TISSUE ENGINEERING: Part B
Volume 21, Number 4, 2015
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DOI: 10.1089/ten.teb.2014.0547

termis
Tissue Engineering
& Regenerative Medicine
International Society

REVIEW ARTICLE

Feeder Layer Cell Actions and Applications

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Cultures of growth-arrested feeder cells have been used for years to promote cell proliferation, particularly with low-density inocula. Basically, feeder cells consist in a layer of cells unable to divide, which provides extracellular secretions to help another cell to proliferate. It differs from a coculture system because only one cell type is capable to proliferate. It is known that feeder cells support the growth of target cells by releasing growth factors to the culture media, but this is not the only way that feeder cells promote the growth of target cells. In this work, we discuss the different mechanisms of action of feeder cells, tackling questions as to why for some cell cultures the presence of feeder cell layers is mandatory, while in some other cases, the growth of target cells can be achieved with just a conditioned medium. Different treatments to avoid feeder cells to proliferate are revised, not only the classical treatments as mitomycin or γ-irradiation but also the not so common treatments as electric pulses or chemical fixation. Regenerative medicine has been gaining importance in recent years as a discipline that moves biomedical technology from the laboratory to the patients. In this context, human stem and pluripotent cells play an important role, but the presence of feeder cells is necessary for these progenitor cells to grow and differentiate. This review addresses recent specific applications, including those associated to the growth of embryonic and induced pluripotent stem cells. In addition, we have also dealt with safety issues, including feeder cell sources, as major factors of concern for clinical applications.

Introduction

Feeder layer cells usually consist of adherent growth-arrested, but viable and bioactive, cells. These cells are used as a substratum to condition the medium on which other cells, particularly at low or clonal density, are grown. Often the cells of the feeder layer are irradiated or otherwise treated so that they will not proliferate. Faced with the lack of a technique that allows large-scale colony production from single cells, Puck and Marcus first reported the use of feeder cells in cell culture in 1955.[1] Feeder cells have the capacity to support *in vitro* survival and growth of some fastidious cells that would require the presence of a variety of known or unknown soluble or membrane-bound growth factors and receptors. While several cell types are completely dependent on physical contact with a feeder layer for survival and expansion, some other feeder-dependent cells can be grown feeder free provided that culture dishes are coated with extracellular matrix proteins such as laminin, collagen, fibronectin, or a mixture of the extracellular matrix components (Matrigel) and supplemented with a medium conditioned by feeder cells. This review covers various aspects of feeder cell properties and applications.

Treatments to Arrest the Proliferation of Feeder Cells

Feeder cells have to provide one or several active signals and factors to support the expansion of cultured target cells, but they have to be prevented from overgrowing the culture.[2] This fact makes necessary to maintain feeder cells in a nonmultiplying, but metabolically active, state allowing them to express specific ligands or cytokines.[3] Although new methods have been developed in recent years[4,5] to growth arrest feeder cells, γ-irradiation (GI) and mitomycin-C (MC) treatments remain the most commonly used methods to avoid feeder cells dividing. The choice of GI or MC treatment is often guided by the availability of GI equipment, because the MC reagent is readily available at low cost and irradiation is expensive and time-consuming.[6] These methods are considered to be equivalent as both treatments inhibit DNA replication, but they do it in a different manner. MC is capable of arresting cells in G1 and S

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and G2 phases of the cell cycle while the cells remain vital.[7] It is a chemotherapeutic agent that avoids DNA double-strand separation during cell replication by forming covalent cross-links between DNA opposite strands, while RNA and protein synthesis continue. The damage for the DNA induced by GI is not fully understood[8] although it is commonly accepted that GI causes DNA double-strand breaks and interferes with DNA replication.[9] High-energy irradiation can completely suppress cell division long before general metabolism is appreciably affected. Although both treatments seem to be qualitatively equivalent, some studies suggest that GI is more suitable and efficient than MC treatment for the preparation of nonreplicating feeder cells. Roy *et al.* compared the ability of GI- and MC-treated feeder cells to support the expansion of normal human B lymphocytes. The results of their study show that MC-treated cells are metabolically altered and subsequently less efficient at maintaining target cell expansion in comparison with GI feeder layer.[3] Fleischmann *et al.*, also found significant differences between GI and MC treatment of feeder murine embryonic fibroblasts (MEF) for the cultivation of embryonic stem cells (ESC). Their study demonstrated that GI-MEF are preferable because of a higher proliferation rate of ESC and less differentiation and higher concentration of soluble factors, in comparison with MC-MEF.[8] On the other hand, some studies have demonstrated that MC seems to be better to maintain the adherence of feeder layers in long-term culture assays than GI-treated fibroblasts as irradiated feeder begin to detach after some weeks of culture.[6]

In view of the disadvantages of both methods traditionally used to prepare growth-arrested feeder cells, other novel treatments have been recently described for the arresting of feeder cell proliferation. On the basis of previous studies that show that ultrashort electric pulses (EPs) can penetrate inside the cells and induce intracellular responses while maintaining the viability of cells,[10,11] Browning *et al.*, described a new method using 10 ns EPs of 40 kV/cm, to prepare high-quality feeder cells to culture and sustain undifferentiated ESC over time.[4]

The use of chemically fixed feeder cells to support the growth and maintenance of hematopoietic stem cells,[12] ESC,[13] and induced pluripotent stem cells (iPSC)[5] has also been reported. Mild treatment with glutaraldehyde (GA) or formaldehyde (FA) leads to growth arrest and immobilization of most of the proteins on the surface of the feeder cells. Chemically fixed feeder can be lyophilized and stored, and thus can be used as culture substrate at any time. After detaching target cells, fixed cells remained immobilized on the dish surface; therefore, the chemically fixed feeder cells could be reused several times without affecting their functions.[5] Another advantage of chemically fixed feeder cells is that they do not detach from plates and do not contaminate target cell cultures after detachment.

Although growth arrest is critical to avoid feeder layer overgrowth, there are situations where fast-growing target cells may be grown in the presence of live feeder cells. It was shown that certain densities of proliferative MEF sustain the prolonged expansion of human ESC (hESC) at least as efficiently as mitotically inactive MEF.[14] Recently, a fully autologous coculture system utilizing nonirradiated autologous fibroblasts to support the expansion of human keratinocytes for clinical use has been described. The study demonstrates that autologous fibroblasts are equally effective as lethally irradiated 3T3 cells indicating that this coculture method is suitable for producing keratinocytes in large quantities and retaining their proliferative undifferentiated phenotype, required for clinical application.[15] These studies are based on the fact that the requirement for mitotic inactivity has never been confirmed and that the different methods employed to growth arrest feeder cells, such as irradiation or chemical treatments, may lead to damage and transformation of the cells and potential transmission of damaged genetic material to the culture.[14–16] A scheme summarizing the different treatments to prepare feeder layer cells is shown in Figure 1.

Mechanism of Action

Experimental evidences indicate that feeder layers support the growth of target cells by releasing growth factors to the culture media. However, this is not the only action as feeder cells have also important roles in other processes such as detoxifying the culture medium or synthetizing extracellular matrix proteins needed to control the cultured cell growth and acting as a substrate for the attachment of cells.[17]

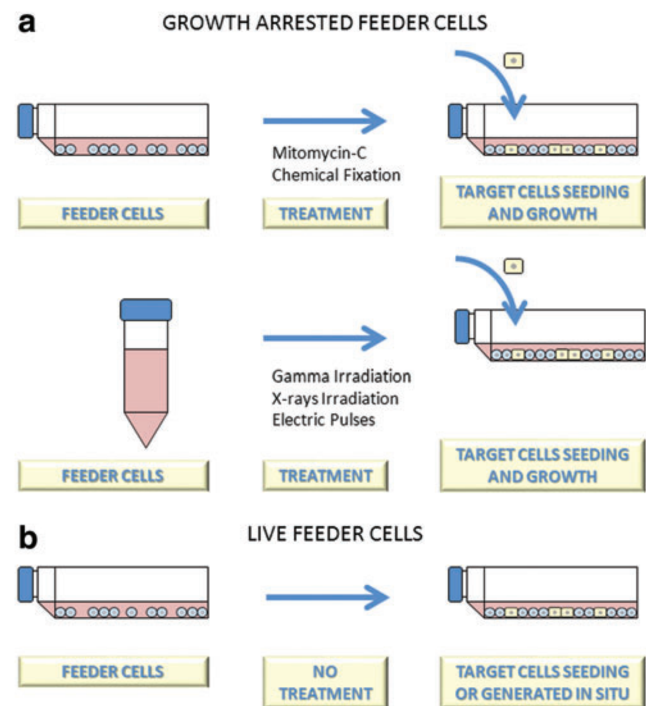

FIG. 1. Different treatments to prepare feeder layer cells. **(a)** Growth-arrested feeder cells. Growth arrest induced by chemicals (*upper panel*: such as mitomycin-C [MC] and glutaraldehyde) can be applied directly onto the culture surface followed by extensive washing. Growth arrest induced by physical methods (*lower panel*), such as GI, EPs, and X-ray irradiation, requires previous feeder cell trypsinization and reseeding. **(b)** Live feeder layer cells. In some cases, target cells may be grown in the presence of feeder cells capable of dividing. Some live feeder cells (such as human fibroblasts) may also become target cells as in the case of iPSC upon reprogramming. EPs, electric pulses; GI, γ-irradiation; iPSC, induced pluripotent stem cells. Color images available online at www.liebertpub.com/teb

Nonreplicating viable and metabolically active feeder cells are capable of stable synthesis of ligands or cytokines needed to support the selective expansion of cultured target cells.[3] Although the growth factor repertoire of a given feeder cell differs from one to another, there are critical common factors. An example of this is the leukemia inhibitory factor (LIF), a cytokine required for maintaining both the proliferation and developmental potential of nonprimate stem cells, which is used as an additive for media of human and mouse ESC.[8] ESC express LIF receptor and the binding of LIF to these receptors activates JAK-STAT pathways, critical for stem cell maintenance.[18]

It is known that purified growth factors and also the conditioned medium of other cell lines can be used in some instances to make fastidious cells independent of the presence of feeder cell layers. The growth factor requirements of the ESC appear to have been elucidated.[19] Transcriptomic and protein analyses comparing supportive and nonsupportive human feeder cells for hESC, revealed that the supportive feeder cells also express higher levels of fibroblast growth factor-2 (FGF2), Gremlin 1 (a bone morphogenetic protein 4 pathway inhibitor), activin, and several extracellular matrix proteins.[20–23] On the contrary, growth factor requirements for epithelial cell culture have not yet been fully established and these cells still remain dependent on feeder cells when grown under high calcium medium concentrations.[24,25] A major role of JNK-dependent soluble factors secreted by murine feeder fibroblasts mediating both differentiation and proliferation of target cells (i.e., keratinocytes) has been, however, recently shown.[26] Despite the knowledge gained on the growth factors expressed by feeder cells, additional mechanisms to explain how pluripotent and multipotent stem cells are supported by feeder layers remain elusive.[27]

As mentioned above, while the addition of some feeder layer secreted factors, either as conditioned medium or as individual recombinant factors may suffice to replace feeder cells in culture, there are cases of mandatory physical contact for target cells to grow. Such contact favors mechanotransduction and niche effects that include juxtacrine signaling pathways such as those mediated by integrins or the Notch system.[28] Richards *et al.* compared the growth of two hESC lines on three different human feeder layers (fetal muscle, fetal skin, and adult fallopian tubal epithelial cells) and on feeder-free matrices with the conditioned medium prepared from the three human feeders and from MEF. In this study, human feeders were clearly superior to feeder-free matrices supplemented with any of the conditioned mediums tested to support the growth of undifferentiated hESC. This result showed that direct feeder contact was important not only due to juxtacrine interactions but also because feeder cells exert additional functions, including removal of toxic or inhibitory factors from the culture medium.[29]

Proximity or direct contact is important also, because feeder cells can synthetize extracellular matrix proteins that act as a substrate for the attachment of cultured cells. Epithelial cells *in vivo* need a close interaction with the surrounding mesenchyme and this relationship between epithelial and mesenchymal cells has to be reproduced *in vitro*. 3T3 feeder fibroblasts constitutively produce hyaluronic acid (HA), a nonsulfated glycosaminoglycan (GAG) widely distributed throughout the extracellular matrix of connective tissue. It has been observed that the production of HA rises locally in a variety of processes involving cell motility.[30] HA becomes hydrated, expanding the extracellular space. This process mechanically weakens intercellular binding facilitating cell detachment and also provides hydrated tracks for the migration of detached cells, as observed in wound repair and embryonic morphogenesis.[31] The proliferation of cutaneous mast cells can be enhanced or restricted depending on the amount of HA synthetized by Swiss strain of 3T3 feeder fibroblasts.[32,33] Mechanical factors also appear important. When rat tracheal epithelial (RTE) cells grown in the 3T3 conditioned medium were compared to RTE cell colonies formed in the presence of feeder cells, it could be observed that the latter contained more cells that were both smaller and more homogeneous, suggesting that the physical presence of 3T3 cells provided an improved substrate. These differences may be explained by the presence of feeder cells acting as a physical barrier preventing RTE cells from spreading out in addition to factors needed for cell growth that cannot be provided by the 3T3 conditioned medium alone.[34] A schematic of feeder layer mechanisms of action is shown in Figure 2.

In recent years, the use of feeder layers for the cultivation of cells with high growth factor and niche requirements such as ESC or keratinocytes has been boosted by the use of a Rho kinase (ROCK) inhibitor. Three independent studies[35–37] demonstrated that the Rho kinase inhibitor, Y-27632, significantly suppresses keratinocyte differentiation and extends

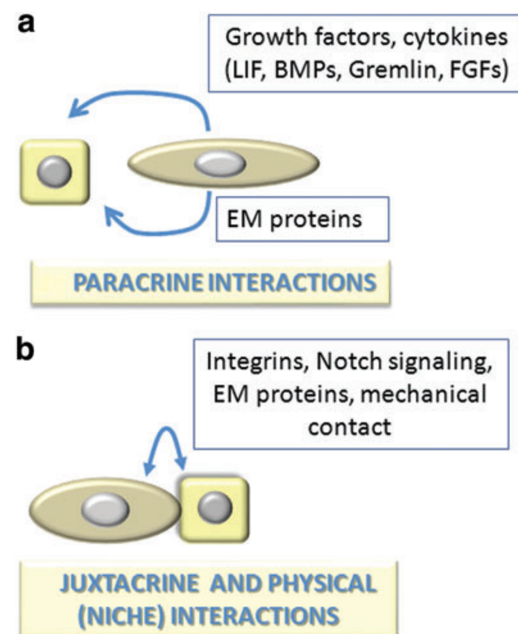

FIG. 2. Feeder cell–target cell interactions. **(a)** Paracrine interactions. Feeder cells produce a number of growth factors and cytokines that could eventually be replaced by added recombinant proteins to the culture medium. **(b)** Juxtacrine and physical interactions. The presence of feeder cells is mandatory for target cells to grow, since cell–cell contact mediate juxtacrine signaling pathways and/or mechanical nest effects. Color images available online at www.liebertpub.com/teb

the life span only in the presence of feeder cells or their conditioned medium. Similarly, inhibition of ROCK enhances post-thaw viability, improves cloning efficiency, and decreases anoikis in hESC cultured with feeder layers. The effects of the ROCK inhibitor are not fully understood but associate with survival signals, including inhibition of apoptosis and cell detachment, mediated by remodeling of the actin cytoskeleton.

Safety Considerations

The feeder layer cells are widely used and their origin (allogeneic or xenogeneic) is not an important issue when the culture has an *in vitro* purpose. However, when the clinical use is being considered, in addition to effectiveness, safety conditions should be taken into account.

The use of animal feeder cells like 3T3 murine fibroblasts runs the risk of cross-transfer of animal pathogens from the animal feeder, matrix, or conditioned medium to the culture, compromising later clinical applications.[38] Moreover, mouse feeder layers express N-glycolylneuraminic acid (Neu5Gc), a nonhuman sialic acid. Sialic acids are a family of acidic sugars expressed on the surface of all cell types. The most common mammalian sialic acids are Neu5Gc and Neu5Ac (N-acetylneuraminic acid), Neu5Ac being the metabolic precursor of Neu5Gc. Human cells cannot produce Neu5Gc from Neu5Ac, but can incorporate Neu5Gc from the animal feeder layer or from the culture medium, leading to an immune response mediated by circulating antibodies against Neu5Gc present in most healthy humans.[19,27,39,40] Xenogeneic feeder cells have also a risk of contamination, as feeder cells also detach from the surface when target cells are digested from culture flasks. Contamination with feeder cells can cause severe problems when target cells are used for clinical purposes.[5] Thus, the source of feeder cells has to be carefully chosen depending on the purpose of the culture. Some examples of feeder cell types and their corresponding target cells are shown in Table 1.

3T3 Feeder Layer and Epithelial Stem Cell Culture

Keratinocytes and other epithelial cells had resisted attempts to serial cultivation until 1975, when Howard Green and his postdoc, James Rheinwald approached the cultivation of human teratomas using feeder layers. Their studies led to the serendipitous finding that lethally irradiated 3T3 mouse fibroblasts, promoted the growth of human epidermal keratinocytes.[41,42] The J2 strain of 3T3 cells, originally developed by Green appears to be the most suited for keratinocyte growth[42] although other strains such as the Swiss 3T3 work equally well but at higher seeding density.[43] Large expansion of human keratinocytes on plastic surfaces under high calcium concentrations (above 0.1 mM) is not feasible without feeder layer. However, human keratinocytes can be efficiently grown in high calcium without feeder layer when the culture substrate is provided by a fibroblast-embedding three-dimensional fibrin dermal matrix.[43–45] Thus, the combined action of the fibrin matrix and the factors secreted by the embedded human fibroblasts are capable to replace the presence of the 3T3 feeder layer. Despite that several clinical-grade keratinocyte culture methods have been developed employing feeder-free conditions (i.e., low-calcium defined media and low/absent components of animal origin),[25,46,47] methods based on the Rheinwald and Green's culture protocol remain as the gold standard for clinical applications.[48] Reasons for this include (i) the high keratinocyte expansion rates achieved,[43] (ii) the long-term maintenance of keratinocyte stemness and regenerative capacity after grafting of not only normal human keratinocytes but also genetically manipulated donor and patient-derived cells,[48–50] and (iii) the fact that the use of 3T3 in the initial stages of the keratinocyte culture has not been associated with any adverse effect in the 30-year history of this system for clinical grafting applications.[42]

As mentioned above, human skin and mucosal keratinocytes are cells that require physical contact with feeder cells.[34] An extreme case for the physical contact requirement with feeder cells is found in the growth of retinal pigment epithelial (RPE) and iris pigment epithelial cells. While growth of these cells takes place in direct contact with 3T3 cells, the 3T3 conditioned medium alone inhibits proliferation of RPE cells, confirming that the feeder cell effect is mediated through cell–cell interaction.[51]

Since the use of animal-derived components in *ex vivo* expansion of epithelial stem cells poses potential risks or disease transfer and contamination with 3T3 cells, culture conditions that minimize such risks provide an advantage when developing a cell therapy product for human transplantation.[52] Within this context, some authors have reported in the last years different human feeder cells as an alternative to the use of mouse 3T3 fibroblasts, such as irradiated dermal fibroblasts,[40,52–54] proliferative fibroblasts,[15,16] and mesenchymal stem cells.[52,55]

MEF versus Human Feeder Layer Cells for Human Pluripotent Cell (hESC and hiPSC) Culture

ESC are cells derived from the inner cell mass (ICM) of the mammalian blastocyst. These cells are pluripotent and immortal and can be continuously cultured in an undifferentiated state.[56] ESC can differentiate into cells of all three germ layers, for this reason, hESC have the potential to be used to treat various diseases, including neurodegenerative diseases, diabetes, myocardial infarction, stroke, and spinal cord injury.[57]

In 1998, the first derivation of hESC lines was published. This work describes the necessity of mitotically inactivated MEF feeder layer to grow hESC continuously in an undifferentiated stage in culture.[58] This protocol remains the gold standard method for hESC culture. There are, however, several factors that limit the clinical applications of hESC. First, as mentioned above, the use of MEF as feeder cells may compromise the therapeutic potential of these stem cells because of the risk of zoonosis and contamination. Almost all the current hESC lines that are NIH-registered have been derived and maintained in coculture with growth-arrested MEF.[59]

Over the following years, researchers have tried to avoid the use of MEF feeder layers. hESC can be cultured in coated surfaces (Matrigel matrix) supplemented with the MEF-conditioned medium.[60] This fact may still expose the hESC to xenopathogens from the conditioned medium. The possibility of growing hESC on human feeder layers (fetal muscle, fetal skin, and adult fallopian tubal epithelial feeder cells) has also been reported.[29] In addition, this last study

Table 1. Overview of Feeder Layer-Target Cell Pair Systems

Target cell	*Feeder cell*	*Arrest treatment*	*Reference*
HeLa (human cervical carcinoma epithelial cells)	HeLa	X-ray irradiation	1
Human keratinocytes	3T3	γ-Irradiation	41–45
	Human dermal fibroblasts	Not arrested	16
	Adipose-derived mesenchymal stem cells	X-ray irradiation	55
	3T3		
	3T3	γ-Irradiation	53
	Human dermal fibroblasts		
	Human dermal fibroblasts	Not arrested	15
	3T3	γ-Irradiation	54
	Human dermal fibroblasts		
	3T3	Mitomycin-C	35
Human oral mucosal epithelium	Adipose-derived mesenchymal stem cells	X-ray irradiation	55
	3T3		
	Human dermal fibroblasts	γ-Irradiation	40
	3T3		
	3T3	Mitomycin-C	52
	Human dermal fibroblasts		
	Human bone marrow-derived mesenchymal cells		
Human limbal epithelium	Human amniotic epithelial cells	Mitomycin-C	27
	Adipose-derived mesenchymal stem cells	X-ray irradiation	55
	3T3		
	3T3	Mitomycin-C	52
	Human dermal fibroblasts		
	Human bone marrow-derived mesenchymal cells		
Iris pigmented and retinal pigmented epithelial cells	3T3	Mitomycin-C	51
Rat tracheal epithelial cells	3T3	γ-Irradiation	34
Human embryonic stem cells	MEF	γ-Irradiation	58
	Human fetal muscle cells	Mitomycin-C	29
	Human fetal fibroblasts		
	Human adult fallopian tubal epithelial cells		
	MEF		
	MEF	Not arrested	14
	Human dermal fibroblasts	Mitomycin-C	7
	MEF	Mitomycin-C	22,39
	Human dermal fibroblasts	Mitomycin-C	23
	MEF		
	Human amniotic mesenchymal cells	Mitomycin-C	57
	Human amniotic epithelial cells		
	MEF		
Murine and primate embryonic stem cells	MEF	Glutaraldehyde fixation	13
	Human amniotic epithelial cells	Formaldehyde fixation	
	MEF	Mitomycin-C	8
		γ-Irradiation	
	Mouse bone marrow stromal cells	Mitomycin-C	18
Zebrafish embryonic stem cells	Zebrafish spleen cell line ZSSJ	γ-Irradiation	2
	Rainbow Trout spleen cell line RTS34st	Electric pulses	4
Mouse iPS	MEF	Glutaraldehyde fixation	5
		Formaldehyde fixation	
	Murine amniocytes	Mitomycin-C	62
Human iPS	MEF SNL line	Mitomycin-C	65
	Human dermal fibroblasts		
	MEF SNL line	Mitomycin-c	61
	Human amniocytes	γ-Irradiation	62
Human cord blood hematopoietic progenitor cells	Human bone marrow stromal cells	Glutaraldehyde fixation	18
Normal human B lymphocytes	L4.5 (modified MEF L929 line)	Mitomycin-C	3
		γ-Irradiation	
Mast cells	3T3	Mitomycin-C	32

The table compiles examples of target cells, the usual feeder cells used for their culture, and the growth-arrest treatment employed in each case. iPS, induced pluripotent stem cells; MEF, mouse embryonic fibroblasts.

describes the derivation and establishment of a new hESC line all the way from the ICM stage on human support systems and xeno-free culture media, in contrast to the NIH-registered hESC lines, which have been established and grown on MEF feeders.[29] Human foreskin fibroblasts as feeder layers for hESC have been also used and are easily obtained from new born babies after circumcision.[56] Likewise, amniotic mesenchymal cells have been used as feeder layers to sustain propagation of hESC in the undifferentiated state. As the human amniotic membrane is usually regarded as postlabor medical waste, this property can avoid ethical problems against clinical application.[57]

Besides ethical problems, a further potential barrier to stem cell therapy with hESC is that of immunological incompatibility causing tissue rejection and requiring the use of immune-suppressing agents.[61] These issues have recently been addressed with the iPSC, which provide a powerful tool for cell therapies based upon autologous patient-specific stem cells and they largely remove the ethical concerns that restrict the use of hESC.[62] iPSC generation needs the expression of several transcription factors in the adult cell, by synthetic modified mRNA, chemical agents, direct protein introduction, or gene transfer to reprogram the differentiated somatic cell into iPSC. iPSC resemble ESC due to their ability to differentiate into all the three germ layers and are capable to form teratomas. In many laboratories, human iPSC (hiPSC) are maintained on animal-derived feeder cells such as MEF. Replacement of allogeneic or xenogeneic feeder layers is another safety issue that must be addressed in establishing hiPSC when a clinical use is intended.[63] The use of pericellular matrix of decidua-derived mesenchymal cells (PCM-DM) as a human-derived substrate to culture hiPSC has been described. Decidual mesenchymal cells show a potent maintenance-supporting activity similar to that of MEF and can substitute animal-derived matrices as Matrigel,[64] but the existence of unidentified pathogens, such as viruses and prions in these nonautologous feeder cells and a potential immune reaction, has to be considered. Human dermal fibroblasts (HDF) have been used as autologous feeder layers to generate and maintain hiPSC derived from HDF.[65] The culture system described in this work demonstrated that fibroblasts from an individual can play dual roles as a source of iPSC and feeder cells. This is an important step toward the establishment of safety cell therapies based upon hiPSC.

Facing with the fact that younger cell types appear to exhibit more efficient reprograming to the iPSC state, the use of human amniocytes as a dual source of iPSC and feeder layer has recently been reported.[62]

Conclusion

While future regenerative medicine is likely to rely on the use of hESC and hiPSC, epidermal and other epithelial stem cells are a reality in the clinics. All these cells have in common their dependency on the feeder layers to be grown and maintained healthy and regenerative. Although this review aims at describing current applications, benefits, and drawbacks of feeder cells, intense investigation is underway to achieve feeder-free and animal product-free conditions for clinical applications combining effective combinations of matrices and culture media. We do not intend to review the literature on this subject, but the interested reader could obtain useful information from recent studies dealing with this important issue not only for the culture of iPSC but also keratinocytes.[46,66,67] Despite these advances, it is important to remark that both the iPSC and their RPE cell derivatives used in the recent clinical trial for macular degeneration conducted in Japan (UMIN000011929) were produced with MEF feeder cells and xenogenic products.[68,69]

We envision that, however, new insight on the supporting chemical and physical factors provided by feeder cells will lead us to the development of perfect synthetic feeder substitutes.

Acknowledgments

E.G.P. was supported by grants CIVP16A1864 from Fundación Ramón Areces. F.L. was supported by grants PI11/01225 and PI14/00931 from Instituto de Salud Carlos III and S2010/BMD-2359 from Comunidad de Madrid. M.D.R. was supported by grant S2010/BMD-2420 from Comunidad de Madrid.

Disclosure Statement

No competing financial interests exist.

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Received: September 17, 2014
Accepted: January 21, 2015
Online Publication Date: March 23, 2015