Available online at www.sciencedirect.com
ScienceDirect
Journal of Biotechnology 134 (2008) 79–87
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Efficient expansion of clinical-grade human fibroblasts on microcarriers: Cells suitable for *ex vivo* expansion of clinical-grade hESCs

Blaine Wesley Phillips [a,b,*], Ricky Yee Meng Lim [b], Thong Teck Tan [a,b], William Lathrop Rust [a,b], Jeremy Micah Crook [a,b]

[a] *Institute of Medical Biology, 8A Biomedical Grove, #05-15 Immunos, Singapore 138648, Singapore*
[b] *ES Cell International Pte. Ltd., 11 Biopolis Way, #05-06 Helios, Singapore 138667, Singapore*

Received 6 September 2007; received in revised form 28 November 2007; accepted 13 December 2007

Abstract

Human embryonic stem cells hold considerable potential for cell-based treatments of a variety of degenerative diseases, including diabetes, ischemic heart failure, and Parkinson's disease. However, advancing research to provide clinical-grade product requires scale-up to therapeutic quantities of stem cells and their differentiated progeny. Most human embryonic stem cell culture platforms require direct support by a fibroblast feeder layer or indirect support using fibroblast conditioned medium. Accordingly, large numbers of clinically compliant fibroblasts will be requisite for stem cell production. Published platforms for feeder production are insufficient for stem cell scale-up, being costly to operate and requiring considerable effort to prepare, maintain and harvest. Here we describe the expansion of cGMP-grade, FDA-approved human foreskin fibroblasts using cGMP-grade reagents and polystyrene-based cationic trimethyl ammonium-coated microcarriers in spinner flasks. Fibroblasts attach rapidly to the microcarriers ($T_{1/2}$ =75 min), and expand with a maximum doubling time of 22.5 h. Importantly, microcarrier-expanded fibroblasts and their conditioned medium support pluripotent stem cell growth through >5 passages, enabling extended self-renewal and expansion while retaining full differentiation potential. In summary, the method described is an economical and cGMP-compliant means of producing human fibroblast cells in support of cGMP human embryonic stem cell culture.

Keywords: Human embryonic stem cells; ES cells; Microcarriers; Scale-up

1. Introduction

Since their initial isolation (Thomson et al., 1998), human embryonic stem cells (hESCs) have been recognized for their potential to treat a variety of diseases, including diabetes (Phillips et al., 2007), ischemic cardiac infarct (Mummery et al., 2003), and Parkinson's disease (Ben-Hur et al., 2004). Meeting this potential, however, will require technical improvements to the large-scale expansion of hESCs and their differentiated progeny, under clinically compliant conditions.

Like their murine counterparts, hESCs are traditionally cultured on a feeder layer of murine fibroblasts (mEFs; Thomson et al., 1998). Contemporary alternatives to mEF-based culture employ non-xenogeneic human fibroblasts equally capable of hESC support (Richards et al., 2003). Most recently, feeder-free (with feeder conditioned medium (CM) but without feeder co-culture) and feeder-independent (without CM or feeder co-culture) platforms for hESC growth have been described (Amit et al., 2004). While feeder-free hESC culture is proving successful, the efficacy of feeder-independent methods remains controversial (Rajala et al., 2007).

Whether "feeder-free" or "feeder-independent", hESC culture has benefited from the identification of growth support matrices, such as matrigel (Sjogren-Jansson et al., 2005; Xu et al., 2001) or fibronectin (Amit et al., 2004), and various growth factor supplements including bFGF (Levenstein et al., 2006). Despite such developments, however, optimal long-term pluripotent hESC self-renewal still remains largely dependent upon fibroblast co-cultures or CM (Ding et al., 2006).

Microcarriers have been used for many years to enable the suspension culture of fibroblastic and adherent cell types (Varani et al., 1996). Early commercial applications using this technology have enabled the mass production of growth factors

* Corresponding author at: Institute of Medical Biology, 8A Biomedical Grove, #05-15 Immunos, Singapore 138648, Singapore. Fax: +65 67745077.
E-mail address: blaine.phillips@imb.a-star.edu.sg (B.W. Phillips).

0168-1656/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2007.12.007

(Giard et al., 1979), hybridomas (Voigt and Zintl, 1999), and vaccines (Frazatti-Gallina et al., 2004). As the types of microcarriers have increased, notably by varying their composition and surface coatings, so too have the potential applications. Examples include adult porcine mesenchymal stem cell expansion on microcarriers in stirred suspension culture (Frauenschuh et al., 2007). To date there have been no reports of hESC culture on microcarriers. Regardless, this approach could be useful for the production of large quantities of fibroblast feeder cells for direct hESC co-culture, or fibroblast CM for feeder-free hESC support.

We present here an efficient method for the bulk expansion of human fibroblasts using cGMP-grade reagents, microcarriers and a stirred suspension platform. Following microcarrier-based expansion, fibroblasts and fibroblast CM maintain the ability to support prolonged hESC expansion in an undifferentiated state. Microcarrier-expanded fibroblasts and CM have similar efficacy to fibroblasts and CM generated by traditional flat-bed culture. This method represents a useful and practical approach to obtaining the large quantities of human fibroblasts needed to support the production of stem cells for R&D and clinical trials of cell-based therapies.

2. Methods and materials

2.1. Microcarrier-based fibroblast culture and conditioned medium production

Microcarrier-based expansion was performed using Hillex-II™ microcarriers (SoloHill Engineering, Inc.). These commercially available microcarriers are polystyrene-based beads with a cationic trimethyl ammonium coating. Prior to inoculation, microcarriers were pre-cultured for 1 h at a density of 10 mg/ml, total volume of 40 ml in feeder medium (FM) composed of phenol red-free DMEM supplemented with 10% fetal bovine serum (FBS), 1% pen/strep and 1× L-glutamine. All cultures were grown in 100 ml internal propeller spinner flasks (Integra Biosciences). For fibroblast inoculation, cGMP-grade human fibroblasts (from passage 7 frozen stocks; Ortec International) were either directly thawed into the culture bottle, or used to seed T175 flasks for future microcarrier studies. The pre-equilibrated microcarriers were inoculated with 5×10^6 cells (thus 125,000 cells were seeded to 10 mg beads/ml). Attachment of the cells to the microcarriers occurred under static (no spinning) conditions overnight, with agitation every 2 h. The next morning, the medium was topped up to a final 80 ml volume, and the bottles were transferred to a magnetic stirring platform (Integra Biosciences, IBS Cell Spin) and stirred at a rate of 40 revolutions per minute. A 50% medium change was performed every 2–3 days. The fibroblasts were passaged approximately every 5 days. To dissociate the cells, the beads were allowed to settle at the bottom of the bottle, and then rinsed two times with PBS (Ca^{2+} and Mg^{2+}-free, Invitrogen). The microcarrier/cell pellet was then resuspended in 5 ml of TrypLE™ (Invitrogen) and incubated for 5 min at 37 °C. 5 ml of FM was added, and the mixture vigorously triturated to release the cells from the microcarriers. The mixture was transferred to a 15-ml conical tube, and the beads allowed to settle for 1 min. The supernatant containing the single cell suspension of fibroblasts was transferred to a new tube and counted by trypan blue exclusion.

For CM production, human fibroblasts were grown to confluence (4 days) on microcarrier beads as described above (approximately 2.5×10^7 cells total). FM was then removed; the cultures were rinsed with 2× 25 ml PBS, and incubated with 100 ml of fresh hESC medium for 24 h. By collecting medium daily, the fibroblasts cells were capable of conditioning up to 6× 100 ml batches of medium before the culture was discarded. The batches of medium were then pooled and passed through a 0.22 μm filter, and stored at 4 °C for up to 3 weeks, or frozen at −20 °C for several months.

2.2. Flask-based (control) fibroblast culture and conditioned medium production

Similar to microcarrier-based cultures, all flask-based fibroblast cultures employed passage 7 stocks of cGMP-grade human fibroblasts and grown as previously described (Crook et al., 2007). Briefly, fibroblasts were thawed and seeded to T225 flasks at 1.2×10^4 cells/cm^2 in 50 ml FM. Medium was refreshed every 3–4 days. For CM production, 8.8×10^6 γ-irradiated fibroblasts were seeded to T225 flasks and allowed to attach over 24 h. FM was removed and cultures were rinsed with PBS. Fibroblasts were incubated for 24 h with 100 ml fresh hESC medium, which was collected and stored at 4 °C. Batches acquired over 6 days were pooled, filtered, and stored as before.

2.3. hESC culture

For feeder-based co-culture, the cGMP-derived hESC line ESI-017 was grown on γ-irradiated human fibroblasts (3×10^5 cells/cm^2) as described previously (Crook et al., 2007). The cells were cultured in hESC medium containing KO-DMEM, 1% non-essential amino acids, 1× penicillin/streptomycin, 2 mM L-glutamine and 20% KO-SR (all reagents were from Invitrogen and cGMP-compliant, Table 1) supplemented with 50 μg/ml cGMP-grade bFGF (Strathman). hESCs were dissociated with 1.25 mg/ml of collagenase NB6 (Serva), streaked using a 2-ml pipette, harvested with a cell scraper, and transferred to new feeder plates. For feeder-free culture, hESCs were grown in feeder CM described above. All tissue culture plates were coated with 5 μg/cm^2 human plasma fibronectin (Crook et al., 2007). Differentiations were performed using protocols described previously (Mummery et al., 2003; Phillips et al., 2007).

2.4. Teratoma formation

hESCs were dissociated into clumps of 50–500 cells as described above, transferred to hESC medium, and injected ($(2–4) \times 10^6$ cells per 50 μl) into the quadricep of the right hind limb of male SCID mice. Mice were maintained under controlled conditions in accordance with the National Institutes of Health (NIH) and National Advisory Committee for Laboratory Animal Research (NACLAR) guidelines, and with approval of the Biopolis Institutional Animal Care and Use Com-