Biomaterial Strategies for Stem Cell Maintenance During *In Vitro* Expansion

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Stem cells, having the potential for self-renewal and multilineage differentiation, are the building blocks for tissue/organ regeneration. Stem cells can be isolated from various sources but are, in general, available in too small numbers to be used directly for clinical purpose without intermediate expansion procedures *in vitro*. Although this *in vitro* expansion of undifferentiated stem cells is necessary, stem cells typically diminish their ability to self-renew and proliferate during passaging. Consequently, maintaining the stemness of stem cells has been recognized as a major challenge in stem cell-based research. This review focuses on the latest developments in maintaining the self-renewal ability of stem cells during *in vitro* expansion by biomaterial strategies. Further, this review highlights what should be the focus for future studies using stem cells for regenerative applications.

Introduction

STEM CELLS, HAVING THE ABILITY to self-renew and give rise to multiple cell types,¹ are the key factors in both developmental biology and regenerative medicine. In the last decade, an increasing interest in research on stem cells and their clinical applications has become apparent. For therapeutic applications, stem cells are first obtained from either early-stage embryo or adult tissues, expanded *in vitro*, and transplanted back into patients in order to treat disease or injury (Fig. 1). The most frequently studied stem cells include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), bone marrow mesenchymal stem cells (BMSCs), and adipose tissue-derived stem cells (ASCs).

Human ESCs, derived from the early-stage embryo, are one of the most widely studied cell sources for regenerative medicine (Fig. 2). ESCs exhibit the ability to differentiate into a variety of specialized cell types and, hence, represent a unique opportunity for tissue engineering and regenerative medicine. For example, controlled human ESC differentiation could result in an improved vision for patients with macular degeneration.² However, their clinical application is mainly limited by their ethical concerns. Recently, human iPSCs, free of ethical and political issues, have been produced by transfection of certain stem cell-related genes into adult somatic cells, and they have gained great interest in regenerative medicine (Fig. 2). These cells are similar to natural pluripotent stem cells in morphology and have the ability to differentiate into a variety of cell types. Nevertheless, one limitation of human iPSCs is that the efficiency of

successfully reprogrammed cells has been incredibly low. The low efficiency rate necessitates *in vitro* expansion before clinical use of iPSCs. Other concerns involve the difficulties in homogeneous cellular differentiation to specific cell types and the *in vivo* properties of immortal cells such as the tumorigenic fate of teratoma-initiating iPSCs.³

Mesenchymal stem cells (MSCs) are relatively safe and have been isolated from a variety of tissues, for example, bone marrow,^{4,5} adipose tissue,⁶ dental pulp,⁷ hair follicles,⁸ dermis,⁹ heart,¹⁰ liver,¹¹ and spleen.¹² There has been an increase in adult BMSC research in tissue regeneration (Fig. 2). Transplanted BMSCs can accelerate healing in human cutaneous wounds,¹³ repair infarcted human myocardium,⁴ chronic lower extremity wounds,⁵ and induce the formation of sufficient new bone to enable the reliable placement of dental implants.¹⁴ Nevertheless, the bone marrow harvest procedure is complex. ASCs have become one of the most popular stem cell populations in stem cell-based regeneration research (Fig. 2), as adipose tissue can be harvested in larger quantities with less invasive methods. The research to date has tended to focus on their potential for clinical applications. For instance, use of expanded ASCs is a safe and effective treatment for complex perianal fistula⁶ and depressed scars.¹⁵

Besides MSCs, other somatic stem cells play essential roles in regenerative medicine. For instance, the transplantation of peripheral blood stem cell is beneficial for acute myeloid leukemia¹⁶; transplantation of neural stem cells (NSCs) can enhance synaptic plasticity, reduce neuronal loss, and improve cognition in animal models of Alzheimer's disease¹⁷;

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FIG. 1. Schematic diagram of stem cell-based therapies. Stem cells can be obtained from either early-stage embryos or adult tissues, expanded *in vitro*, and transplanted back to patients. Color images available online at www.liebertpub.com/teb

hematopoietic stem cell (HSC) transplantation leads to rapid improvement of clinical symptoms and quality of life in interleukin (IL)-10- and IL-10 receptor-deficient patients; and corneal epithelial stem cell therapy using *ex vivo* expanded autologous cells proves successful in the treatment of unilateral limbal stem cell deficiency.¹⁸

FIG. 2. Increasing research in developing new therapies with stem cells in tissue regeneration by using keywords “ESCs, iPSCs, BMSCs or ASCs” and “tissue regeneration” in Web of Science. ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; BMSCs, bone marrow mesenchymal stem cells; ASCs, adipose tissue-derived stem cells. Color images available online at www.liebertpub.com/teb

Despite promising clinical applications, stem cells are usually found in low numbers, and their response to aging typically diminishes their ability to self-renew and proliferate.¹⁹ To be effective for therapeutic applications, large numbers of stem cells are needed. For example, for bone tissue engineering, 160 million cells would be required for 20 cm³ of tissue engineered implants based on using 8 million cells/cm³ scaffold^{20,21} to gain substantial bone formation in the case of large bone defects. In the case of treating chronic ischemic heart disease by stem cell injection, lack of diffusion of the transplanted cells could also result in low cell delivery efficiency,²² thus high numbers of cells are required. Nevertheless, the fate of stem cells is doomed in the same way: The pluripotency of ESCs is affected by the number of passages²³ and mitochondrial dysfunction has been found to occur with prolonged culture of ESCs²⁴; hemangioblasts/blast cells derived from human iPSCs have been shown to exhibit limited growth and expansion capability and early senescence with decreased hematopoietic colony-forming capability²⁵; significant decreases in the proliferation and differentiation potential of murine and human BMSCs were observed during *in vitro* expansion^{26–28}; and the expression of stemness biomarkers in human ASCs decreased significantly during long-term manipulation, along with the decrease of differentiation ability (adipogenesis, osteogenesis, and neurogenesis).²⁹

Taken together, the main question is how to maintain the stemness of stem cells during *in vitro* culture. If this problem can be solved, then a large number of high quality cells could be obtained for clinical purposes. Control of stem cell fate has

FIG. 3. Stem cell fate is regulated by varied factors, including genetic influences, cell–cell communications, growth factors and cytokines, extracellular matrix (e.g., component contents, topography/architecture), and physiochemical environment (e.g., matrix stiffness, oxygen tension, mechanical forces, and electrical cues). Color images available online at www.liebertpub.com/teb

been well reviewed,^{30–35} but unfortunately, there is limited research on how to increase stem cell expansion while maintaining their potential. As shown in Figure 3, stem cell fate is regulated by varied factors, including genetic influences, cell–cell communications, growth factors and cytokines, extracellular matrix (ECM; e.g., component contents, topography/architecture), and physiochemical environment (e.g., matrix stiffness, oxygen tension, mechanical forces, and electrical cues). This review mainly focuses on how to maintain the stemness of stem cells by exploiting biomaterial properties.

Miscellaneous Approaches for Stem Cell Maintenance

Figure 3 and Table 1 show that stem cell fate is regulated by various factors and accordingly, the stemness of stem cells can be maintained by a variety of approaches, including but not limited to gene transduction, growth factors, and cell–cell interactions.

Transduction of various genes can regulate the fate of tissue-specific adult stem cells and embryonic pluripotent cells.^{36–41} For example, MSCs transduced with human telomerase can undergo more than 260 population doublings without losing their osteogenic potential, whereas control cells became senescence after 26 population doublings.^{42,43} Despite promising results, several challenges remain to be overcome. First, genetically modified cells lack the long-term expression of the transgene,^{44,45} which will significantly limit the clinical and research applications. Second, most current gene delivery strategies are based on viral vectors in order to achieve the stable delivery of genetic information into eukaryotic genomes, which may pose a significant risk to the patients' immune system.⁴⁶ Third, along with gene delivery strategies, there exists the risk of interrupting intrinsic genes by random insertion of vector sequences.^{47,48} Moreover, the procedure of gene transduction is relatively complex.

Besides gene transduction, the external signals that control stem cell fate collectively make up the stem cell microenvironment or niche. The niche saves stem cells from depletion, while protecting the host from over-exuberant stem-cell

proliferation.⁴⁹ Therefore, *in vitro* recapitulation of the *in vivo* stem cell niche, which constitutes secreted factors, cell–cell interactions and ECM,⁵⁰ provides a promising approach in reprogramming stem cells for therapeutic purposes.

Growth factors and cytokines hold promise for the maintenance of stem cells due to their ease of application. Wnt proteins, basic fibroblast growth factor (bFGF), transforming growth factor-beta's and their family members, and IL-6 have shown their effects on stem cell maintenance.^{51–60} However, to maximize their clinical potential, the dosage should be optimized before their clinical administration, because the effects of growth factors on stem cells are dose dependent. For example, low doses of BMP-4 significantly increase the survival of human ASCs and retain their stemness as well as multipotency, whereas high doses increase apoptosis and reduce cell proliferation.⁶¹ In another regard, growth factors and cytokines may influence the genetic events of normal cells and, eventually, lead to malignancy.⁶² Moreover, high production costs of growth factors still exist and safe alternatives to expensive growth factors are desirable for clinical use.

The interactions between stem cells, as well as interactions between stem cells and neighboring differentiated cells, could regulate stem cell fate.^{63,64} However, there are still several distinct drawbacks of using cell–cell interaction strategies. First, there is a potential of infectious agent contamination when using animal-derived cells for clinical use.^{65,66} Second, feeder cells used for ESCs also undergo senescence and lose stem cell supportive properties after several passages.⁶⁷ Third, this process is cumbersome, expensive, and time consuming. Last but not least, the specific mechanisms by which feeder cells support the undifferentiated growth of stem cells has not been defined. This supporting effect is likely not caused by direct cell–cell contact but by certain growth factors secreted by feeder cells. For instance, human ESCs could not be maintained by using human feeder cells lacking the ability to synthesize bFGF, as indicated by the significant decreased expressions of Oct-4 and Nanog in human ESCs cultured in bFGF-knockout group compared with those in the control.⁶⁵

Due to the limitations of gene transduction, growth factor, and cell–cell interaction approaches, increasing emphasis has

TABLE 1. MISCELLANEOUS APPROACHES FOR STEM CELL MAINTENANCE

<i>Stimuli parameters</i>	<i>Cell types</i>	<i>Observations</i>	<i>References</i>	<i>Limitations</i>
Genetic modifications	Human and murine ESCs	The differentiation potential of ESCs can be maintained by the over-expression of Oct4, Sox2, Nanog, Ronin and Zfx	148–152	<ul style="list-style-type: none"> • Lack of the long-term expression of the transgene • Risk to the patients' immune system • Risk of interrupting intrinsic genes complex
	Human MSCs	Knockdown of p21 enhances proliferation, and osteogenic capacity in human MSCs; MSCs transduced with hTERT can undergo more than 260 population doublings without losing their osteogenic potential	42,43,153	
Cell-cell communications	Human ESCs	Various types of feeder cells can support the undifferentiated growth of human ESCs	65,154	<ul style="list-style-type: none"> • Risk of infectious agent contamination • Senescence of feeder cells • Cumbersome, expensive, and time-consuming process
	Murine HSCs	Endothelial cells can retain their self-renewal and repopulation ability	63,64	
Growth factors and cytokines	Human ESCs	Wnt3a and bFGF can retain human ESCs in an undifferentiated state	55,57,58,155	<ul style="list-style-type: none"> • Risk of interrupting the genetic events of normal cells • Risk of malignancy • High production costs
	Human MSCs	Wnt3a increase proliferation and inhibit osteogenic differentiation, and IL-6 preserves the undifferentiated state of human MSCs	52,60	
	Murine HSCs	Wnt3a can sustain the self-renewing fate of HSCs with reduced differentiation	51	
Biomaterials mimicking ECM components	See Tables 2 and 3			<ul style="list-style-type: none"> • Co-operation among many disciplines, including biomaterials, nanotechnology, medicine, biomedical engineering, cell and molecular biology for the development of various forms of advanced biomaterials
Topography/architecture	Murine ESCs	Nanofibers morphologically mimicking the ECM support the proliferation and self-renewal of murine ESCs	131–133	
	Human MSCs	Nanostructured surface with square lattice symmetry can retain stem-cell phenotype and maintain the growth of human MSCs	134	
Matrix stiffness	Murine ESCs and muscle stem cells, human MSCs	Substrates that match the stiffness of their own microenvironments promote their self-renewal ability and maintain their stemness	139–141	
Oxygen tension	Varied types of cells	Hypoxia maintains undifferentiated states of embryonic, mesenchymal, hematopoietic, and neural stem cell phenotypes	Reviewed in 129	

(continued)

TABLE 1. (CONTINUED)

<i>Stimuli parameters</i>	<i>Cell types</i>	<i>Observations</i>	<i>References</i>	<i>Limitations</i>
Mechanical forces	Human ESCs	Biaxial cyclic strain promotes self-renewal and retains pluripotency of human ESC	142	
Electrical cues	Murine NSCs	Continuous and defined levels of electric current promote NSC proliferation (twofold) ¹³⁸	145	
	Human MSCs	Pulsed electromagnetic field exposure could enhance cell proliferation; polyelectrolyte hydrogel matrices can support long-term survival and enhance proliferation (1.5-fold) of human MSCs	146,147	

ESCs, embryonic stem cells; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; bFGF, basic fibroblast growth factor; NSCs, neural stem cells; ECM, extracellular matrix; hTERT, human telomerase; IL-6, interleukin-6.

been focused on stem cell ECM for stem cell maintenance. Decellularized ECM from porcine synovium-derived stem cells (SDSCs), a three dimensional (3D) matrix consisting of nanostructured fibers with collagen I as one of the major structural proteins, facilitated SDSCs regaining stem cell phenotypes.⁶⁸ An increase in cell number from 2.7- to 14.6-fold and an enhanced chondrogenic capacity were observed

compared with SDSCs plated on plastic flasks.⁶⁸ Similar results were observed in human BMSCs and tendon stem cells when the cells were expanded using decellularized ECM from human BMSCs and tendon tissues, respectively.⁶⁹⁻⁷¹ The ECM that exists as a mix of several different proteins provides instructive cues for cell fate decisions, primarily via the integrin family of cell surface adhesion receptors.⁷² Those instructive

TABLE 2. BIOMATERIALS IN PLURIPOTENT STEM CELL EXPANSION

	<i>Biomaterials</i>	<i>Cell types</i>	<i>2D/3D</i>	<i>Feeder layer</i>	<i>Conditioned medium</i>	<i>Undifferentiated cells can be maintained</i>	<i>References</i>
Natural	Matrigel, vitronectin	Human ESCs and human iPSCs	2D	No	No	25 passages	77
	Matrigel, a combination of collagen IV, fibronectin, laminin, and vitronectin	Human ESCs	2D	No	No	20 passages	57
	Matrigel	Human ESCs	2D	No	No	9 and 22 passages	78
	Matrigel, Collagen IV	Human ESCs	2D	No	Yes	130 population doublings	76
	Collagen I	Human ESCs	2D	No	Yes	7 passages	80
	Laminin-111, -332 and -511	Human ESCs	2D	No	Yes	10 passages	82
	Laminin-511	Murine ESCs	2D	No	No	31 passages	84
		Human ESCs and iPSCs	2D	No	No	20 passages	85
	Vitronectin	Human ESCs	2D	No	No	7 passages	87
		Human iPSCs	2D	No	No	10 passages	90
Synthetic	Calcium alginate hydrogels	Human ESCs	3D	No	No	260 days without passaging in the undifferentiated stage	112
	PAS	Human ESCs	2D	No	No	10 passages	93
	Heparin-binding peptides	Human ESCs	2D	No	No	17 passages	94
	APMAAm	Human ESCs	2D	No	No	20 passages	97
	PMEDSAH	Human ESCs	2D	No	No	10 passages	98
	sIPN	Human ESCs	3D	No	Yes	5 days	104
	HA	Human ESCs	3D	No	Yes	20 days	107

2D, two dimensional; 3D, three dimensional; PAS, peptide-acrylate surfaces; APMAAm, aminopropylmethacrylamide; PMEDSAH, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]; sIPN, semi-interpenetrating polymer network; HA, hyaluronic acid.

TABLE 3. BIOMATERIALS IN ADULT STEM CELL EXPANSION

	Biomaterials	Cell types	2D/3D	Observations	References
Natural	Matrigel	Human MSCs	2D	Improved cell expansion (1.6-fold) and enhanced neuronal differentiation	79
	Collagen types I and IV together with laminin and fibronectin	Human haematopoietic progenitors	2D	Increased CD34 ⁺ (1.3-fold) and CD41 ⁺ (1.2-fold) cell expansions	81
	Laminin	Murine HSCs	2D	Amplified HSCs compartment in BMSCs during culture	86
	Decellularized ECM from human BMSCs	Human BMSCs	2D	Enhanced proliferation capacity (2 to 4-fold) and increased chondrogenic and osteogenic potentials	69,70
	ECM from tendon tissue	Human TSCs	2D	Reduce TSC population doubling time (15%) and preserve the stemness of TSCs	71
	ECM generated by MSCs from young mice	Aged murine MSCs	2D	Replication and osteogenesis of aged MSCs can be rescued	156
	Decellularized ECM from porcine SDSCs	Porcine SDSCs	3D	An increase in cell number (2.7 to 14.6-fold) and an enhanced chondrogenic capacity	68
	Human platelet lysate gel	Human MSCs	3D	Increased proliferation rates (around twofold) and enhanced colony-forming units outgrowth	108
Synthetic	Connecting segment-1	Human HSCs	2D	Higher CD34 ⁺ cell expansion (sixfold)	95
				Greater expansion of HSCs (2 to 5-fold) and more pluripotent colony-forming units	96

BMSCs, bone marrow mesenchymal stem cells; TSCs, tendon stem cells; SDSCs, synovium-derived stem cells.

cues can be categorized as biochemical signals (e.g., ECM components) and biophysical cues (e.g., topography and stiffness).^{30–32,34,35} Thus, biomaterials designed by controlling parameters, including components, material architecture, surface topography, mechanical and electrical properties, may act as an artificial ECM to direct stem cell fate.

Biomaterials for Stem Cell Maintenance

The use of biomaterials, from both natural and synthetic origins, to develop two-dimensional (2D) and 3D cell culture systems for the expansion of pluripotent stem cells (Table 2) as well as adult stem cells (Table 3) is summarized in this section. The modifications of biomaterials in terms of topography/architecture, mechanical properties, and biochemical factors are also described (Fig. 4).

Overview of biomaterials

Natural biomaterials. *In vitro* cell culture is usually carried out on flat substrates, which hold the advantages as a simplified approach to identifying the effect of individual niche components on stem cell fate. The common technique of growing cells on tissue culture polystyrene is gradually being replaced by culturing cells on substrates with a more appropriate composition. Stem cells can sense and respond to ECM molecules through the interaction between integrins

on the cell surface and cognate ligand-binding motifs on ECM molecules. The ECM is mainly composed of collagens, laminins, and glycoproteins serving as substrates for a variety of adhesion molecules such as integrins.⁷³ The integrins are a major family of ECM receptors that transmit information from the matrix to cells, thereby playing a key role in the regulation of cell behavior, including cell survival, adhesion, proliferation, and differentiation.⁷⁴ Designing biomaterials mimicking ECM is a relatively economic and safe approach to regulate the fate of stem cells. Considering that most cells require adhesion to an ECM for survival and growth through ECM–integrin interaction, natural biomaterials consisting of ECM components have been tested for stem cell expansion.

Matrigel. Matrigel is a gelatinous protein mixture and commercially available product comprising several ECM components, such as laminin, type IV collagen, and heparan sulfate proteoglycan.⁷⁵ Matrigel served as the first feeder-free culture system in which undifferentiated human ESCs could be maintained for 130 population doublings.⁷⁶ Human ESCs showed successful expression of pluripotency genes and the surface markers of pluripotency proteins during passaging. It should be noted that the stemness of human ESCs can be maintained on Matrigel in mouse embryonic fibroblast conditioned medium, but ESCs on Matrigel without conditioned medium completely differentiated after two passages.

FIG. 4. Biomaterial strategies *in vitro* expansion of stem cells. 2D and 3D cell culture systems developed by the use of biomaterials, including both natural and synthetic origins, for the expansion of stem cells. The modifications of biomaterials include topography/architecture, and biochemical factors. 2D, two dimensional; 3D, three dimensional. Color images available online at www.liebertpub.com/teb

Several other chemically defined culture media formulations have been developed to support the undifferentiated proliferation medium of human ESCs on Matrigel substrate in feeder-free conditions.^{57,77,78} Still, a variety of growth factors and cytokines are required in these defined culture mediums.

For adult stem cells, Matrigel significantly improved cell expansion of MSCs by 1.6-fold through surface modification of tissue-grade polystyrene.⁷⁹ Nevertheless, Matrigel is derived from mouse tumor cells and may cause pathogen transmission to humans. Growth of stem cells under xeno-free conditions will benefit the later clinical applications.

Collagen. Collagen, the main component of connective tissues, is a classic natural material for tissue engineering and regenerative medicine. Collagen I, which is well defined, a component of several FDA-approved products and widely available, has recently been tested as a potential human ESC growth biomatrix. Collagen I is reported to support the undifferentiated growth of human ESCs for seven passages.⁸⁰ Human ESCs grown on Collagen I processed a comparable population doubling rate as human ESCs grown on Matrigel. These cells expressed pluripotency proteins and retained a normal karyotype.⁸⁰ Furthermore, collagen IV has been shown to support the undifferentiated growth of human ESCs, although the cultures on collagen IV did not contain as many undifferentiated colonies as the cultures on Matrigel.⁷⁶ It should be noted that these culture systems still need the presence of conditioned medium from feeder layers, which is full of growth factors.^{76,80}

For adult stem cells, tissue culture surfaces coated with collagen types I and IV along with laminin and fibronectin increased CD34⁺ (1.3-fold) and CD41⁺ (1.2-fold) cell expansions, which provide a better environment for the *ex vivo* expansion of hematopoietic progenitors.⁸¹

Laminin. Laminin, a major component of the ECM, is found in the basal lamina. Recombinant human laminins have been investigated for stem cell culture due to the advantage that they are abundantly available and well-characterized human-origin proteins.⁸² More than 15 laminin isoforms have been

identified⁸³ and human ESCs, commonly expressed abundant integrin $\alpha_6\beta_1$, bind predominantly to laminin-111, -332, and -511.⁸² Consequently, human ESCs cultured on laminin-111, -332, and -511 substrates can retain an undifferentiated state for 10 passages without feeder layers.⁸² However, similar as for collagen substrates, these culture systems need the presence of conditioned medium, for which they are feeder free but not xeno free.⁸² Without conditioned medium or feeder cells, Domogatskaya *et al.* reported that recombinant human laminin-511 but not -332, -111 alone was sufficient to maintain the self-renewal of mouse ESCs for approximately 31 passages.⁸⁴ Later, the same research group reported that this xeno-free system could also support the undifferentiated growth of human ESCs and iPSCs for at least 20 passages, indicating great potential for therapeutic purposes.⁸⁵

In addition, laminin also benefits *ex vivo* culture of murine HSCs, as it amplified HSCs compartment in BMSCs during culture.⁸⁶

Vitronectin. Vitronectin, an abundant secreted glycoprotein found in serum and the ECM, was shown to promote human ESC attachment through interaction with $\alpha_v\beta_5$ integrin.⁸⁷ Braam *et al.* reported that recombinant vitronectin was a functional alternative to Matrigel, supporting human ESC sustained self-renewal and pluripotency in defined, nonconditioned medium for at least 1 month (seven passages).⁸⁷ Later, other groups also reported that vitronectin could support the undifferentiated expansion of human ESCs with a variety of defined medium.^{88,89} The expanded human ESCs maintained differentiation capacity and expressed pluripotency markers as strongly as the human ESCs cultured on feeder layers.^{88,89} More recently, human iPSCs successfully maintained their stemness for 10 passages when cultured on vitronectin-coated dishes in xeno-free medium.⁹⁰

However, the response of MSCs and ESCs is different in fate determination when cultured on vitronectin substrate. For example, contact with vitronectin promotes the osteogenic differentiation of human MSCs.⁹¹ Thus, the results obtained from one cell type cannot simply be transferred directly to other types of cells.

Although the results show great potential, natural biomaterials in general present several limitations when used as cell culture systems. First, they are usually not well-defined and difficult to control at the molecular level, and, therefore, may contribute to the variability in results.⁹² Second, the difficulty in purification and sterilization makes the manipulation more elaborate and complicated. Third, natural biomaterials may invoke immunogenic responses after implantation due to the structure similarity to biological substances. Thus, well-defined synthetic materials with controlled properties may be safer for clinical applications.

Synthetic biomaterials. Widespread clinical applications of stem cells require chemically defined materials that are low in cost, with tunable physical properties and long-term stability. For that reason, defined synthetic biomaterials have been developed to replace the complicated natural materials due to the advantages of ease of scale-up and decreased risks of disease transmission from unknown pathogens. Recently, synthetic peptides and polymers have been developed for stem cell expansion.

Synthetic peptides. ECM molecules are usually quite large and present diverse domains for cell adhesion. Biologically active peptides are organic compounds that serve as a synthetic alternative to ECM proteins. Synthetic peptides hold the advantages of stability, ease of synthesis, and conjugation to materials. Melkounian *et al.* reported that human ESCs could be successfully maintained on synthetic peptide-acrylate surfaces (PAS) in chemically defined xeno-free medium for more than 10 passages with similar phenotypic marker expression as cells cultured on Matrigel.⁹³ Here, amine-containing peptides derived from active domains of ECM, including bone sialoprotein, vitronectin, fibronectin, and laminin, were conjugated to PAS to provide a synthetic alternative to complex ECM. At the same time, Klim *et al.* screened more than 500 unique surfaces based on 18 bioactive peptides by using an array to present bioactive peptides either alone, in different combinations, or at varying surface densities.⁹⁴ The results showed that the surfaces presenting heparin-binding peptides which recognize cell surface glycans supported the long-term growth of human ESCs and maintained their pluripotency markers for more than three months with a defined medium.⁹⁴

For adult stem cells, surface immobilization of adhesion peptides on substrate benefits *ex vivo* expansion of HSCs from umbilical cord blood.^{95,96} HSCs cultured on synthetic substrates surface-immobilized with peptides containing the connecting segment-1 binding motif (EILDVPST) showed 2- to 5-fold greater expansion of HSCs and more pluripotent colony-forming units than those on fibronectin-grafted and polyamine-grafted dishes, suggesting that the specific interaction between HSCs and connecting segment-1 helps maintain the pluripotency of HSCs during the *ex vivo* expansion.⁹⁶

Synthetic polymers. Preadsorption of proteins or peptides to substrates increases cost and limits scalability. Recently, the long-term self-renewal of human ESCs was reported to be successfully maintained on hydrogel interfaces of aminopropylmethacrylamide (APMAAm) for more than 20 passages in chemically defined media without the previous

attachment of any biological coatings such as peptides, proteins, or Matrigel™.⁹⁷ Since the interface was not functionalized with proteins or peptides to promote cell adhesion, it was identified that bovine serum albumin adsorbing from the culture media played a key role in human ESC attachment to APMAAm interfaces.⁹⁷ Villa-Diaz *et al.* developed another standardized and fully defined synthetic polymer coating, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH), which could sustain the undifferentiated growth of human ESCs in commercially available defined medium for 10 passages.⁹⁸ In this case, six polymer coatings by surface-initiated graft polymerization were synthesized and tested, and only PMEDSAH supported attachment and proliferation of human ESCs. However, the mechanism of this phenomenon is not clear, and the contribution of various physicochemical properties (such as wettability, stiffness, surface topography, and zeta potential) of PMEDSAH still needs to be determined. Those findings demonstrate that chemically defined, synthetic biomaterials can be applied to propagate ESCs. However, no report is available with regard to their effects on the self-renewal of adult stem cells based on our literature survey. Moreover, the mechanisms by which the substrates function remain largely unclear.

Biomaterials for 3D cell culture

The 2D culture systems mentioned earlier present useful methods for stem cell expansion, but there are certain limitations. Cells grown on 2D substrates exhibit an unnatural dorsal/ventral polarized state that is in contrast to the situation *in vivo*, where cells are completely embedded in a 3D microenvironment. Accordingly, 3D cell culture models, which elicit a more physiological state, provide a promising alternative for monolayer cell culture. Cells grown in a 3D environment display more similarities to *in vivo* cells in morphology and molecular regulation.⁹⁹ Compared with the 2D culture, 3D culture provides another dimension for external mechanical inputs and cell adhesion, which affects integrin ligation, cell contraction, and associated intracellular signaling.^{100,101} Thus, developing 3D culture systems have been evaluated for stem cell maintenance. In 3D cell culture models, cells are embedded in biomaterials, cultured as cellular spheroids, or grown on 3D scaffold materials.¹⁰² Biocompatible 3D scaffolds have been well reviewed by Moroni *et al.* for tissue engineering applications.¹⁰³ However, no such scaffold has been reported to be utilized as a 3D system for stem cell expansion. This is most likely because the pattern of cell seeding, attachment, and growth on these 3D scaffolds is similar to that seen on 2D surfaces. Thus, this review mainly focuses on cell encapsulation and cellular spheroid methods for the expansion of stem cells.

Cell encapsulation. Embedding cells in semi-permeable materials is the most common approach for 3D cell culture. A completely synthesized ECM hydrogel composed of a semi-interpenetrating polymer network (sIPN) was prepared from poly(*N*-isopropylacrylamide-coacrylic acid) [p(NIPAAm-co-AAc)].¹⁰⁴ To promote cell adhesion, the polymer network was interpenetrated by polyacrylic acid-graft-Ac-CGGNGE PRGDTYRAY-NH2 [p(AAc)-g-RGD] linear polymer chains, which binds to several integrin receptors, including α_1 , α_v , β_1 ,

and $\alpha_v\beta_3$.¹⁰⁵ By varying the polymer components and the density of the network cross-linker, sIPNs with a range of cell-adhesion ligand densities and matrix stiffness could be created.¹⁰⁴ Although specific cell-matrix interactions were promoted by the peptide sequences presented in the sIPN, conditioned medium from fibroblast feeder layers was still needed to maintain self-renewal of human ESCs.¹⁰⁴ In another study, hyaluronic acid (HA) hydrogel has been tested for controlling human ESC self-renewal. The rationale to choose HA was that the HA content is highly present in undifferentiated cells and decreases at the onset of differentiation.¹⁰⁶ It was found that human ESCs cultured in HA maintained their undifferentiated state, preserved their normal karyotype, and maintained their full differentiation capacity as indicated by embryoid body formation for 20 days.¹⁰⁷ Afterward, as the cells remodeled the hydrogel, colonies near the surface were released, making it difficult to accurately quantify cell proliferation as well for the long-term maintenance of stem cells.

For the expansion of adult stem cells, Walenda *et al.* demonstrated a novel 3D matrix fabricated from human platelet lysate (HPL) gel for the expansion of MSCs.¹⁰⁸ Since HPL is of human origin, it can be used as a substitute for fetal calf serum without the risk of xenogeneic immune reactions or transmission of bovine pathogens. This gel culture system facilitates a twofold increase in proliferation rate and enhanced colony-forming units outgrowth compared with tissue culture plastic for culture expansion of MSCs.¹⁰⁸

Cellular spheroids. Cellular spheroids that often mimic the *in vivo* situation more closely than a cell encapsulation system provide an alternative 3D culture system.^{109–111} Cellular spheroids are aggregate cells that grow free of foreign materials.¹⁰² Spheroids may have contact with culture materials, but no foreign material is present within spheroids. For example, human ESCs formed spheroids by encapsulation in calcium alginate hydrogels.¹¹² The encapsulated human ESCs retained an undifferentiated state, and their pluripotency in a feeder layer-free and xeno-free environment for approximately 260 days. ESCs increased in size and number without escaping the confines of the hydrogel for the duration of the cultures. Furthermore, these encapsulated ESCs were easily recovered from the hydrogels by using a dissolution buffer.¹¹²

For adult stem cells, the methods that have been reported to induce cellular spheroids include spinner flasks and rotating wall vessel bioreactor,¹¹³ microwells,¹¹⁴ nonadherent surface,¹¹⁵ hanging drops,¹¹⁶ forced-aggregation technique,¹¹⁷ and micropatterned surfaces.^{118,119} Moreover, spheroid formation of several cell types has been reported on chitosan films.^{109,120–123} The gene expression of self-renewal markers (i.e., Oct4, Sox2, and Nanog) in MSCs and ASCs was significantly higher for the cells in spheroids in comparison to the monolayer cells,^{109,120} indicating that the cells in spheroids experienced a probably de-differentiating and returned to a more primitive state.¹⁰⁹

The 3D culture systems provide promising results for the *in vitro* expansion of stem cells. However, the mechanisms of cellular interaction within these systems are not clear. It is often difficult to clarify the direct effects of the biomaterial itself from the diffusional limitations that are common to all 3D culture. Limitations in the diffusion of soluble factors

might account for some of the differences frequently observed between 2D and 3D culture systems.¹²⁴ For instance, the stem cell maintenance might be caused by the oxygen gradients (owing to the low solubility of oxygen in aqueous media) that could attribute to the higher expression of self-renewal marker genes.^{125,126} All human nucleated cells can sense oxygen tension and respond to a reduced O₂ concentration (hypoxia).¹²⁷ The conventional *in vitro* cell culture is performed under 21% oxygen tension.¹²⁸ On the other hand, stem cell niches are usually located in regions of low oxygen tension.^{125,126} For example, the oxygen concentrations of mesenchymal, neural, and HSC niches are between 1% and 8%.¹²⁹ It has been well reviewed by Mohyeldin *et al.* that hypoxia maintains undifferentiated states of embryonic, mesenchymal, hematopoietic, and NSC phenotypes.¹²⁹ These studies indicate that optimization of oxygen tension for each type of stem cells is essential for their stemness maintenance during *in vitro* expansion. Future work is directed to clarify the influence of biophysical and biochemical cues of biomaterials on stem cell fate determination, which may help reduce the complexity and develop advanced biomaterials for stem cell expansion.

Modification of biomaterials for stem cell maintenance

Topography/architecture. The cell/material interface has been shown to exert considerable influence on stem cell function and differentiation.¹³⁰ Recently, polymeric nanofibers morphologically mimicking the ECM/basement membrane were found to support the proliferation and self-renewal of mouse ESCs.^{131,132} In addition, Markert *et al.* screened 504 different types of topographical surface microstructures in order to identify specific topography that can support the expansion of undifferentiated murine ESCs without feeder cells or conditional medium.¹³³ The results of this study indicated that stem cells are affected mainly by the lateral and vertical dimensions of the microstructures, while the distribution of the topographical patterns is less important.¹³³ This finding is of great importance for the design of biomaterials for stem cell maintenance.

For adult stem cells, a nanostructured surface with square lattice symmetry has been identified to retain stem-cell phenotype and to maintain the growth of adult MSCs.¹³⁴ It is likely that such nanoscale features alter the interaction of integrin receptors within cell adhesions, resulting in changes in intracellular tension. Significant progress has been made in designing biomaterials to enhance stem cell differentiation.^{33,135} From the opposite point of view, one can take advantages from these studies and use them to fabricate novel biomaterials to inhibit stem cell differentiation and facilitate stem cell maintenance. For example, disordered nanoscale structures can stimulate human MSCs to produce bone mineral *in vitro*, even in the absence of osteogenic supplements,¹³⁶ while the ordered square nanostructures can retain their stem-cell phenotype and multipotency.¹³⁴ Thus, the designing of novel biomaterial platforms based on the current knowledge on biomaterials and cell differentiation may enhance the *in vitro* expansion of stem cells.

Taken together, these studies demonstrate that nanoscale patterning can serve as a powerful tool for the manipulation of stem cells. Since most studies focused on ESCs, more research work on how to maintain the potential of tissue-specific adult

stem cells by controlling the topography of their substrates should be explored in future.

Biomechanical factors. Stem cells can “feel” and act in response to the stiffness of the substrates.¹³⁷ Tissue culture plates, usually used for culturing adherent cells, are much stiffer than the cell microenvironments *in vivo*. Substrate stiffness has been identified as a vital parameter to determine stem cell fate. For instance, rigid matrices that mimic collagenous bone are osteogenic,¹³⁸ while soft substrates which match the intrinsic stiffness of ESCs promote self-renewal and pluripotency of ESCs.¹³⁹ Substrates mimicking muscle stiffness regulate self-renewal, enhance survival, prevent differentiation, and promote stemness of muscle stem cells.¹⁴⁰ Polyacrylamide gels that mimic the elasticity of bone marrow can maintain MSCs in a quiescent state.¹⁴¹ Accordingly, it is safe to hypothesize that stem cells cultured on substrates which imitate the stiffness of their own microenvironments may be a promising system to promote their self-renewal ability and maintain their stemness.

In addition to stiffness, there is mounting evidence that external mechanical stimulation plays critical roles in stem cell fate determination.³⁵ For instance, Saha *et al.*¹⁴² applied biaxial cyclic strain to a deformable elastic substratum on which the human ESC colonies were cultured. Results showed that human ESC self-renewal was promoted as measured by an increase in self-renewal gene expression (Oct4 and SSEA-4); the pluripotency of human ESCs was retained as evidenced by their ability to differentiate to cell lineages in all three germ layers.¹⁴² Thus, the application of mechanical forces may be useful toward stem cell maintenance for therapeutic applications.

Biochemical factors. Some biochemical factors have also been reported for the efficient *in vitro* expansion of stem cells. For example, short-wave UV/ozone radiation treatment on conventional tissue culture plates yields chemically defined surface with high intensities of several secondary ions (e.g., $C_2H_5O^+$ and $C_2H_6N^+$) that are favorable to human ESC colony formation.¹⁴³ This chemically defined surface generates more than thrice the number of cells than feeder-containing substrates per surface area, which will finally facilitate cell therapeutic applications.¹⁴³ Another example is that human MSCs maintained a stem cell phenotype when cultured on $-CH_3$ -modified glass surfaces, but processed osteogenic differentiation when cultured on $-NH_2$ - and $-SH$ -modified glass surfaces.¹⁴⁴

Bioelectrical factors. The behavior of *in vitro* cultured cells could also be influenced by bioelectrical cues through application of electrical or electromagnetic currents/fields to stimulate substrate and/or cell construct. For this purpose, Chang *et al.*¹⁴⁵ developed a biphasic current stimulator chip for stem cell culture. This stimulator chip could generate both positive and negative currents in the same culture chamber. Further experiments indicated that this culture system could double NSC proliferation by applying continuous and defined levels of electric current.¹⁴⁵ For human MSCs, pulsed electromagnetic field exposure could enhance cell proliferation during the exponential phase and it possibly resulted from the shortening of the lag phase.¹⁴⁶ Recently, Lim *et al.* developed polyelectrolyte hydrogel-based

multifunctional matrices, which provide not only 3D structural support to the embedded cells but also dynamic electrical and mechanical cues to the human MSCs.¹⁴⁷ These anionic hydrogels could undergo reversible, anisotropic-bending dynamics in an electric field by changing the concentration of anionic groups within the hydrogel. Thanks to their close resemblance to the native cellular environment in multiple aspects, these new 3D electro-mechanical matrices were shown to support human MSC survival for at least 21 days and to enhance cell proliferation by 1.5-fold.

Conclusions

To facilitate the translation of stem cells from bench to bedside, this article reviews recent developments regarding the diverse properties of biomaterials for stem cell expansion. It should be noted that cell fate is influenced by a variety of factors in combination. Robust, fast, safe, and cost-effective methods using defined biomaterials may reconstruct the *in vivo* niche of stem cells, which will finally benefit both research and routine clinical purpose. The development of 3D culture systems by using biomaterials recapitulating the *in vivo* stem cell niche will probably provide better microenvironments for stem cells compared with the traditional 2D monolayer culture systems. New insights should be gained into the modifications of biomaterials such as the topography, stiffness, and surface chemistry. Though progress has been made to maintain the self-renewal ability of stem cells, we are still far from the large-scale expansion of undifferentiated stem cells for therapeutic purpose. In the future, biomaterials should be designed to present a combination of physical and chemical factors to expand stem cells. Moreover, the ultimate function of expanded cells requires an *in vivo* evaluation to confirm their regenerative capacity, for example, by transplantation of expanded cells into animal models. This needs collaboration among many disciplines, including cell and molecular biology, biomaterials, pharmacology, nanotechnology, and medicine.

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