microwell systems have been developed for efficient cell aggregation.

### B. Hanging drop

By placing cell suspension drops under a lid, the hanging drop method allows gravity to concentrate cells at the base of the drop. This method is appreciated for its simplicity and the ability to regulate spheroid size.[115] However, an increased cell number or an extended culture duration can lead to higher risks of cell necrosis and apoptosis.[116] To address these challenges, a high-throughput culture system featuring an array of 384 hanging drops on a plate has been developed. This system is specifically designed for cancer stem cell spheroid culturing and pharmaceutical analysis, offering a more efficient and scalable solution.[117]

### C. Low attachment culture surface

Low-attachment strategies reduce cell adherence to culture surfaces, promoting spheroid formation through enhanced cell–cell interactions. Compounds such as polydimethylsiloxane[118] and agarose[119] have been used to create non-adhesive surfaces. Additionally,

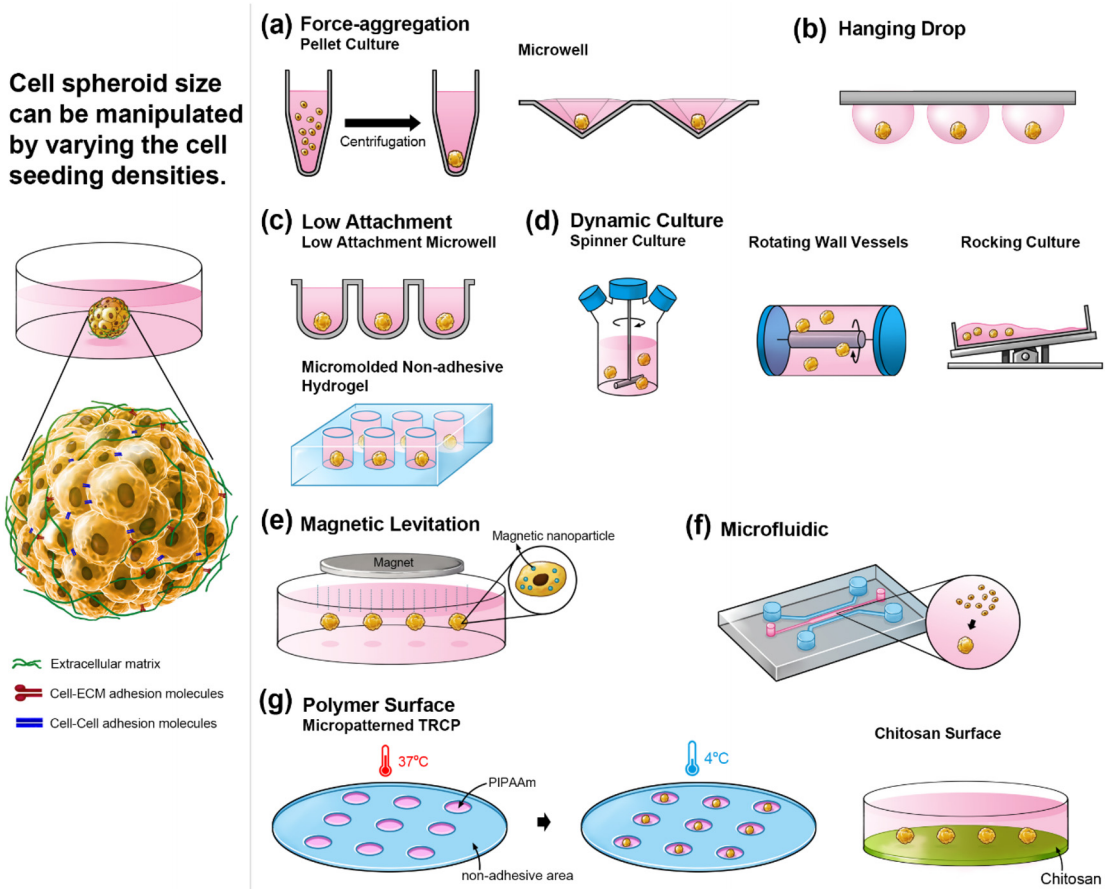


**FIG. 2.** Cell spheroid culture and fabrication techniques. (a) Force aggregation: utilizing pellet culture and centrifugation to promote cell aggregation. (b) Hanging drop: drops containing cells are suspended to facilitate spheroid formation. (c) Low attachment microenvironment: creating spheroids using ultra-low attachment surfaces or agarose-coated wells to prevent cell adhesion. (d) Dynamic culture: incorporating rotating wall vessels and rocking culture systems to enhance nutrient and gas exchange. (e) Magnetic levitation: employing magnets and magnetic nanoparticles to levitate cells, promoting spheroid formation without physical support. (f) Microfluidic: leveraging microfluidic channels to control the cell culture environment precisely. (g) Polymer scaffolds: using micropatterned thermally responsive cell culture platforms (TRCP) or chitosan surface to provide structural support for spheroids.

micromolded non-adhesive hydrogels, composed of agarose[120] or polyacrylamide,[121] facilitate the spontaneous formation of spheroids within low-attachment micromolds. These micromolds can be machine-crafted into various shapes for array-based, high-throughput spheroid cultures, offering a versatile and scalable approach for generating uniform spheroids.[118]

### D. Dynamic culture

The spinner flask method is a dynamic culture technique for spheroid formation that utilizes an impeller, which facilitates cell collisions, evenly distributes nutrients, and prevents cell sedimentation. Cells initially form loose aggregates through integrin binding and compact over time via E-cadherin interactions.[122] The stirring rate is critical, as low rates may cause sedimentation, while high rate may induce shear stress.

Rotating wall vessels, similar to spinner flasks, reduce cell settling by rotating along the x-axis, creating a microgravity environment with minimal shear forces.[123] This dynamic approach ensures uniform spheroid size and shape, enhancing spheroid yield.[124] Rocking culture systems, employing shakers or rockers, create a dynamic environment where cell suspensions gradually form spheroids, typically over three to four weeks. However, the prolonged culture period may limit their practical use.[6,125]

### E. Magnetic levitation

Magnetic levitation is an innovative spheroid construction method that utilizes magnetic forces to counteract cell sedimentation. The process involves incubating cells overnight with magnetic nanoparticles to enhance particle attachment, followed by resuspension in a low-attachment microwell plate placed above a magnet. This setup levitates the cells to the air–liquid interface, facilitating their spontaneous aggregation into spheroids. While this technique effectively forms spheroids, it requires specialized equipment, such as biocompatible magnetic nanoparticles and appropriate magnetic setups, which may increase the complexity and cost of the process.[126,127]

### F. Microfluidic

Microfluidic technology significantly enhances the production and culture of cell spheroids on a chip. This technology excels in managing dynamic flow and gradients of oxygen, nutrients, and growth factors, providing advantages over other spheroid formation methods.[111] When integrated with biosensors, microfluidic devices enable real-time imaging and monitoring, facilitating precise disease modeling and drug testing.[111,128] However, the complexity of these systems may necessitate specialized equipment and expertise, potentially limiting accessibility and increasing operational costs.

### G. Polymer surface

Chitosan polymer surface provides an alternative to traditional low-attachment approaches for spheroid formation.[38,129] The process involves coating culture surfaces with a chitosan solution, sometimes augmented with hyaluronan, which is then dried. Cells initially adhere to these modified surfaces, proliferate, and subsequently migrate to form spheroids.[129] However, variability in spheroid size can arise due to differences in cell adhesion and migration rates.

TRCD dishes with PIPAAm enable consistent spheroid creation by featuring specific zones for cell adhesion and a non-adhesive polyethylene glycol (PEG) perimeter. Lowering the temperature makes the PIPAAm hydrophilic, simplifying spheroid harvest.[130,131] Nevertheless, this method requires precise temperature regulation for effective spheroid recovery, adding complexity to the process.

## VI. APPLICATIONS IN TISSUE REGENERATION

### A. Preclinical animal models of stem cell sheets

Cell sheet technology enables the transplantation of a substantial number of stem cells to the target tissues, either through direct application or with the assistance of devices. This approach has shown promising therapeutic potential in several large animal models, including rabbits,[132] dogs,[133] pigs,[134] and monkeys.[135] Particularly, MSC sheets have shown significant pro-angiogenic, anti-fibrotic, and immunomodulatory effects *in vivo*. BM-MSC sheets have been reported to enhance angiogenesis and accelerate wound healing.[86,136] They also exhibited regenerative potential for bone and cartilage repair, particularly in growth plate injury.[96] After transplantation, BM-MSC sheets continuously released VEGF and HGF, thus reducing tubular and endothelial injury and curbing renal fibrosis via microvascular protection.[137]

ASC sheets have been thoroughly studied in myocardial infarction models, demonstrating their ability to promote angiogenesis, suppress remodeling and fibrosis, and improve cardiac function.[9,132,138] The composite construct made of ASC sheets and embryonic stem cell-derived cardiac progenitors has demonstrated cardiomyogenic potential and trophic support, significantly enhancing myocardial angiogenesis.[135] ASC sheet engineering is also used for cutaneous wound healing and limb ischemia, primarily through neovascularization and anti-fibrosis mechanisms.[11,44,56,82,139,140] Treatment of articular cartilage with ASC sheets result in reduced expression of MMP-1, MMP-13, and aggrecanase-1.[141] Additionally, ASC sheets have been employed to reinforce anatomical barriers and inhibit fibrosis in applications such as intestinal anastomosis,[142] colorectal anastomosis,[143] pancreatic transection stumps,[88] and arterial injuries.[134] Subcutaneous grafting of ASC sheets has been shown to increase adiponectin levels, decrease TNF-$\alpha$ levels, and improve glucose intolerance, indicating potential for diabetes management.[144] Synovial MSC sheets, known for their efficacy in chondrogenesis, have been utilized to repair cartilage defects in porcine models.[12,145] Furthermore, tissue engineers have explored the potential of amniotic MSC sheets for bone and cartilage regeneration,[59,60] while urine-derived stem cell sheets have enhanced rotator cuff repair in a canine model by stimulating the formation of enthesis-like tissue.[66]

### B. Clinical trials of stem cell sheets

To summarize clinical trials involving stem cell sheets, we searched the WHO international clinical trials registry platform (ICTRP), ClinicalTrials.gov, Japan primary registries network (JPRN) portal, and European Union Clinical Trials Register. We identified both unpublished and published trials conducted up to June 2024. Clinical studies on stem cell sheets and spheroids were compiled in Table II.

The periodontal ligament (PDL) is rich in stem cells, which hold promise for autologous transplantation in patients with periodontitis.[61,62] In a randomized controlled trial involving 30 subjects with

**TABLE II.** A summary of clinical trials/studies employing 3D scaffold-free stem cell culture methods.

| Target tissue | Stem cell construct | Model tested | References |
|---|---|---|---|
| Periodontal tissue | PDL-derived stem cell sheet | Periodontitis | 146 |
| | PDL-derived cell sheet | Periodontitis | 147 |
| | PDL-MSC sheet | Periodontitis | jRCT2090220391, UMIN000034310 |
| Corneal epithelium | Limbal epithelial cell sheet | Unilateral limbal stem cell deficiency | [216]NCT04773431, KCT0004741 |
| Articular cartilage | ASC spheroid | Knee osteoarthritis | NCT04773431, KCT0004741, jRCTb050200097, jRCTb050220148 |
| Lumbar disc | ASC spheroid | Discogenic low back pain | 156 |
| Retinal tissue | UC-MSC spheroid | Retinitis pigmentosa | 159 |

periodontitis, one group was treated with artificial bone alone, while the other group received PDL-derived stem cell sheets combined with artificial bone. The trial confirmed safety over a 12-month period but found no significant difference in clinical improvement between the two groups.[146] In another uncontrolled trial, ten subjects receiving PDL-derived cell sheets showed sustained improvement in clinical parameters at 6 months and no adverse effects over an average follow-up period of 55 months.[147]

Autologous limbus tissue containing limbal epithelial stem cells has demonstrated efficacy in managing limbal stem cell deficiency.[148,149] In a single-arm trial, autologous limbal epithelial cell sheets were transplanted in 10 subjects with unilateral limbal stem cell deficiency. Successful reconstruction was achieved in 60% of subjects at one year, increasing to 70% at two years, significantly surpassing outcomes observed with allogeneic limbal transplantation. Regarding visual acuity, 50% of the subjects showed improvement at one year, with this figure rising to 60% at two years. No clinically significant adverse events related to cell sheet transplantation was reported.[216]

### C. Preclinical animal models of stem cell spheroids

Stem cell-based spheroid therapy has emerged as a potent tool in regenerative medicine, demonstrating superior therapeutic effects. The safety and efficacy of this approach have been rigorously evaluated and compared to single-cell suspensions in various animal models, including rabbits and pigs. These 3D structures not only exhibit enhanced therapeutic potential but also demonstrate efficacy in enhancing wound healing, treating bone and osteochondral defects, and managing cardiovascular diseases. Integrating these innovative approaches into translational research holds immense promise for enhancing clinical outcomes, offering a more reproducible and effective strategy for cell-based therapies. For instance, studies have successfully highlighted the regenerative potential of BM-MSC spheroids in cartilage and subchondral bone repair, offering accelerated healing in osteochondral defect models.[150,151] Similarly, synovial MSCs, renowned for their chondrogenic capabilities, have been shown useful in repairing cartilage defects in animal models.[152] Additionally, ASC spheroids have been effectively delivered to the myocardium via transcatheter injection to enhance cardiac repair.[153]

### D. Clinical trials of stem cell spheroids

Based on our review of clinical trials focusing on MSC therapies, particularly MSC spheroids, it is evident that while intravascular infusion has been extensively studied for various MSC therapies, trials specifically involving MSC spheroids are relatively limited. One significant concern in the clinical application of MSC spheroids is the thrombotic risk associated with intravascular MSC infusion.[154] In a preclinical trial involving monkeys, human MSC spheroids, approximately 450 $\mu$m in diameter, were intravenously administered to assess safety and distribution. Remarkably, these spheroids persisted in the bloodstream for over one hour, contrasting with dissociated cells, which tended to accumulate primarily in the lungs. Importantly, during a 60-day observation period, none of the 19 monkeys experienced fatalities or detectable physiological changes, suggesting a potential safe application of MSC spheroid therapy in humans.[155]

In a phase I uncontrolled trial, researchers examined the efficacy and safety of intradiscal ASC spheroids combined with hyaluronic acid injection in a cohort of eight patients with discogenic low back pain.[156] These spheroids showed promising outcomes when initially primed with matrilin-3, a protein known to enhance the expression of collagen II and aggrecan. Six subjects reported reduced pain scores at six months, and four showed radiological improvements by the modified Pfirrmann grading system. Importantly, no adverse events were reported during the study period.[156]

Moreover, in the realm of degenerative retinal diseases, clinical trials involving suprachoroidal injection of umbilical cord-derived MSCs (UC-MSCs) have demonstrated effectiveness and safety in patients with retinitis pigmentosa (RP).[157,158] In an uncontrolled trial involving 15 RP patients, suprachoroidal implantation of UC-MSC spheroids led to improvements in best-corrected visual acuity (BCVA), visual field (VF), and multifocal electroretinogram (mfERG) recordings over a six-month follow-up period. Significant improvements in BCVA were observed compared to fellow eyes at one, three, and six months.[159]

## VII. RECENT ADVANCES AND EMERGING TECHNOLOGIES

### A. Bioprinting and bioreactors

Bioprinting technologies have been applied in tissue engineering to enable precise deposition of cells and biomaterials to construct complex structures for personalized implants.[160] Techniques like micro-extrusion, inkjet, and laser assistance allow for the creation of microtissues, which offer greater regenerative potential compared to single-cell suspensions, though residual biomaterials remain in the spheroids.[161] Bioprinting more sophisticated and large tissues with robust mechanical properties requires the combination of multiple

bio-fabrication tools and materials.[162] Micro-extrusion printing, driven by pneumatic or mechanical forces, is particularly effective in dispensing spheroids to form these microtissues. Innovative approaches involve placing spheroids in molds to create columnar structures for repairing bone defects, aided by computer-aided mold manufacturing.[163]

Bioinks, composed of bioprintable materials like hydrogels, biodegradable polymers, cell aggregates, synthetic polymers, and decellularized extracellular matrix components, play a critical role in modulating cellular behavior within tissue-engineered scaffolds.[164] Over the past decade, composite approaches using 3D printing have been extensively explored to fabricate vascularized organoids. For instance, Liu *et al.* developed cardiac microtissues by combining early vascular cells (EVCs) and cardiomyocytes, facilitating *in situ* vascular differentiation and self-assembly to form vascular networks within engineered tissues.[165] This EVC spheroid-laden cardiac patch presents an innovative therapeutic option for myocardial infarction. Similarly, Puistola *et al.* used decellularized ECM in corneal stroma-specific bioink, integrating it with ASC-derived corneal stromal keratocytes to create corneal stroma structures without needing donor corneas. The cost-effective approach is expected to enhance the accessibility and efficiency of treatments for corneal blindness.[166]

The Kenzan bioprinting method represents a high-resolution, scaffold-free approach to cell-only 3D bioprinting, where spheroids serve as the printing units. In this method, a Kenzan array, consisting of needles arranged in a cuboid formation, acts as a 3D framework for spheroid deposition. Each spheroid is precisely impaled onto a needle at a specific depth, allowing it to fuse with neighboring spheroids in a defined spatial layout. Post-printing, the needle array is removed, leaving the 3D structure to mature in bioreactors, which also help maintain the integrity of the fused spheroids. The Kenzan method has successfully printed gingival MSC spheroids for osteogenic applications and constructed 3D nerve structures for nerve regeneration.[167,168] However, challenges persist, particularly in preventing the disintegration of partially fused spheroids during needle array removal.

Bioreactors play a pivotal role in tissue engineering by providing a dynamic culture environment that supports stem cell growth and differentiation.[169] These biomimetic conditions optimize oxygen and nutrient delivery, overcoming diffusion barriers and facilitating tissue maturation and functionality of 3D stem cell constructs. This approach offers an optimal microenvironment for developing large-scale engineered tissues with enhanced therapeutic potential.

### B. Integration with biomaterials

Biomaterials play a crucial role in mimicking the native ECM and providing mechanical strength. Biodegradable transfer membranes such as Seprafilm®[136] and PGA sheets,[61,62,133] have been used in animal studies to aid in the transplantation of cell sheets. For instance, artificial bone combined with stem cell sheets has been successfully employed to regenerate bone and periodontal tissues.[61,170] Similarly, combining BM-MSC spheroids with basement membrane matrices or artificial bones has shown benefits in bone regeneration.[171]

The injection of spheroids often results in a low rate of cell retention at the targeted sites. Biomaterials address this challenge by facilitating the grafting of stem cells into target tissues, ensuring sustained delivery of critical cytokines and growth factors. For example, a biodegradable cardiac patch composed of poly (ester carbonate urethane) urea, in conjunction with ECM hydrogels and ASC sheets, enhanced stem cell engraftment, cardiac function, and angiogenesis in a myocardial infarction model compared to ASC alone.[172] Biodegradable pocketed patches constituted of PEG and polycaprolactone, combined with bio-sealant, supported the transplantation of ASC spheroids onto the heart surface.[173] Moreover, acellular dermal matrix, a biocompatible material specializing in skin repair, can effectively deliver ASC spheroids *in vivo*.[174] It provides a supportive scaffold that enhances the viability and integration of ASC spheroids, improving therapeutic outcomes in wound healing applications.

### C. Genetic modifications

Specific genes play pivotal roles in enhancing cellular functions and amplifying therapeutic outcomes in tissue engineering. For instance, researchers have engineered BM-MSCs to overexpress trophic factors like HGF or VEGF, significantly boosting their paracrine effects.[175] This genetic modification not only increases proliferation and enhances the expression of these factors *in vitro* but also stimulates angiogenesis, improves cardiac function, and prevents hypoxia-induced apoptosis when cell are delivered intramyocardially. Similarly, BM-MSC sheets overexpressing VEGF-A via lentiviral transduction have shown enhanced angiogenesis and reduced fibrosis.[176] Baculovirus-mediated gene delivery for VEGF overexpression in ASC sheets also demonstrated therapeutic benefits in myocardial infarction and ischemic limb models.[132,177] HGF-overexpressing ASC sheets delivered via adeno-associated viral vector serotype DJ (AAV-DJ) have promoted vascularization and neuroprotection while attenuating necrosis and fibrosis in ischemic limb models.[178] AAV-DJ is also effective in introducing multiple trophic factors into ASC sheets, leading to better preservation of viable myocardium compared to non-transduced ASC sheets in myocardial infarction models.[179]

In spinal cord injury (SCI) models, MSC spheroids engineered to express brain-derived neurotrophic factors (BDNF) through pCAGGS vector incorporation have shown improved motor function recovery and reduced neuronal loss.[180] Moreover, BM-MSCs derived from the SB623 cell line, known for increased production of angiogenic and neuroprotective factors compared to unprocessed BM-MSCs, have been used in clinical trials for ischemic strokes (NCT02448641).[181] Transplantation of SB623 cell sheets in ischemic cardiomyopathy models has demonstrated improved cardiac function by promoting angiogenesis.[182]

### D. Biophysical modifications

Biophysical modifications, such as hypoxic conditions and photo-biomodulation, significantly influence stem cell behavior and enhance tissue regeneration. For instance, BM-MSCs cultured under 3% oxygen exhibit decreased replicative senescence and enhanced chondrogenesis, mediated by the PI3K/AKT pathway and HIF-1α.[183] ASCs under similar hypoxic conditions also showed increased viability, prevention of apoptosis, and elevated expression of growth factors like VEGF, FGF2, and HIF-1α.[184,185] Hence, hypoxia-preconditioned BM-MSC sheets have demonstrated improved therapeutic efficacy in myocardial infarction models, enhancing cardiac function, angiogenesis, and reducing infarct size compared to unconditioned sheets.[136] Hypoxic preconditioning of ASC sheets enhanced the expression of pro-angiogenic factors such as VEGF and FGF2,[186] while modulating HGF expression and α1-adrenergic receptor sensitivity.[187] Similarly,

both BM-MSC and ASC spheroids exhibit enhanced chondrogenesis and increased secretion of ECM proteins, VEGF, and FGF2 under hypoxic conditions.[188] Hypoxia-preconditioned UCB-MSC spheroids, specifically, upregulated anti-apoptotic gene *Bcl-2*,[189] while ASC spheroids exhibited higher HIF-1α expression, indicative of their pro-angiogenic potential.[8]

Photo-biomodulation, a noninvasive light therapy that uses specific wavelengths of light to enhance cellular functions, has been shown to strengthen the biological properties of ASC spheroids, including survival rate after transplantation, secretion of pro-angiogenic factors, and promotion of angiogenesis in an ischemic limb model.[190] Consistent results were observed in another skin flap ischemia model, where ASC spheroids were exposed to 660 nm LED light for 10 min before grafting.[191]

## VIII. CHALLENGES AND FUTURE PROSPECTS

Generating 3D organoids for tissue engineering has made tremendous progress in the past decades, but these organoids are often confined to thin-layer tissues such as corneas and cartilage.[192] The abundant ECM within stem cell spheroids or sheets can offer suitable microenvironment for cellular morphogenesis and regeneration. A critical challenge is ensuring angiogenesis in host tissues post-transplantation to provide a stable and continuous supply of nutrients and oxygen. Pre-vascularization, achieved by seeding human umbilical vein endothelial cells on MSC sheets, has shown potential for forming and integrating new vessels with host vasculature.[193,194] Various strategies for constructing pre-vascularized tissues have been employed to mimic the physiological microvascular network,[195] establishing substantial foundation for long-term functionality following transplantation. Moreover, the addition of multiple angiogenic growth factors to 3D-cultured MSCs showed synergistic effects on angiogenesis for improving tissue repair.[196] Similarly, the integration of spheroids of the same source on ASC sheets can augment the angiogenic capability of sheets, providing scaffold-free composites for ischemic tissue regeneration.[119]

Achieving reproducible and cost-effective stem cell products is crucial for translational medicine. Large-scale production requires efficient cell expansion systems, such as cell suspension cultures in bioreactors, and automated manufacturing processes.[197] Clinical-grade cell products must comply with good manufacturing practices and regulatory standards, with potency assessments ensuring their functional effectiveness.[198] Currently, the high cost and time involved in manufacturing autologous cell sheets and spheroids limit their clinical use. Increasing evidence supports the efficacy of allogenic stem cell transplantation, paving the way for ready-to-use products in regenerative medicine.[198] Cryopreservation of 3D stem cell constructs may further facilitate their clinical application, but maintaining cell viability remains a significant hurdle.[93]

By integrating heterotypic cells or innovative technology, such as nanomedicine and microfluidics, we can significantly enhance their limited potential and accurately simulate the microenvironment of targeted lesions, thus bringing promising future in 3D tissue engineering.[195,199,200] Moreover, genetic and biophysical modifications of stem cells have unlocked their therapeutic potential but raise ethical and technical challenges. Genetic modifications can lead to instability and ethical concerns, while biophysical adjustments require specialized expertise and equipment. Continued research to address these limitations will be essential for the future of scaffold-free culture systems in regenerative medicine and biomedical research. After overcoming these challenges, stem cell-based 3D tissue engineering can be advanced to offer new options for effective and efficient regenerative therapies.

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## AUTHOR DECLARATIONS

### Conflict of Interest

The authors have no conflicts to disclose.

### Ethics Approval

Ethics approval is not required.

### Author Contributions

Ke-Chun Liu and Yueh-Chen Chen contributed equally to this work.

**Ke-Chun Liu:** Visualization (equal); Writing – original draft (equal). **Yueh-Chen Chen:** Visualization (equal); Writing – original draft (equal). **Chi-Fen Hsieh:** Writing – original draft (supporting); Writing – review & editing (supporting). **Mu-Hui Wang:** Writing – original draft (supporting). **Meng-Xun Zhong:** Writing – original draft (supporting). **Nai-Chen Cheng:** Conceptualization (lead); Funding acquisition (lead); Supervision (lead); Writing – review & editing (lead).

## DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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