Chapter 12
Feeder Cell Sources and Feeder-Free Methods for Human iPS Cell Culture

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Abstract Induced pluripotent stem cells (iPSCs) hold great promise for regenerative medicine and disease modeling. The original methods to grow human iPSCs utilized methods developed for human embryonic cells (ESCs), in which mitotically inactivated mouse-derived fibroblasts are mainly used as a "feeder" cell layer to maintain the undifferentiated status of pluripotent stem cells. However, these methods still require further consideration to facilitate cell expansion and to maintain the undifferentiated state of human iPSCs and/or ESCs for a longer period of time. In addition, the use of animal-derived feeders should be avoided for eventual clinical application of iPSC therapies. Therefore, human-derived feeder culture systems or feeder-free culture systems are currently being developed to prevent exposure to animal pathogens. In this review, existing mouse and human feeder culture systems for human ESCs and iPSCs are first introduced, and then previously reported feeder-free culture methods using extracellular matrix-associated products or synthetic biomaterials are outlined to discuss an appropriate culture system for clinical application of iPSCs.

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12.1   Introduction

Human induced pluripotent stem cells (iPSCs) generated by the introduction of defined factors from somatic cells exhibit pluripotency similar to that of human embryonic stem cells (ESCs) [1]. The iPSC technology offers great promise for regenerative therapies and disease modeling in both the medical and dental fields [2]. iPSCs have pluripotency to differentiate into almost all cell types and superior self-renewal capacity that enables unlimited expansion [3], which has prompted researchers to apply them as a cell source for transplantation therapies to regenerate various types of missing, diseased, or defective tissues/organs. However, before iPSCs can be used in the transplantation therapy, several technical limitations of the culture methods must be addressed. For instance, human ESCs and iPSCs cannot sustain their original characteristics in monoculture on standard tissue culture plates without supporting factors. In an in vitro culture system, human ESCs/iPSCs typically require "feeder cells", which produce specific stemness-supporting factors, to prevent spontaneous differentiation [4, 5]. Feeder cells also produce adhesion molecules and extracellular matrix (ECM) to improve ESC/iPSC attachment, thereby supporting the growth and survival of ESCs/iPSCs. The most commonly used feeder cells are mitotically inactivated mouse-derived fibroblasts [4, 6–8]; however, these animal-derived feeder cells pose an increased risk of transferring unknown viruses and zoonotic pathogens in addition to immune rejection. Alternatively, human-derived cells have also been shown to effectively function as feeder cells for ESCs/iPSCs. Furthermore, "feeder-free" culture systems, such as culture using cell-free ECM proteins or synthetic biomaterials as substrates, have recently received increasing attention. This review assesses various ESC/iPSC culture methods with regard to feeder cells and feeder-free methods to help identify an appropriate culture condition for future clinical applications of iPSCs.

12.2   Feeder Cells for ESC/iPSC Culture

12.2.1   Mouse-Derived Feeder Cells

Since mouse ESCs were first established in 1981 [9, 10], mitotically inactivated fetal mouse fibroblasts have been used as feeder cells for mouse ESC culture. This feeder culture method developed for mouse ESCs was applied to human ESCs when Thomson group first established human ESC lines in 1998 [4], showing that mouse feeder cells could be used to facilitate proliferation and prevent differentiation of human ESCs. Subsequently, several types of mouse-derived feeder cells have been used in human ESC/iPSC studies, such as mouse embryonic fibroblasts (MEFs), STO cells, and SNL 76/7 cells.

MEFs are the most commonly used feeder cells used to support the pluripotent status of human ESC cultures [4, 11, 12]. Primary MEFs are not homogeneous, as

they contain several types of cells other than fibroblasts [13]. To maintain ESC proliferation and pluripotency, MEFs produce various proteins, including transforming growth factor beta 1 (TGF-β1), activin A, bone morphogenetic protein (BMP)-4 [14], and pleiotrophin (heparin-binding growth factor) [15]. When used as in feeder cell layers, MEFs are proliferation-inactivated by chemical (mitomycin C) treatment or gamma irradiation prior to seeding of ESCs/iPSCs. It should be noted that the mitotic inactivation or irradiation of MEFs stimulates the expression of several signaling proteins, such as Wnt-3 [16], which may participate in the molecular mechanisms underlying the maintenance of pluripotency in co-cultured human ESCs/iPSCs. The outbred mouse CF-1 strain may be the most widely used donor for MEF feeder cells for ESC and iPSC culture [13, 17], as CF-1 MEFs have been shown to produce TGF-β1, activin A, BMP-4, gremlin, and noggin [14, 18, 19] but not bFGF [14]. One disadvantage of using primary MEFs is their limited proliferation capacity, which requires repeated isolation from embryonic mice to supply feeder cells [20]. In addition, repeated passaging causes MEFs to lose their capacity to support the proliferation of ESCs/iPSCs [21]. To solve this problem, Choo et al. [22] generated an immortalized primary MEF line (ΔE-MEF) through infection with retrovirus vectors encoding the E6 and E7 genes from human papillomavirus (DNA tumor virus) and demonstrated the consistent and reproducible feeder capacity of these cells for hESC culture.

STO cells were isolated by Bernstein from Sandoz inbred mouse (SIM)-derived fibroblasts as a thioguanine- and ouabain-resistant sub-line [13]. STO cells are more easily maintained than MEFs for preparation of feeder layers because STO cells are a spontaneously transformed cell line. In 1998, Shamblott et al. [23] showed that STO cells can be used in a feeder layer for establishing of human embryonic germ cells. In 2003, Park et al. [20] first demonstrated that STO cells have the potential to support the establishment and maintenance of human ESC lines. Thereafter, STO cells have been widely used as feeder cells for human ESC and iPSC culture [24, 25]. Proteome analyses have revealed that STO cells produce unique protein species, such as insulin-like growth factor binding protein 4 (IGFBP-4), pigment epithelium-derived factor (PEDF) and secreted protein acidic and rich in cysteine (SPARC, also known as osteonectin), which may be associated with differentiation and cell growth [26]. Talbot et al. [17] showed from quantitative immunoassays that STO cells express lower levels of activin A, interleukin-6, IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 than CF-1 cells but higher levels of hepatocyte growth factor (HGF) and stem cell factor (c-kit ligand). The difference in the growth factor production among feeder cell types may result in different abilities to support the growth of undifferentiated human ESCs/iPSCs. Indeed, conditioned media from primary MEF cultures but not STO cell cultures support the undifferentiated status of human ESCs on Matrigel- or laminin-coated culture plates [27]. One of the main mechanisms by which STO cells support ESC/iPSC pluripotency appears to be laminin expression because laminin on the STO cell surface interacts with specific receptors (integrin α6, β1 dimer) on human ESCs to maintain their undifferentiated status [20]. Therefore, compared to MEFs, STO feeder cells may require more direct cell contact with human ESCs/iPSCs to support pluripotency.
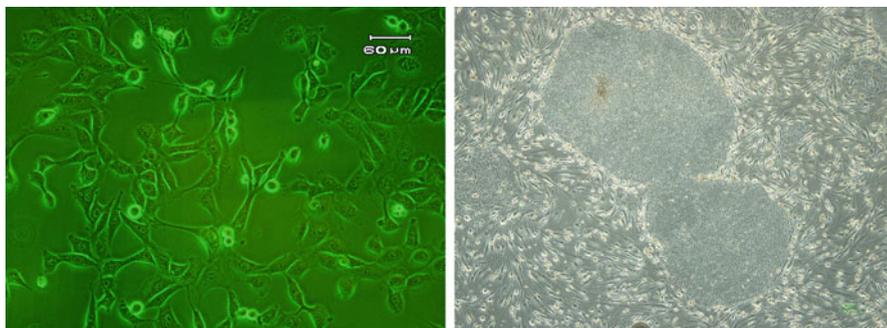
Fig. 12.1 (*Left*) Mouse-derived SNL76/7 cells. (*Right*) Undifferentiated human iPSC colonies on the SNLP76/7-4 feeder cells

SNL76/7 cells were clonally derived by Bradley [28] from STO cells transformed with neomycin resistance and murine leukemia inhibitory factor (LIF) genes. SNL76/7 cells abundantly express the pleiotropic cytokine LIF, which promotes long-term maintenance of mouse ESCs by suppressing spontaneous differentiation [29]. In contrast to the case with mouse ESCs, LIF does not satisfactorily support the self-renewal of human ESCs [4]. Nonetheless, SNL76/7 cells basically inherit the nature of STO cells; therefore, SNL 76/7 cells can be used as feeder cells for both mouse [6, 30, 31] and human [1, 28, 30] ESCs/iPSCs. Bradley also established a puromycin-resistant derivative of SNL76/7 cells (SNLP76/7-4 cell line: Fig. 12.1) that is useful for drug selection of transfected ESCs/iPSCs [32]. An immortalized mouse fetal liver stromal cell line (KM3 cells) was also reported to support the growth and maintenance of human ESCs when co-cultured in with the ESCs in a feeder cell layer [33].

These feeder cell lines may be useful for laboratory experiments because they enable large-scale expansion of human ESCs/iPSCs at low cost; however, it is important to note that mouse feeder methods are associated with a high possibility of contamination by the feeder cells during human ESC/iPSC isolation. Kim et al. [34] described a unique and less labor-intensive method to reduce contamination by feeder cells by culturing human ESCs on porous membranes of transwell inserts that have mouse feeder cells attached to the other side of the membrane.

12.2.2   Human-Derived Feeder Cells

Although mouse feeder systems are convenient for laboratory experiments, such xenobiotic support systems are associated with the risk of cross-transfer of animal pathogens and are thus not favorable for future clinical application of iPSCs. To solve this problem, many studies to date have demonstrated the utility of human-derived cells as feeders for human ESCs and iPSCs (Table 12.1).

Table 12.1 Summary of human feeder cells for human ESC/iPSC culture

Feeder cell sources	Cells	Culture medium	References
Foreskin fibroblasts	iPSCs	KSR medium	[35, 36]
	ESCs	KSR medium	[35–42]
	ESCs	HEScGRO	[43]
	ESCs	FBS-hES/KSR medium	[44]
Bone marrow-derived MSCs	ESCs	KSR medium	[37, 39, 45]
Adipose-derived stromal cells	iPSCs	mTeSR1	[46]
	ESCs	KSR medium	[47]
	iPSCs		
Amniotic MSCs	ESCs	–	[48]
Amniotic epithelial cells	ESCs	KSR medium	[49]
	ESCs	DMEM	[50]
	iPSCs	DMEM–F12 medium	[51]
Placental fibroblasts	ESCs	KSR medium	[37]
	ESCs	X-VIVO 10	[52]
Umbilical cord stromal cells	ESCs	KSR medium	[53]
Transgenic fetal fibroblasts	ESCs	KSR medium	[54]
Fetal muscle cells	ESCs	H1/H2 medium	[55]
Fetal skin cells	ESCs	H1/H2 medium	[55, 56]
Fetal liver stromal cells	ESCs	KSR medium	[57]

MSCs mesenchymal stem cells, *ESCs* embryonic stem cells, *iPSCs* induced pluripotent stem cells, *KSR* KnockOut™ Serum Replacement, *HEScGRO medium* animal-component-free medium (Millipore), *FBS* fetal bovine serum, *hES medium* standard human ESC culture medium, *mTeSR1* chemically defined xeno-free human ESC culture medium (Stemcell Technologies), *DMEM* Dulbecco's modified Eagle medium, *X-VIVO 10* chemically defined and serum-free medium (Lonza)

Primary human foreskin fibroblasts (FFs), which can easily be prepared from infant foreskin, are among the most frequently used human feeder cells for ESCs and iPSCs [35, 37–44]. Similar to primary MEF cells, human FF feeder cells show limited expansion in culture; therefore, fresh batches of FFs have to be prepared on a routine basis. To overcome this drawback, Unger et al. [36] established a conditionally immortalized human FF line that secreted bFGF and showed that the generated cells could support the culture of both human ESCs and iPSCs.

Mesenchymal stem cells (MSCs) are easily accessible postnatal human cells that include bone marrow-derived MSCs (BMSCs) [3]. Culture-expanded BMSCs can support prolonged expansion of human ESCs in culture [37, 39, 45]. Adipose-derived stromal cells (ASCs), which are another type of MSCs, also have the ability to serve as feeder cells for human ESCs/iPSCs [46, 47]. Sugii et al. [58] showed that ASCs express high levels of bFGF, TGFβ, fibronectin, and vitronectin and can thus serve as feeder cells for both autologous and heterologous human iPSCs. Because ASCs can be easily isolated by surgery or lipoaspiration from adults, their use as feeder cells is expected to provide an important step toward establishing safe, clinical-grade human iPSC lines.

The amniotic fluid contains MSCs that are easily obtained and relatively exempt from ethical problems. Zhang et al. [48] reported that human amniotic MSCs can be used as feeder cells for effective growth of human ESCs. Human amniotic epithelial cells (AECs) can be isolated from the surface membrane of fresh placentas, and they express many growth factors including EGF, bFGF, TGF-β, and BMP-4 [49, 59, 60] in addition to stem cell markers such as Oct-4 and Nanog [50]. Additionally, Liu et al. [51] recently showed that microRNA-145-mediated regulation of Sox2 expression in human AECs maintains the self-renewal and pluripotency of human iPSCs. Human placental fibroblasts also showed comparable or superior efficacy to MEFs as feeder cells for human ESCs [37, 52]. Because the human placenta and amnion are discarded as medical waste, they may be promising tissue sources for human feeder cells for iPSCs. Umbilical cord stromal cells, which can be obtained through noninvasive procedures, also support the self-renewal of human ESCs in serum-free conditions [53]; therefore, they may be still another promising source of human feeder cells for iPSC culture.

Other previously reported human feed cells for ESC/iPSC culture include dermal fibroblasts [61], adult fallopian tube epithelial cells [55], adult lung cells [56], transgenic (puromycin resistant) fetal fibroblasts [54], fetal muscle cells [55], fetal skin cells [55, 56], and fetal liver stromal cells [57]. However, although these human feeder cells can be used in laboratory experiments, they are not suitable for clinical use because the harvesting of the source tissue is invasive and may pose ethical issues.

Nearly all human feeder cells require supplementation with bFGF to sustain human ESC/iPSC potential; therefore a bFGF-dependent pathway may be crucial for maintaining the pluripotency of human pluripotent stem cells. Park et al. [37] evaluated the feeder ability of several types of human feeder cells (placental cells, BMSCs, and FFs) and showed that these cells support the undifferentiated growth of human ESCs through bFGF synthesis. In contrast, Bendall et al. [62] demonstrated that human ESCs autologously produce fibroblast-like cells around their colonies that act as a supportive niche for the survival and self-renewal of human ESCs through IGF-II production in response to bFGF.

12.3   Feeder-Free Methods for ESC/iPSC Culture

To achieve reliable and safe production of human iPSCs, it is desirable to use reagents that are defined, qualified, and preferably derived from a non-animal source. Although the use of human feeder cells circumvents the use of animal-derived feeder cells, the function of the feeder cells in the human iPSC co-culture system is still not fully understood. In addition, the preparation of the feeder cells is highly laborious, which limits the large-scale production of human iPSCs for future clinical applications. Therefore, development of feeder-free human iPSC culture systems has been an important focus of recent iPSC research.

Table 12.2 Summary of extracellular matrix-related biomaterials for feeder-free human ESC/iPSC culture

Matrix/biomaterial	Cells	Culture medium	References
Matrigel™	ESCs	hES medium	[27, 63, 64]
		MEF-CM	
Human MSC-derived matrix	ESCs	SBX medium	[65]
Human serum	ESCs	hES-dF-CM	[66]
Laminin	ESCs	hES medium	[27, 67]
		MEF-CM	
		NC-SFM	
Recombinant laminin E8 fragments	ESCs/iPSCs	mTeSR1	[68]
Fibronectin	ESCs	KSR medium	[69]
Vitronectin	ESCs	mTeSR1	[70]
Collagen (type 1)	ESCs	mTeSR1	[71]
Hyaluronic acid hydrogels	ESCs	MEF-CM	[72]

MSCs mesenchymal stem cells, *ESCs* embryonic stem cells, *iPSCs* induced pluripotent stem cells, *hES medium* standard human ESC culture medium, *MEF-CM* mouse embryonic fibroblast-derived conditioned medium, *SBX medium* chemically defined xeno-free medium, *hES-dF-CM* conditioned medium of fibroblasts derived from differentiated human ESCs, *NC-SFM* non-conditioned serum-free medium, *KSR* KnockOut™ Serum Replacement, *mTeSR1* chemically defined xeno-free human ESC culture medium (Stemcell Technologies)

12.3.1 ECM-Related Materials

The ECM is a uniquely assembled three-dimensional (3-D) molecular complex that varies in composition and diversity, and consists of basic components such as laminin, fibronectin, vitronectin collagen, cadherin, elastin, hyaluronic acid, and proteoglycans. In the absence of feeder cells, human ESCs/iPSCs require attachment factors to promote their survival and proliferation. In this regard, the ECM and its soluble factors support the adhesion, growth, and maintenance of ESCs/iPSCs. To date, various ECM-related materials have been evaluated as a substitute for feeder cells for human pluripotent stem cell culture (Table 12.2).

12.3.1.1 ECM Components

Matrigel™ [27, 63, 64], which is a commercially available protein mixture extracted from the Engelbreth-Holm-Swarm mouse tumor [73], is one of the most frequently used matrices for feeder-free growth of undifferentiated human pluripotent stem cells. Matrigel™ contains a complex and poorly defined mixture of fibronectin, laminin, type IV collagen, entactin, and heparan sulfate proteoglycans, in addition to various growth factors such as bFGF, EGF, PDGF, NGF, and TGF-β. To maintain human ESCs/iPSCs in an undifferentiated state, Matrigel™-coating requires soluble stemness-supporting factors produced by MEFs or other feeder cells (conditioned medium) [27]. Despite its availability and ease of use, Matrigel™

is not ideal for potential clinical application of human iPSCs because it is animal-derived and xenogenic pathogens can be transmitted through culture even though no feeder cells are present. However, Peiffer et al. [65] demonstrated that matrices derived from human MSCs could advantageously replace MEF or hMSC feeder cells. Furthermore, human serum can be also used as a matrix to support the undifferentiated growth of human ESCs [66].

12.3.1.2   Recombinant ECM Products

Human ESCs express integrin receptors for major ECM proteins (laminin, fibronectin, collagen, and vitronectin) and all of these receptors functionally mediate cell adhesion [70]. Laminin, which is a major component of the ECM of all basal laminae in vertebrates, can support the pluripotency of human ESCs when used together with the conditioned medium of MEFs [27]. The MEF conditioned medium can also be replaced, however, as the combination of a human laminin coating with defined medium supplements, such as recombinant bFGF and the additional growth factors flt3-L, SCF, and LIF, was shown to support the growth and maintenance of undifferentiated human ESCs [67]. It has also been shown that recombinant laminin E8 fragments (LM-E8s), which are truncated peptides composed of the C-terminal regions of the α, β, and γ chains of laminin, enable robust propagation of human ESCs and iPSCs in an undifferentiated state in cultures with defined and xeno-free media [68]. We have confirmed that human gingiva-derived iPSCs [30] can be maintained in an undifferentiated state on LM-E8-coated plates after dissociation and passaging (Fig. 12.2).

Fibronectin, vitronectin, and gelatin (a hydrolyzed product of collagen) are rich in arginine-glycine-aspartate (RGD) peptide sequences that are required for integrin-mediated cell adhesion and growth through activation of cellular signaling pathways [74]. Amit and Itskovitz–Eldor [69] reported that a human fibronectin coating and medium supplementation with TGFβ and bFGF provide a feeder-free and serum-free culture system for human ESCs. Vitronectin is the major ECM protein but is not present in Matrigel™; thus, Braam et al. [70] reported that recombinant vitronectin was a defined functional alternative to Matrigel™ for supporting sustained self-renewal and pluripotency of human ESC lines. Liu et al. [75] also demonstrated that nanofibrous gelatin substrates can provide an alternative to Matrigel™ for long-term expansion of human ESCs.

Type 1 collagen is the most abundant structural protein of the human body. Furue et al. [71] reported that a substrate composed only of type I collagen could be combined with a defined medium supplemented with heparin, bFGF, insulin, transferrin, and fatty acid-free albumin conjugated with oleic acid for culture of human ESCs. E-cadherin, a cell adhesion molecule, is essential for intercellular adhesion [76] and colony formation among mouse ESCs [77]. Nagaoka et al. [78] generated a fusion protein consisting of human E-cadherin and the IgG Fc domain and demonstrated that this protein could be substituted for Matrigel™ and could support the pluripotency of human ESCs and iPSCs under completely defined
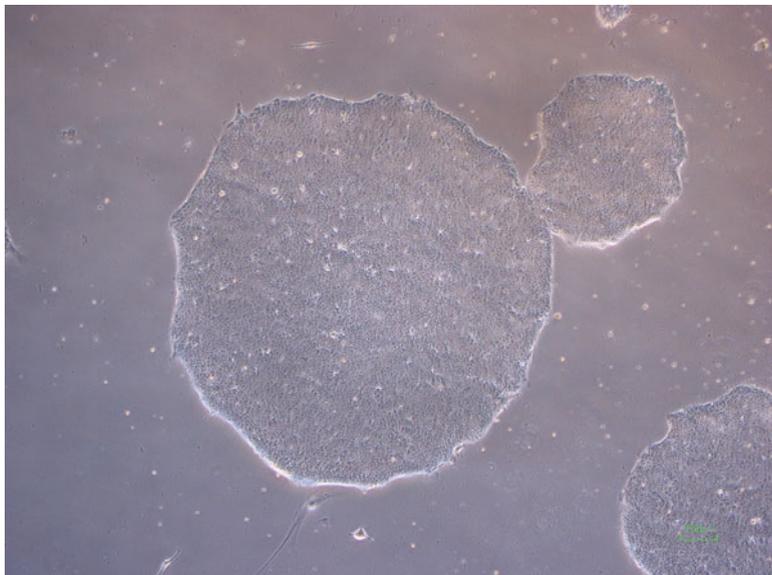
Fig. 12.2 Undifferentiated human gingiva-derived iPSC colonies on a recombinant laminin E8 fragment-coated plate (feeder-free culture)

culture conditions. Hyaluronic acid (HA) is an anionic, nonsulfated glycosaminoglycan that is distributed widely throughout connective, epithelial, and neural tissues. Gerecht et al. [72] demonstrated that HA-based hydrogels maintain the undifferentiated state of human ESCs in the presence of conditioned medium from MEFs.

12.3.2   Synthetic Materials

Although human-sourced and recombinant ECM materials can be used in animal-component-free and effective culture systems for human ESCs/iPSCs, they are still associated with high product cost and possible batch-to-batch variation. In contrast, synthetic biomaterials and chemical coating technologies (Table 12.3) may offer a fully defined culture system with lower cost and higher consistency.

Because most cells are cultured on tissue culture-treated polystyrene, the development of a chemical treatment for standard tissue culture-treated polystyrene is desirable for a 2-dimensional culture system for ESCs/iPSCs. Along these lines, Mahlstedt et al. [79] demonstrated that oxygen plasma-etched tissue culture-treated polystyrene could maintain the pluripotency of human ESCs in MEF conditioned medium.

However, the 3-D microenvironment has recently been appreciated for its ability to influence the behavior of pluripotent stem cells. For example, a 3-D porous

Table 12.3 Summary of synthetic materials for feeder-free human ESC/iPSC culture

Synthetic materials	Cells	Culture medium	References
Oxygen plasma-etched tissue culture-treated polystyrene	ESCs	MEF-CM	[79]
		hES medium	
Trimethyl ammonium-coated polystyrene microcarriers	ESCs	hES medium	[80]
Calcium alginate hydrogels	ESCs	hES medium	[81]
Chitosan and alginate scaffolds	ESCs	hES medium	[82]
Polymer scaffolds/poly-D-lysine	ESCs	mTeSR1	[83]

ESCs embryonic stem cells, *iPSCs* induced pluripotent stem cells, *MEF-CM* mouse embryonic fibroblast-derived conditioned medium, *hES medium* standard human ESC culture medium, *mTeSR1* chemically defined xeno-free human ESC culture medium (Stemcell Technologies)

natural polymer scaffold prepared from a chitosan and alginate complex was reported to sustain the self-renewal of human ESCs without the support of feeder cells or conditioned medium [82]. Similarly, Carlson et al. [83] reported the combination of poly-D-lysine, which is a synthetic positively charged amino acid chain commonly used as a coating to enhance cell adhesion, with synthetic polymer scaffolds with a 3-D fibrous architecture promote the adhesion, proliferation, and self-renewal of human ESCs. Additionally, microcarrier particles have also been used as substrates to amplify various types of adherent cells [84]. In particular, Phillips et al. [80] showed that seeding on trimethyl ammonium-coated polystyrene microcarriers enabled feeder-free 3-D suspension and-single cell culture for human ESCs, thus providing a low-cost, practical feeder-free method for large-scale human ESC/iPSC production. Furthermore, Siti-Ismail et al. [81] demonstrated that human ESCs encapsulated in calcium alginate hydrogels could be maintained in an undifferentiated state for more than 260 days without requiring feeder cells or passaging.

12.4 Conclusions

Feeder methods and ECM-related/synthetic materials for human ESC culture are relatively well established in the literature as outline above; however, the appropriate methods and materials for human iPSC culture, especially for clinical use, still need to be established. Most previous ESC/iPSC studies of animal-component-free methods focused on the growth and maintenance of iPSCs; however, few studies have focus on the generation and expansion of human iPSCs. Because iPSCs are generated from one reprogrammed somatic cell, xeno-free methods to efficiently promote the clonal growth of single human ESCs are necessary. Bigdeli et al. [85] reported that human ESC lines can be adapted to matrix-independent growth, even on plastic plates, by using a specified conditioned medium derived from human embryonic fibroblasts. This finding implies that it may be possible to develop a more effective defined culture medium that eliminates the need for a

substrate and thus achieves a feeder-free and xeno-free culture system for iPSCs. Investigators should therefore accumulate fundamental data for feeder- and xeno-free culture technologies by using both synthetic substrates and defined culture medium components. The establishment of cost-effective, easy-to-handle synthetic, defined, and stable xeno-free culture systems for human iPSCs will expedite the use of iPSCs in biomedical applications.

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