

## Functional Cells Cultured on Microcarriers for Use in Regenerative Medicine Research

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Microcarriers have been successfully used for many years for growing anchorage-dependent cells and as a means of delivering cells for tissue repair. When cultured on microcarriers, the number of anchorage-dependent cells, including primary cells, can easily be scaled up and controlled to generate the quantities of cells necessary for therapeutic applications. Recently, stem cell technology has been recognized as a powerful tool in regenerative medicine, but adequate numbers of stem cells that retain their differentiation potential are still difficult to obtain. For anchorage-dependent stem cells, however, microcarrier-based suspension culture using various types of microcarriers has proven to be a good alternative for effective *ex vivo* expansion. In this article, we review studies reporting the expansion, differentiation, or transplantation of functional anchorage-dependent cells that were expanded with the microcarrier culture system. Thus, the implementation of technological advances in biodegradable microcarriers, the bead-to-bead transfer process, and appropriate stem cell media may soon foster the ability to produce the numbers of stem cells necessary for cell-based therapies and/or tissue engineering.

Key words: Microcarriers; Stem cell; Expansion; Transplantation

### INTRODUCTION

Cell transplantation has been widely used in regenerative medicine or tissue engineering applications for many years (32,37,57,100). The most well-established therapeutic applications of cells grown in suspension are blood transfusion and transplantation of hematopoietic stem cells (52,58,66). The key to successful transplantation using cell-based technologies is the preparation of large enough numbers of high-quality cells. Similar to cells grown in suspension, anchorage-dependent cell-based transplantation, such as the use of islet cells for diabetes (6,78), bone marrow cells for myocardial infarct (31,47,79), or critical limb ischemia (3), requires at least  $1-2 \times 10^9$  cells for an adult patient (50–100 kg). Thus, the development of a practical and scalable bioprocess that allows expansion and transplantation of functional cells would potentiate cell-based therapies.

Many culture systems have been designed to expand

cells with identical properties (36,87). The key to successful large-scale and long-term cell culture is a well-defined and controlled bioreactor design. Stirred bioreactors (for suspension culture) can overcome several drawbacks of static cultures, including the presence of concentration gradients and difficulties in monitoring and control. Except for hematopoietic cells (38), however, mammalian cells are usually expanded as anchorage-dependent cells, and therefore development of a culture system with suitable support is necessary. The stirred microcarrier culture system can facilitate the expansion of cells that require attachment, and provides an environment that can easily be controlled and monitored.

In addition, owing to their capacity for self-renewal and differentiation, stem cells such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), and other adherent stem/progenitor cells have been recognized for their

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great potential in regenerative medicine. To realize the potential of stem cell-based therapies, however, technical improvements in the large-scale expansion of stem cells and their differentiated progeny are required. Microcarriers may be an alternative culture system for producing significant quantities of cells that are required for future clinical product development.

### MICROCARRIERS

The microcarrier culture system was introduced by van Wezel in 1967 and has been applied successfully to the growth of anchorage-dependent cells for the production of vaccines or pharmaceuticals, and to expand cell populations (88). This culture system offers the advantage of providing a larger surface area for the growth of anchorage-dependent cells in a suspension culture system, and overcomes several problems with static cultures, including the requirement for large quantities of culture media, space expense, inefficient gas–liquid oxygen transfer, presence of concentration gradients, and difficulties in monitoring and control.

Although the microcarrier culture system has many advantages, the limitation of cell attachment to the microcarrier surface depends on the carrier diameter (typically in the range of 100–400  $\mu\text{m}$ ), density (typically between 1.02 and 1.10 g/ml), and chemical composition (48). Today, microcarriers are commonly composed of a dextran matrix, with diethylaminoethyl (DEAE) anion exchange groups being widely used. This group of microcarriers includes Cytodex™ 1, Cytodex 2, and Hillex™. In addition, Cytodex 3 is also a member of the DEAE-dextran family, but the Cytodex 3 microcarriers are coated with a thin layer of collagen (48). The other materials that are used for microcarriers include plastic, glass, and cellulose. However, the aforementioned microcarriers are less amenable in tissue engineering or direct transplantation because they cannot be decomposed by the human body. Trypsin, collagenase, and dispase have been used to retrieve viable cells from nonbiodegradable microcarriers for use in cell-based transplantation studies. However, perfect separation of the cells from nonbiodegradable microcarriers after enzyme treatment is still difficult. Therefore, development of microcarriers composed of biodegradable chemicals may be an ideal answer.

Recently, microcarriers made of biodegradable substances including gelatin (CultiSpher™ family) (49), collagen (Cellagen™) (30), poly-lactic-co-glycolic acid (PLGA) (85), poly-L-lactic acid (PLLA) (19), and hydroxyapatite (26) have raised great interest and been extensively studied for their clinical application in the field of regenerative medicine. In addition, cell-seeded microcarriers can be used to transport cells to the tissue(s) that needs to be repaired.

In this article, the microcarrier culture systems used for primary cells, adult stem cells, and embryonic stem cells are reviewed as follows.

### PRIMARY CELLS

Microcarriers have the benefit of delivering cells to damaged or degenerated tissue for repair and restoration of function. Several studies have shown that primary cells cultured on microcarriers and implanted into animal models can cure several disorders for many years. Table 1 summarizes a number of examples of microcarriers that have been used in animal models. These studies have provided crucial information about liver insufficiency (20–22), cartilage repair (35,49,85), skin repair (44,89,90), and Parkinson's disease (12,14,73).

In 1986, Demetriou et al. first reported a new method for expansion of primary hepatocytes with Cytodex 3 (dextran matrix with a layer of collagen) and the subsequent hepatocyte transplantation that results in prolonged hepatocyte viability and function in vivo (20,22). In addition, transplantation of microcarrier-attached hepatocytes had a curative effect and provided sufficient metabolic support, detoxification of ammonia, and an increase in the survival rates of liver-damaged rats (54,98). Recently, Xu et al. described the positive effects of intraperitoneal transplantation of Cytodex 3 microcarrier-attached hepatocytes in rats with acute liver failure induced by D-galactosamine (98). Additionally, a microcarrier-attached hepatocyte-based bioartificial liver also facilitated metabolic support and modified the humoral response in an experimental therapy for liver failure (81,97).

For skin repair, Voigt et al. first reported that Cytodex 3 microcarrier-attached keratinocytes provide a new multilayered and keratinized epithelium in a full-thickness wound healing model, but the microspheres could not be degraded and induced an inflammatory response (90). Therefore, this group investigated another biodegradable substance, PLGA microcarriers, which were coated with gelatin and recombinant human epidermal growth factor. At 14 days posttransplantation, a new stratified epithelium was detected in the full-thickness wound healing model (89). Recently, Liu et al. produced autologous melanocytes, keratinocytes, and fibroblasts on a large scale using the CultiSpher-G microcarrier culture system (41–44), and showed a decrease in the size of recalcitrant venous leg ulcers using autologous keratinocytes (44). Using the novel bioreactor microcarrier cell culture system (Bio-MCCS) of Liu et al., autologous human keratinocytes can be rapidly expanded on one specific type of modified CultiSpher-G microcarriers, which are first cultured with autologous dermal fibroblasts that are killed when they reach confluency (43). For cartilage repair, Malda et al. reported that human

**Table 1.** Microcarrier-Expanded Primary Cells for Transplantation

Cell Type	Microcarrier	Model	Curative Effect	Ref.
Hepatocytes				
Rat	Cytodex™ 3	Liver insufficiency (rat)	Transplantation of microcarrier-attached normal hepatocytes reduces serum bilirubin levels of Gunn rats.	22
Rat	Cytodex 3	Liver insufficiency (rat)	Plasma albumin concentration of albumin-deficient rats progressively increases for 6 days and then declines after transplantation of normal hepatocytes that are microcarrier attached.	20
Rat	Cytodex 3	Acute liver insufficiency (rat)	Significantly higher blood glucose levels are observed compared to the levels in control rats that receive injections of microcarriers, liver cells, or medium alone.	21
Rat	Cytodex 3	Acute liver ischemia (rat), Subtotal hepatectomy (rat)	A higher caffeine clearance, a higher urea production, and a significantly smaller loss in body weight in comparison to sham-transplanted control rats.	13
Rat	Cytodex 3	Acute liver insufficiency (rat)	Survival rate: microcarriers group > vacant microcarriers group	98
Chondrocytes				
Human	CultiSpher™-G	Cartilage repair (subcutaneous dorsum) (nude mice)	Microporous gelatin microcarriers are effective matrices for nasal chondrocyte expansion while maintaining the ability of chondrocyte differentiation.	49
Rabbits	PLGA	Cartilage repair (subcutaneous dorsum) (mice)	Nine weeks after implantation of chondrocytes with PLGA microcarriers, solid or white cartilaginous tissues form at subcutaneous dorsum.	35
Sheep	PLGA	Cartilage repair (nude mice)	Chondrocytes cultured on PLGA microspheres were further assessed for cartilage tissue formation in collagen type I gels in nude mice.	85
Keratinocytes				
Human	Cytodex 3	Full-thickness wound healing model (nude mice)	Transplantation of microcarrier-attached keratinocytes results in a reconstituted epithelium that is multilayered and keratinized.	90
Human	PLGA	Full-thickness wound healing model (nude mice)	Approach to expand cultured human keratinocytes and reconstitute the epidermis in full-thickness wounds using a new microspherical transport system.	89
Human	CultiSpher-G	Skin transplants (human)	Transplantation of microcarrier-attached keratinocytes decreases the size of recalcitrant venous leg ulcers.	44
Human	Cytoline 1™ (polyethylene and silica)	Skin transplants (human)	Wounds heal in a shorter time and a better condition is achieved with the treatment. Microcarriers only do not have a curative effect.	9
Human	CultiSpher-G	Skin transplants (in vitro human skin equivalent model)	Microcarrier-expanded keratinocytes seeded onto the 3D in vitro human skin equivalent model are able to produce a viable epidermis. When keratinocytes are cocultured with feeder cells on microcarriers, a slightly greater cell yield is observed compared to keratinocytes cultured on microcarriers without feeder cells.	11

(continued)

**Table 1.** Continued

Cell Type	Microcarrier	Model	Curative Effect	Ref.
Human/ fibroblasts (human and porcine)	PLLA	Living skin replacement bi- otherapy (pig)	Allogeneic and xenogeneic fibroblasts neither have nor elicit any noticeable inflammatory or immune reaction during the 14-day experimental period. Both porcine and human dermal living skin re- placement provide cells and/or factors that acceler- ate dermal tissue regeneration.	40
Fibroblasts Human and rat	Cytodex-3	Hemophilia B (gene ther- apy) (rat)	Intraperitoneal transplantation of microcarrier- attached transduced fibroblasts forms aggregates in the peritoneal cavity and exhibits positive immuno- histochemical staining for human factor IX up to 8 weeks following transplantation.	55
Adrenal chromaffin cells				
Rat	Cytodex 3 glass beads	Parkinson's disease (rat)	Microcarrier-attached cells maintain their effect in reducing rotation for at least eight months, but ani- mals implanted with cells alone show a reduction in rotation that lasted less than 3 months.	16
Rat	Cytodex 3 glass beads	Parkinson's disease (rat)	Animals that receive adrenal chromaffin cells- beads show significant behavioral recovery that is sustained over the 12-month test period, but the group that received adrenal chromaffin cells alone only exhibited a significant reduction in the first month.	12
Ventral mesen- cephalon cells				
Human and rat	Cytodex microcar- riers (no article)	Parkinson's disease (rat)	Microcarriers provide enhanced survival of both rat allograft and human xenograft fetal mesence- phalic cells in the rat striatum.	73
Retinal pigment epithelial cells				
Human	Gelatin microcar- rier	Parkinson's disease (rat)	These results further support the possibility that implantation of cultured hRPE cells may be a promising therapeutic option for patients with Par- kinson's disease.	14

PLLA, poly-L-lactic acid; PLGA, poly-lactic-co-glycolic acid; hRPE, human retinal pigment epithelium.

nasal chondrocytes could be expanded on CultiSpher-G microcarriers, and that the collagen content of microcarrier-expanded chondrocytes 14 days after implantation was slightly higher than T-flask-expanded cells (49). In addition to expansion on gelatin-based microcarriers, expansion of chondrocytes on PLGA microcarriers demonstrated the feasibility of articular cartilage repair in a mouse model (35,85). In addition, gelatin microcarrier-based cell delivery has shown positive clinical results in Parkinson's disease using human retinal pigment epithelial cells (14,27,93).

Furthermore, other biodegradable natural polysaccharides and FDA-approved hydrogel-based microcarriers have been shown to facilitate adhesion, proliferation,

and differentiation of anchorage-dependent cells (e.g., fibroblasts, osteoblasts, and MSCs), and may be useful in clinical regenerative medicine in the musculoskeletal or dermatological fields (91,92).

#### ADULT STEM CELLS

The technologies described above have encouraged the use of the microcarrier culture system as a powerful tool for expansion of stem cells for clinical use. Moreover, the use of microcarriers in expansion, differentiation, and transplantation has only been explored to a limited extent. Tables 2 and 3 list currently available information about stem cells cultured on microcarriers. Moreover, this system also makes possible the use of

**Table 2.** Expansion of Adult Stem Cells Using Microcarrier Culture

Cell Type	Microcarriers (Bioreactor)	Cell Recovery	In Vitro Differentiation	Notes	Ref.
Mesenchymal stem cells					
Human (bone marrow)	CultiSpher™-G (spinner flasks)	Trypsin-EDTA	Osteogenesis	After 7 days of cultivation, a 10-fold increase in viable cells is obtained in spinner culture. After microcarrier culture for 12 days, the MSCs maintain surface antigens and osteo-lineage potential.	95
Porcine (bone marrow)	Cytodex™ 1, Cytodex 2, Cytodex 3 (spinner flasks)	Trypsin-EDTA	Osteogenesis, chondrogenesis	Bead-to-bead transfer. Bead-to-bead transfer by 1:1 medium with beads after 14 days of cultivation. The osteogenic and chondrogenic differentiation of MSCs was confirmed after enzymatic harvest from microcarriers.	29
Human (adipose)	CultiSpher-G (spinner flasks)	Collagenase, osteogenesis, adipogenesis	After microcarrier culture for 1 week, direct differentiation to osteo-lineage or adipo-lineage cells on microcarriers is seen.	71	
Rat (bone marrow stromal cells)	CultiSpher-S, Cytodex 1, Cytopore 2 (spinner flasks)	Not tested	Osteogenesis, chondrogenesis, adipogenesis	Transplantation. Beads coated with 5 ng/ml fibronectin. Significantly less apoptosis in the short term in vivo. After 3 weeks of microcarrier culture, higher osteocalcin and alkaline phosphatase gene expression of undifferentiated MSCs compared to plate cultures. Induced bone formation in vivo in the long term after direct subcutaneous transplantation of BMMSCs-CultiSpher S.	99
Goat (bone marrow)	Cytodex 1 (spinner flasks)	Not tested	Not tested	Bead-to-bead transfer. Bead-to-bead transfer by 30% medium with beads per 3 days.	76
Rat (outer ear)	CultiSpher-S (spinner flasks)	Dispase	Osteogenesis, chondrogenesis, adipogenesis	Bead-to-bead transfer. Bead-to-bead transfer by 1:1 medium with beads when MSC culture reached confluence. Lower in vitro function for osteogenesis and adipogenesis compared to passage 0.	75
Human (bone marrow)	Gelatin-grafted-gellan (TriG) microcarriers (shaker)	Not tested	Osteogenesis	Transplantation. The formation of osteogenic matrices is better achieved in hydrogel-encapsulating cell-laden TriG microcarriers than in hydrogels.	92
Human (bone marrow)	Plastic microcarriers (rotary wall vessels)	Not tested	Osteogenesis, adipogenesis	Microgravity. Microgravity inhibits osteogenic differentiation of MSCs but induces adipogenic differentiation.	102
Human (bone marrow)	Plastic microcarriers (rotary wall vessels)	Not tested	Osteogenesis, Adipogenesis	Microgravity. Microgravity significantly reduces RhoA activity and phosphorylation (osteogenesis), but increases G-actin and cellular lipid content (adipogenesis).	51

(continued)

**Table 2.** Continued

Cell Type	Microcarriers (Bioreactor)	Cell Recovery	In Vitro Differentiation	Notes	Ref.
Mesenchymal progenitor cells					
Goat (bone marrow)	Hydroxyapatite (bacteriological grade 6-well plates)	Not tested	Not tested	Transplantation. Subcutaneous implantation in nude mice shows abundant bone formation after 4 weeks with a 212–300- $\mu\text{m}$ diameter particle range, but no bone formation is apparent with the 500–706- $\mu\text{m}$ diameter range particles.	26
Bone marrow stromal cells					
Rat (bone marrow)	Calcium titanium phosphate (untreated 96-well plates)	Not tested	Osteogenesis	Direct proliferation and osteo-differentiation of bone marrow stromal cells on calcium titanium phosphate microcarriers.	7
Bone marrow cells					
Rat (bone marrow)	Hydroxyapatite (spinner flasks)	Trypsin	Osteogenesis	The cells on the hydroxyapatite microcarriers have a higher glucose consumption than those grown in T-flasks at the early stages of culture, and maintain the potential to differentiate into osteoblasts.	80
Chondro-progenitor cells					
Bovine (cartilage slices)	CultiSpher-G (spinner flasks)	Not tested	Chondrogenesis	Bead-to-bead transfer. Bead-to-bead transfer by 1:15 medium with beads after 5 days of cultivation. Direct proliferation and chondro-differentiation of chondro-progenitor cells on microcarriers.	50
Pancreatic stem cells					
Rat (exocrine pancreas)	Cytodex 1, Cytodex 3 (spinner flasks)	Trypsin	Adipogenic	Cytodex 3 provides a better substrate for the promotion of cell adherence and growth. After cells are enzymatically harvested from the carriers, cells maintain their adipo-lineage potential.	39

continuous bioprocesses to expand and recover human stem cells for subsequent use in clinical cellular therapies or tissue engineering. As illustrated in Figure 1, there are three suitable bioreactors for stem cell expansion or differentiation on microcarriers and three prospective methods of stem cell recovery as well as direct injection for the repair of tissues such as brain, skin, liver, bone, and cartilage.

#### *Mesenchymal Stem Cells*

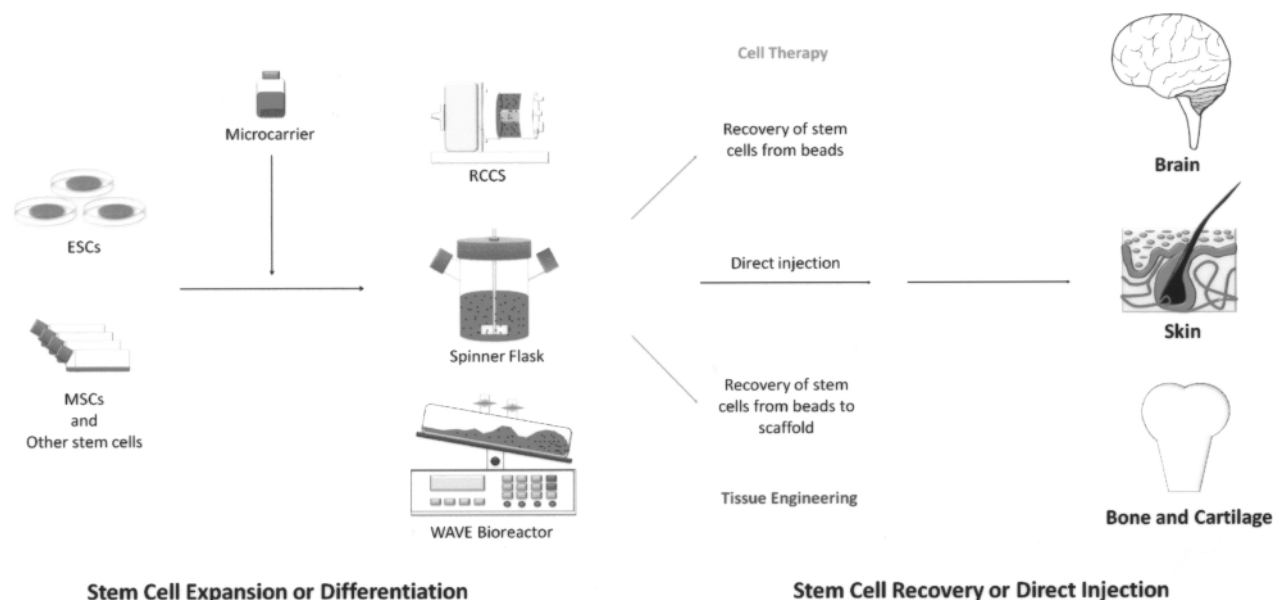
Owing to their high plasticity (8,15,61,65,68,83), high expansion capacity (65,82,83), and ability to avoid rejection after transplantation (72), these adult stem cells are likely to play a prominent role in regenerative medicine. Although some clinical studies have demonstrated

successful tissue engineering with MSCs (8,61,68), technological problems remain, such as a time- and money-consuming expansion phase due to a long expansion period and a labor-intensive process. Therefore, the development of bioreactors that allow monitoring, control, and automation of the cell culture process will be decisively important.

The ability of MSCs to attach, proliferate, and differentiate on macroporous microcarriers in spinner culture has been confirmed (71,95,99) (Fig. 2). The culture of MSCs on microcarriers was first explored by Wu et al. in 2003 (95); the results showed that 7 days of cultivation yielded a 10-fold increase in viable cells in spinner culture, and that the average lactate yield from glucose consumption in the spinner culture was only 1.63, which

**Table 3.** Expansion of Embryonic Stem Cells (ESCs) Using the Microcarrier Culture System

ESC Cell Line	Microcarriers (Bioreactor)	Cell Recovery	In Vitro Differentiation	Notes	Ref.
Mouse (CCE, R1, M8, 9J)	Glass-coated styrene microcarriers, Cytodex <sup>TM</sup> 3 (spinner flask)	Trypsin-EDTA	Cardiomyogenesis, hematogenesis	Feeder free. Developed microcarrier and aggregate suspension culture systems for the propagation mESCs. Analysis of E-cadherin <sup>-/-</sup> and E-cadherin <sup>+/+</sup> cells using both systems provides insight into the mechanisms behind the role of cell aggregation control. After 3 days of culture, the ESCs can differentiate via embryoid body formation.	28
Mouse (S25)	Cytodex 3 (spinner flask)	Trypsin	Neurogenesis	Microcarriers were coated with collagen. Cells grown in spinner flasks consistently show high viability (above 90%), and at three different cell seeding densities, expansion is several fold higher than on control plates. With addition of only 1 mM all <i>trans</i> -retinoic acid at days 4 and 6 of culture, the ESCs can be induced to differentiate into neural precursors.	1
Mouse (46C-GFP)	Cytodex 3, Cultispher <sup>TM</sup> -S (spinner flask)	Collagenase	Cardiomyogenesis	Feeder free. ESCs expanded on macroporous microcarriers using serum-free medium retain the pluripotency and neural commitment potential of the expanded cells.	24
Human (ESI-017)	Hillex II <sup>TM</sup> (spinner flask)	Collagenase	Osteogenesis	Microcarriers were pretreated with MEF-conditioned medium. Efficient expansion of cGMP-grade and FDA-approved human fibroblasts for hESC ex vivo cGMP culture. Using fibroblast conditioned medium, supported ESC growth through >5 passages and retained their pluripotency.	64
Human (ESI-017), mouse (R1 <sup>β<sub>2</sub>-lacZ<sup>KO</sup></sup> )	Hillex-II (spinner flask)	Collagenase	Not tested	Feeder free. Tested the hESC aggregate and single-cell culture together and also screened nine different microcarriers. ESCs are cultured on microcarriers for over five passages, and prolonged self-renewal is observed.	63
Human (H9)	Cytodex 3 (spinner flask)	Trypsin	Osteogenesis, neurogenesis	Feeder- and Matrigel <sup>TM</sup> free. After 14 days of cultivation, a sevenfold increase in cell concentration is obtained with the spinner culture. ESCs expanded in stirred conditions using serum-free medium retain their pluripotency and the ability to commit to the neural lineage.	25
Human (H1, H9)	HyQSphere <sup>TM</sup> (spinner flask)	Collagenase type IV	Endoderm differentiation	Collagen-coated microcarriers were coated with Matrigel. Definitive endoderm differentiation. More than 80% of differentiated ESCs coexpress endoderm markers (FOXA2 and SOX17).	45
Human (H1, H9)	Cytodex 3 (spinner flask)	Collagenase, dispase	Not tested	The beads were coated with Matrigel or seeded mouse embryonic fibroblasts. Karyotype is normal. Screened 12 kinds of commercially available suitable microcarriers and claimed hESC recovery improves when cryopreserved on microcarriers.	56
Human (HES-2, HES-3)	Cellulose-coated Matrigel (ultralow attachment six-well plates)	TrypLE enzyme, collagenase, dispensing and aspirating	Cardiomyocytes	The beads were coated with Matrigel. Authors have developed an easy and robust method for maintaining undifferentiated ESCs in three-dimensional suspension cultures achieving two- to fourfold higher cell densities than those in two-dimensional colony cultures.	59



**Figure 1.** Schematic of stem cells grown in different microcarrier culture systems and the use of stem cell-seeded microcarriers. After detachment from culture dishes or T-flasks, stem cells can be cultured on microcarriers in spinner flasks, a WAVE bioreactor, or a microgravity system such as a rotary cell culture system (RCCS). After expansion or differentiation, cell-seeded microcarriers can be directly injected into the human body, or cells can be enzymatically recovered from microcarriers for use in cell therapy or tissue engineering. ESCs, embryonic stem cells; MSCs, mesenchymal stem cells.

is lower than that in stationary culture (2,44). Additionally, after growth in the spinner culture for 12 days, the MSCs maintained the characteristics of stem cells (95). Recently, the effect of porcine bone marrow mesenchymal stem cell (BMMSC) proliferation using three types of commonly available Cytodex microcarriers was examined by Frauenschuh et al. The results showed that Cytodex 1 microcarriers promoted a higher level of cell attachment and proliferation compared with Cytodex 2 and 3 (29). In addition, this group also demonstrated increased colonization of microcarriers after carrier addition in a 1:1 ratio after almost all microcarriers had become confluent (bead-to-bead transfer) (29). Recently, the microcarrier culture scalability of ear MSCs was expanded with bead-to-bead transfer, and cells maintained their potential for differentiation (75). Also, addition of 30% fresh medium containing microcarriers every 3 days provided decreased production of lactate from glucose and continuous proliferation of goat MSCs on microcarriers (76). In addition, a more homogenous cell distribution on the microcarriers was obtained as a result of bead-to-bead transfer (76). Based on the use of bead-to-bead transfer, the available area can be extended and the culture of anchorage-dependent cell can be prolonged by free cells colonizing from old beads to fresh beads (60).

Recently, adult MSCs have been proposed as attractive candidates for regenerative therapy of connective tissues. MSC-seeded microcarriers may simplify and en-

hance the performance of tissue engineering approaches for cartilage and bone repair. Rubin et al. reported a strategy for the proliferation and differentiation of adipose-derived stem cells on Cultispher-G microcarriers that would allow induction of differentiation *ex vivo*, precise placement of cells, and correct tissue architecture (71). In addition, Yang et al. reported that short-term direct subcutaneous transplantation of BMMSCs expanded on Cultispher-S microcarriers was associated with significantly less apoptosis than trypsinized control cells, and that induction of *de novo* trabecular bone formation *in vivo* occurred in the long term (99).

In addition to the Cultispher family, hydroxyapatite microparticles—another type of biodegradable microcarrier that varies in size and microporosity—were evaluated *in vitro* and *in vivo* for their suitability for use as a carrier in an injectable tissue-engineered bone filler. Fischer et al. showed that both the size and microporosity of nonporous hydroxyapatite microparticles affect *in vivo* bone formation by cultured mesenchymal progenitor cells. The diameter particle size range of 212–300 nm is optimal for use as carriers of cultured goat mesenchymal progenitor cells for the *in vivo* production of bone tissue (26).

In some specific cases, MSCs can be cultured on microcarriers in a microgravity environment modeled by a rotary cell culture system (51,102). Zayzafoon et al. first indicated that microgravity modeled by a rotating wall vessel bioreactor inhibits osteoblastic differentiation of



human MSCs on plastic microcarriers and induces the development of an adipocytic lineage phenotype (102). After 1 year, Meyers et al. confirmed that reduced osteoblastogenesis and enhanced adipogenesis of human MSCs on plastic microcarriers in modeled microgravity were mediated by RhoA and cytoskeletal disruption (51). These reports suggest a possible reason for the reduced bone mass of the human body after prolonged time in space, but also imply that this culture system cannot maintain the differentiation potential of MSCs.

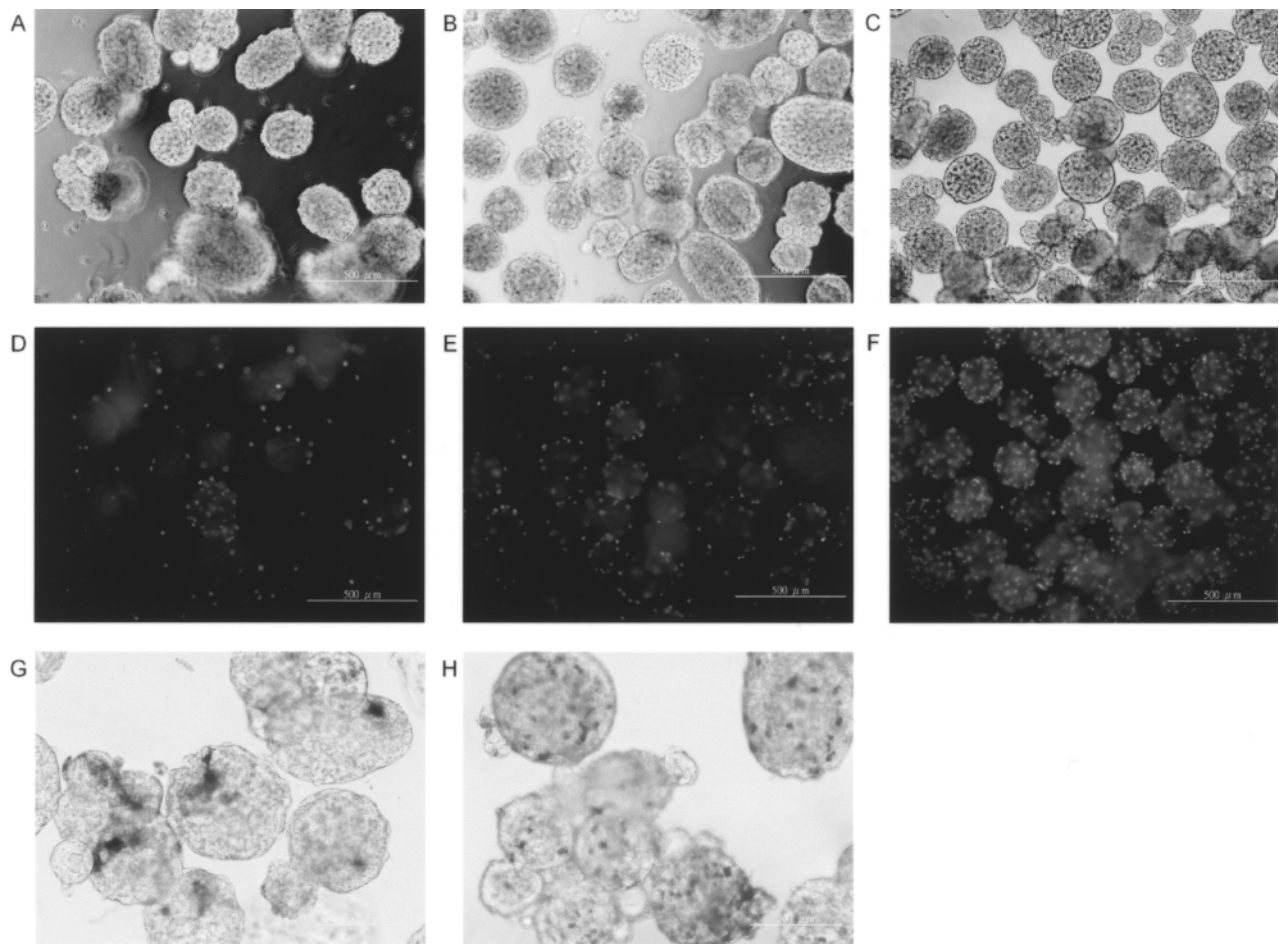
#### Neural Stem Cells

Kallos and Behie first reported the growth and inoculation conditions for the large-scale expansion of mammalian neural stem cells in suspension bioreactors (33). These investigators showed that optimization of pH of

the medium and inoculation conditions are critical for robust cell proliferation. The best growth characteristics for mammalian neural stem cells were obtained with a pH range of 7.1–7.5, a high oxygen level (20%), and low osmolarity of the medium (below 400 mOsm/kg). Kallos and coworkers also proposed optimal serial passaging protocols that would allow mammalian neural stem cells to be grown effectively in suspension culture (34). For the first time, this group was able to grow mammalian neural stem cells on a larger scale in suspension culture and maintain their stem cell characteristics.

#### Pancreatic Stem Cells

Adult pancreatic stem cells can be grown for more than 140 passages while maintaining the expression of typical stem cell markers (39). Serra et al. evaluated two



**Figure 2.** The location and proliferation of BMMSCs on CultiSpher-G microcarriers after 7 days with an initial density of  $5 \times 10^4$  cells/m and a microcarrier concentration of 3 g/L. Samples from (A, D) 5 h, (B, E) 1 day, and (C, F) 7 days stained with Hoechst 33342 dye (D, E, F). Starting on day 3, 50% medium changes were performed every 3 days. Scale bar: 500  $\mu$ M. The alkaline phosphatase (ALP) activity of bone marrow mesenchymal stem cells (BMMSCs) undergoing direct osteo-differentiation in stirred microcarrier cultures under osteogenic conditions is shown by ALP staining (G). The lipid spheres in BMMSCs undergoing direct adipo-differentiation in stirred microcarrier cultures under adipogenic conditions is shown with Oil Red O staining (H). Scale bar: 200  $\mu$ M.

different culture strategies, including cell aggregated culture and the microcarrier culture system. The use of a microcarrier support (Cytodex 1 or Cytodex 3) promoted the expansion of cell populations that retained their self-renewal ability, cell markers, and differentiation potential, allowing them to differentiate into adipocytes (77). In addition, although both Cytodex 1 and Cytodex 3 could sustain cell expansion, Cytodex 3 was a better substrate for the promotion of cell adherence and growth.

#### *Progenitor Cells*

Melero-Martin et al. reported that the use of Culti Spher-G microcarriers resulted in a 17-fold expansion of chondro-progenitor cells grown in batch cultures (50). In addition, chondro-progenitor cells were capable of undergoing bead-to-bead migration, which allowed subcultivation without a harvesting step. In addition to chondro-progenitor cells, Sugo and Ogawa also reported that rat bone marrow cells grown on hydroxyapatite microcarriers maintained the potential to differentiate into osteoblasts, and the cell proliferation was more rapidly in the case of the hydroxyapatite microcarrier than in the case of hydroxyapatite disk or T75 flasks (80). Barrias et al. also reported that bone marrow stromal cells could grow and differentiate into osteoblastic lineage cells when expanded on calcium titanium phosphate microspheres (7).

#### *Hematopoietic Stem/Progenitor Cells*

Hematopoietic stem/progenitor cells have been expanded or differentiated with or without coculture with bone marrow cells grown in microcarrier cultures (23, 74,101). Recently, Chiu et al. demonstrated that CD34<sup>+</sup> human umbilical cord blood progenitors are capable of transdifferentiation to yield a vascular endothelial cell phenotype and that they could assemble into three-dimensional tissue structures in rotary wall vessels when expanded with or without microcarrier beads (17).

### **EMBRYONIC STEM CELLS**

Recently, owing to the pluripotent differentiation potential of human ESCs that are derived from the inner cell mass of blastocysts (86), many groups have suggested that human ESCs have the potential to treat many diseases, including myocardial infarct (53), diabetes (62), and Parkinson's disease (10,18,70). However, both mouse and human ESCs are adherent cells and uniquely grow as colonies. In addition, ESCs are typically grown on a layer of inactivated mouse embryonic fibroblasts (MEFs) that function as feeder cells and maintain the ESCs in an undifferentiated state, so feeder cells are commonly realized as necessary for ESC culture (86). Replacement of MEFs with nonxenogeneic human fibroblasts was shown to support human ESC growth as

well (67). In addition, recent studies have described the successful use of feeder-free (with feeder-conditioned medium but without feeder cell coculture) and feeder-independent systems (without conditioned medium or feeder coculture) with appropriate extracellular matrix molecules, such as Matrigel<sup>TM</sup>, laminin, fibronectin, collagen IV, and vitronectin, for human ESC culture (4,5,46,69,96). Thus, the current methods for scaling up ESC culture using microcarriers have focused on the extracellular matrix molecules present on the surface of microcarriers.

In 2005, Fok and Zandstra first reported a method in which glass-coated styrene microcarriers and Cytodex 3 were used to establish a feeder-free microcarrier culture system for mouse ESCs, which resulted in shorter doubling times than substrate-free aggregate cultures grown in suspension (28). The effects of inoculation of mouse ESCs and the concentration of Cytodex 3 on cell growth and metabolism were analyzed by Abranches et al. (1). After seeding, the cells typically exhibited a growth curve consisting of a short death or lag phase followed by an exponential phase leading to a maximum cell density of  $2.5\text{--}3.9 \times 10^6$  cells/ml and a maximum specific growth rate of  $1.2 \text{ day}^{-1}$  (1). In addition, 46C mouse ESCs cultured on two different microcarriers were compared by Fernandes et al. (24). The maximal fold increase in the population of these mouse ESCs cultured on macroporous CultiSpher-S was higher than that of cells cultured on Cytodex 3, but the maximum specific growth rate was lower when cells were grown in serum-containing conditions (24).

It is possible to establish an economical and c-GMP-compliant microcarrier system to produce human fibroblasts to support clinical-grade human ESC culture. Phillips et al. recently demonstrated the efficient expansion of clinical-grade human fibroblasts on polystyrene-based cationic trimethyl ammonium-coated microcarriers (Hillex II, SoloHill Engineering, Inc.) using c-GMP reagents (64). Notably, while retaining pluripotency, the extended self-renewal and expansion (for more than five passages) of human ESCs were supported by microcarrier-expanded fibroblasts and their conditioned medium. The feeder-free microcarrier system can be used to expand human ESCs as well. Oh et al. demonstrated that Matrigel-coated cellulose microcarriers allow routine passaging and stable propagation of human ESCs without differentiation and maintenance of pluripotent markers such as oct-4, SSEA4, and TRA-1-60 (59). Recently, Fernandes et al. established a feeder- and Matrigel-free microcarrier system for growth of human ESCs with continuous agitation (25). This report describes a promising scale-up system for expansion of human ESCs for use in clinical therapy and research. In addition, Lock et al. established a Matrigel-coated HySphere<sup>TM</sup> (collagen-coated microcarriers)-based microcarrier bioreactor for

generating therapeutically useful quantities of endoderm progeny including pancreatic islet cells and liver cells that were differentiated from human ESCs (45).

Moreover, iPSCs derived from several differentiated cell types via ectopic expression of transcription factors such as Oct4, Sox2, Klf4, and C-Myc have been developed over the past 3 years (84). Generally, these cells exhibit a normal karyotype, are similar to ESCs, and maintain the potential to differentiate into lineages of all three germ layers (2). Recently, mouse iPSCs were used in an animal model of Parkinson's disease (94). Although there is no information regarding iPSCs cultured in the microcarrier system, ex vivo expansion of iPSCs using the microcarrier system for autotransplantation may be possible.

### FUTURE DIRECTIONS AND CONCLUSION

Microcarrier technology can be used to produce large numbers of functional cells that maintain desired properties. Based on the continuing study of biodegradable microcarriers and continuous bioprocesses such as bead-to-bead transfer, further scale-up to produce cells for cell therapy or tissue engineering is possible. Currently, information from in vivo studies is limited and has mostly been focused on adult MSC-seeded microcarriers for regenerative therapy of cartilage or bone defects. Although the in vivo use of ESC-seeded or iPSC-seeded microcarriers has not yet been described, these studies are likely imminent. In conclusion, the microcarrier culture system has great potential as a powerful tool for efficient ex vivo stem cell expansion for future autotransplantation approaches.

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