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A simple cell transport device keeps culture alive and functional during shipping

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Abstract

Transporting living complex cellular constructs through the mail while retaining their full viability and functionality is challenging. During this process, cells often suffer from exposure to suboptimal life-sustaining conditions (e.g. temperature, pH), as well as damage due to shear stress. We have developed a transport device for shipping intact cell/tissue constructs from one facility to another that overcomes these obstacles. Our transport device maintained three different cell lines (Caco2, A549, and HepG2 C3A) individually on transwell membranes with high viability (above 97%) for 48 h under simulated shipping conditions without an incubator. The device was also tested by actual overnight shipping of blood brain barrier constructs consisting of human induced pluripotent brain microvascular endothelial cells and rat astrocytes on transwell membranes to a remote facility (approximately 1200 miles away). The blood brain barrier constructs arrived with high cell viability and were able to regain full barrier integrity after equilibrating in the incubator for 24 h; this was assessed by the presence of continuous tight junction networks and *in vivo*-like values for trans-endothelial electrical resistance (TEER). These results demonstrated that our cell transport device could be a useful tool for long-distance transport of membrane-bound cell cultures and functional tissue constructs. Studies that involve various cell and tissue constructs, such as the “Multi-Organ-on-Chip” devices (where multiple microscale tissue constructs are integrated on a single microfluidic device) and studies that involve microenvironments where multiple tissue interactions are of interest, would benefit from the ability to transport or receive these constructs.

Introduction

Cell and tissue culture techniques are vital to life science research. The ability to share cells and tissue engineered constructs with other investigators is important. The shipping of frozen cell cultures on dry ice and cell cultures in closed flasks is common practice, however, the shipping of cellular constructs on membranes is not. This is unfortunate because the ability to share culture cells on permeable support membranes that has

facilitated studies involving permeability, migration, diffusion, active transport, drug metabolism, absorption, and secretion¹⁻⁷ is still difficult.

Transwell inserts are a type of permeable support that fit into conventional multi-well cell culture plates and feature a thin porous membrane (available in various pore sizes and various plastics). The transwell is designed to hang from the well's top edge to keep the membrane about a millimeter off the bottom of the well. The lid of the well plate is similar to that of a petri dish providing gas exchange in the incubator. These inserts are commonly used to study both anchorage-dependent and/or anchorage-independent cell lines.⁸⁻¹¹ Transwell inserts provide an updated technique for culturing polarized cells because the membrane permits the cells to uptake and secrete molecules on both their basal and apical surfaces and fosters metabolic activities that better mimic those that occur in the body. Also, monolayers or tissue-like constructs can be attached to the bottom and/or top of a transwell making them versatile and they can be inserted in microfluidic systems.

Models of multi-organ interactions have been created using microfluidic systems that connect a variety cellular chambers with fluidic channels.¹²⁻¹⁴ In some of these systems porous membranes are used to provide support for cellular constructs while allowing for diffusion, transport and other interactions of nutrients and signal molecules on metabolites.¹⁵⁻¹⁷ Collectively these techniques can create dynamic systems or models that better resemble normal human or mammalian physiology, however, obtaining prepared cellular constructs that are functional, not contaminated and viable has been difficult.

Some institutes have attempted to ship the cellular constructs on inserts in tissue culture multiwell plates wrapped with parafilm. However, often the cultures have been received dead, damaged, of poor quality, contaminated and/or the medium has leaked out (personal communications with Brian Davis (GE Global Research, Niskayuna, NY) and Jay Hickman (University of Central Florida, Orlando, FL). The present hanging design of the transwell in a well makes it impossible to obtain the sufficient seal in that plate that would be required to transport efficiently. To our knowledge there are no products available that can transport intact cellular constructs on transwell membranes through the mail successfully. If the ability to transport intact cellular constructs was feasible, constructs would be more readily available to collaborators.

There are various methods of cell preservation that are available and are used to transport cells. Cryogenic vials are designed to store frozen cell suspensions (at subzero temperatures ranging from -80 to -196°C). Hypothermic preservation is another method where cells and tissues are chilled between 4 and 10°C in a cold storage solution for short term storage¹⁸. The use of hypothermic preservation using extracellular-matrix-mimetic microparticles has enhanced cell survival and could improve cell therapy and diagnostic accuracy¹⁹. While these methods are capable of long or short term preservation, none of these methods are used to transport cellular/tissue constructs that are on transwell membranes.

We designed a transport device (consisting of a transwell carrier and a sealing lid). We decided that the transwell carrier could be made from a variety of materials (e.g. polycarbonate, polypropylene, polystyrene etc.) taking into consideration certain key

concepts. The material that the device was made of had to be compatible with the cells or tissues and needed to maintain culture sterility. We wanted the device to be made of a material that could be cleaned and autoclaved making it reusable. The outer surface of the transwell carrier could be made of a variety of shapes (i.e. cubic, prism, cylinder etc.), however, the inner dimensions of this container had to be specific depending upon the type of transwell insert used.

The sealing lid of the transport device could be made out of a variety of materials as well. This material could be the same as the carrier of the transport device or it could be made of a more flexible material (e.g. rubberlike). The requirements for the selected material were that the material was compatible with cells or tissues and that it could be sterilized. Another important concept for the lid design was that it needed to completely seal the device to prevent leaking and secure the transwell in place. Some lids could be a push top design or made so they screwed into the device. The lid needed an o-ring to help seal the container and prevent the unit from leaking.

Our lid design resembles the design used in some cryogenic vials. Cryovials are vials that are designed to maintain sample integrity (e.g. cell suspensions) in ultra-low temperature storage. Some of these vials are manufactured from polypropylene to withstand temperatures down to -196°C . The cryovials are certified sterile (by λ radiation), non-cytotoxic, non-pyrogenic and commercially available. Also, some of the cryovial caps have a silicone washer or o-ring to secure a seal (e.g. Corning Inc., Corning, NY and Nalge Nunc, Rochester, NY).

The purpose of our device is to provide a means to transport cellular constructs on transwell membranes to other facilities by suspending the transwell in medium. The transporting device was designed to sustain viability of the cellular constructs, provide support, and keep the transwell secure within the carrier to prevent damage to the cellular/tissue layer. When transporting attached cells in a tissue culture flask it is important that the flask be totally filled with medium to avoid medium exhaustion, prevent detachment, and minimize potential shear damage to the cells.^{20, 21} Excess medium volume protects cells during shipping from drying out, minimizes bubble damage, helps manage pH and reduces temperature fluctuation. Similarly we fill our device full of medium.

Our transport device was designed specifically to transport complete living cellular/tissue constructs attached on transwell inserts at room temperature and should not to be confused with hypothermic preservation methods. In our transporting device, we are addressing the shipping problems that occur when trying to transport transwell inserts in liquid medium to other facilities while retaining high viability and function.

Materials and Methods

Cell Lines

All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in a humidified incubator at 5% CO_2 and 37°C unless otherwise indicated. The human colorectal adenocarcinoma cell line Caco2 was maintained in Eagle's Minimum

Essential Medium (ATCC #30-2003) plus 20% fetal bovine serum (FBS, Invitrogen, Grand Island, NY #26140). The human lung carcinoma cell line A549 was maintained in F12-K Medium (ATCC #30-2004) plus 10% FBS. The human hepatocellular carcinoma cell line (HepG2 C3A) was maintained in Eagle's Minimum Essential Medium (ATCC #30-2003) plus 10% FBS.

For the first set of experiments, the cell lines were expanded in culture flasks, and subcultured by trypsinization onto 24 mm transwell inserts (Corning, Corning, NY #3412) for 6-well plates at a density of 1.11×10^5 cells/cm² (Day 0). The top and the bottom chambers of each well separated by the transwell insert were filled with 0.5 mL and 3 mL, respectively. The medium was refreshed on Day 3. The cultures were used for the simulated shipping experiments on Day 5 when the cultures were fairly confluent.

Another set of experiments was set up to see how the transporting device handled cells that were actively dividing. For these experiments 6.5 mm transwell inserts (Corning #3413) were plated from a concentration of 6.06×10^4 cells/cm² (Day 0). The top and the bottom chambers of each well separated by the transwell insert were filled with 0.2 mL cells and 0.8 mL medium, respectively. Wells without transwells were also plated with 0.2 mL cells and 0.8 mL medium to monitor the cells and determine when to complete the experiment. Cells were used for these experiments on Day 1.

Preparation of the Blood Brain Barrier (BBB) Constructs

Human induced pluripotent stem cell (iPSC) line IMR90-4 (WiCell, Madison, WI #WB0088), developed by Yu et al.,²² was maintained and differentiated into brain microvascular endothelial cells (BMECs) as previously described^{23, 24}. Briefly, the iPSCs were plated on Matrigel (Corning Inc., Corning, NY) coated culture dishes and maintained in mTeSR1 medium (STEMCELL technologies, Vancouver, BC, Canada) until colonies were at close range of each other. The cells were then switched to a differentiation medium DMEM/F12 medium with 15 mM HEPES (Life Technologies, Grand Island, NY) containing 20% KnockOut Serum Replacement, 0.1 mM MEM Non-Essential Amino Acids Solution, 1 mM L-glutamine and 0.1 mM β -mercaptoethanol (Sigma, St. Louis, MO). After 6 days of differentiation, the medium was changed to Human Endothelial Serum Free Medium (Life Technologies) supplemented with 1% platelet poor derived bovine serum (Biomedical Technologies, Baltimore, MD), 20 ng/mL basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN), and 10 μ M retinoic acid (Sigma). Cells were then cultured for two more days before passaging for BBB cocultures. All media were replenished daily throughout the culturing period.

The BMEC-astrocyte cocultures²⁴ were prepared on 6.5 mm transwell inserts (Corning #3413) or 12 mm snapwell inserts (modified Transwell permeable support insert that contains a removable membrane section that easily separates from its support, Corning #3407) (for viability assays) with 0.4 μ m pore polycarbonate membranes (Corning). Before plating cells, the top sides of the transwell inserts were coated with a mixture of 400 μ g/mL collagen IV (Sigma) and 100 μ g/mL fibronectin (Sigma) in Dulbecco's Phosphate Buffered Saline (DPBS) at 37°C for at least 4 h and were then rinsed with DPBS. Primary rat astrocytes (Life Technologies) were seeded onto the non-coated side of the inserts and

cultured in astrocyte growth medium (85% DMEM with high glucose plus 15% FBS) for 48 h. Human iPSC-derived BMECs were then dissociated with Versene Solution and plated onto the coated side of these inserts. The differentiated cells from one well of a 6-well plate (9.6 cm² cell growth area) could seed 12 inserts. The assembled BBB constructs were maintained in a coculture medium (Human endothelial serum free medium supplemented with 1% platelet poor derived serum, 10 μ M retinoic acid, 100 units/mL penicillin and 100 μ g/mL streptomycin) before use for the transport experiments. All reagents not specified were purchased from Life Technologies.

Design of the Transport Devices

The transport devices consisted of a transwell carrier and a sealing lid (Figure 1), both made of polycarbonate. This material was chosen because it is fairly inert, durable, autoclavable, biocompatible and used in many tissue culture supplies. The dimensions of the transwell carrier were specifically designed to accommodate and secure a transwell/snapwell insert for 6-well plates or for 24-well plates (Figure 1D). Both carriers were designed with a 3 mm clearance beneath the insert membrane to keep the living cellular/tissue constructs suspended in medium. The sealing lid was made to fit snugly with the threads of the carrier (height of 10 mm). The devices were sketched in SolidWorks (SolidWorks Corp., Waltham, MA, USA) and milled out of a polycarbonate sheet on a Computer Numerical Control (CNC) Bridgeport Milling Machine. A viton o-ring (McMaster-Carr, Aurora, OH) was added to the lid to ensure sealing. Approximately 35 mL of medium was needed to fill a transporting device for a 6 well plate and 6 mL to fill the 24 well plate device. Examples of a transwell and transport devices were photographed (see Figure 1A–C).

The transwell carrier, sealing lid and forceps were sterilized in a steam autoclave (gravity/dry cycle) before use and handled carefully to prevent contamination.

Cell Line Shipping Experiments

The shipping experiments for three different cell lines were carried out after 5 d culture in the transwells. (3 experiments for each cell line). We wanted to simulate the shipping of fully-developed cellular constructs of three different cell lines. For each experiment, four transwells of a 6-well plate were inserted into a pre-autoclaved transwell transport devices using sterile forceps. Warm medium (approximately 37°C) was pipetted into the bottom of each transport device and into the transwell itself. The device's lid was screwed in place, as the lid was screwed on some of the medium was pushed out and helped to release any bubbles that might have formed (Figure 2). The device was cleaned off, wrapped with parafilm (VWR, Radnor, PA #52858-000), placed into doubled ziplock bags with paper towels in the bottom of the bags, surrounded with Styrofoam packing material in a styroform box, and taped as one would do if you were planning on shipping this package by an overnight carrier.^{25, 26} Then two of researchers tossed the box around in the laboratory for 15 minutes to simulate the handling of a package and then allowed the package to sit at room temperature for 48 h. We assumed that a 48 h study would be sufficient for testing because one would send samples using an overnight shipping company. The other 2 transwells with attached cells from the 6 well plate had their medium refreshed and were

placed back into the humidified incubator at 5% CO₂ and 37°C; these wells were used as our viability controls.

After 48 h of having the cells in the device, the box was tossed around in the laboratory again and the transporting devices were removed from the box (simulating a shipping process). Then, the transwells were removed from the transport devices and placed into a new 6 well plate with fresh DPBS. The transwells from the incubator were also placed into this new 6 well plate. The cells were stained for viability using Invitrogen's live/dead staining kit (Invitrogen #L3224) following the manufacturer's guidelines. Briefly, the cells were washed with DPBS, incubated with staining solution (2 µM calcein AM and 2 µM ethidium homodimer-1 in DPBS) at 5% CO₂ and 37°C for 45 min, washed with DPBS, and then imaged under a fluorescent microscope using FITC filter (494 nm excitation/517 nm emission) and RFP filters (528 nm excitation/617 nm emission). The Live/Dead Stain discriminated live from dead cells simultaneously because green-fluorescent calcein-AM indicated intracellular esterase activity (live) and red-fluorescent ethidium homodimer-1 indicated loss of plasma membrane integrity (dead). Fluorescent micrographs were taken at 100× magnification. Micrographs were assessed using ImageJ to determine viability based on areas of cells that displayed the fluorescent dyes.

The second set of experiments were done with the cell lines (same as above) where the transwells were plated at low densities, grown in the incubator for one day and then placed into the experiment. These experiments were designed to address the device's capability to handle sub-confluent cell cultures. For each experiment, four transwells of a 24-well plate were inserted into a pre-autoclaved transwell transport devices (Shipped) (see above for details), four transwells were placed into a new plate with fresh medium and stored in the dark at room temperature (RT), and four transwells were placed into a new plate with fresh medium and incubated at 5% CO₂ and 37°C (Control). The RT group was not tossed around like the Shipped group because a perfect seal for each individual transwell was difficult to obtain. The top of a transwell rests on the edge of its well in order to suspend the membrane in medium. This necessary feature compromises the ability to create a perfect seal and would cause leaking if inverted. Also, four "wells" (without transwells) were plated at the same density and volumes as the transwells to monitor confluency of the transwell plating. On Day 2 or Day 3, cells were stained for viability in one of two methods: 1. Invitrogen's live/dead staining kit (see above). Fluorescent micrographs were taken at 100× magnification. Micrographs were assessed using ImageJ to determine viability based on areas of cells that displayed the fluorescent dyes. 2. Viability was determined using trypan blue staining. Briefly, cells were dissociated by following the manufacturer's guidelines for trypsinizing transwells (Corning). Then 0.1 mL of 0.4% trypan blue stock solution was added to 0.1 mL of cells, loaded into a hemocytometer, and the blue stained cells and total number of cells were counted. Trypan blue is a vital stain where dead cells absorb the blue color and the live cells are excluded from the stain. Cell numbers were calculated.

Blood Brain Barrier Shipping Experiments

BBB constructs on transwell inserts were transferred to the transport devices on Day 3 of coculture. The transport devices were then filled with the coculture medium and prepared for

shipping as described above for the cell line shipping experiments. The package was shipped to the University of Central Florida (UCF) in Orlando, Florida or shipped back to Cornell University in Ithaca, New York via overnight delivery (24 h in the devices without an incubator). After receiving the package, the inserts with BBB constructs were immediately transferred to a culture plate with fresh coculture medium and placed back into the cell culture incubator or stained for viability. The control BBB constructs were continuously maintained in the incubator without going through the shipping process. The barrier tightness measured by trans-endothelial electrical resistance (TEER) was monitored throughout the process. TEER values were recorded right before the shipping, immediately after the inserts were transferred to a culture plate post shipping, and after they being incubated at 5% CO₂ and 37°C for equilibration for 1 h and 24 h. Cell viability and tight junction proteins were analyzed post shipping. Details for the assays and measurements are described below. The control BBB constructs were measured at the same timepoints as the shipped constructs and analyzed by the same assays.

1) TEER measurement—TEER was monitored using a Millicell ERS-2 Voltohmmeter (Millipore, Billerica, MA) with a “chopstick” STX01 electrode. The meter applied an AC square-wave current of $\pm 10 \mu\text{A}$ at 12.5 Hz and recorded the electrical resistance. The STX01 electrode tips had a silver/silver chloride pellet and the small size of the electrode enabled the measuring of transendothelial voltage and the resistance of cells grown on porous membranes. The electrode tips were sterilized in 70% ethanol for 15 minutes, air dried and rinsed with DPBS before use. The shorter and the longer tips of the electrode were inserted into a transwell insert and the outer well, respectively, to enabled the measurement of the electrical resistance of the BBB construct on the porous membrane. An electrical resistance reading of a collagen-fibronectin coated membrane was recorded as the baseline level and subtracted from each experimental reading. TEER values were normalized to unit area by multiplying the resistance by the effective cell growth area of the insert membrane. The unit of measure was $\Omega \text{ cm}^2$.

2) Cell viability assay—The BBB cells were stained for viability with Invitrogen’s live/dead staining kit and imaged with a fluorescence microscope as described above with a slight modification. BBB constructs were grown on snapwell inserts so that both sides of the membrane could be evaluated separately. The staining reagents were diluted in the BBB coculture medium instead of DPBS to best maintain BBB cell viability during staining incubation. The cells were incubated for 45 minutes, washed with DPBS and then both sides of the membrane were imaged under a fluorescent microscope. Micrographs were assessed using ImageJ to determine viability based on areas of cells that picked up the fluorescent dyes as described above.

3) Immunofluorescence staining of tight junction proteins—BMEC-astrocyte cocultures were analyzed by immunofluorescence staining for zonula occludens protein-1 (ZO-1) and claudin-5 after the 24 h incubation at 5% CO₂ and 37°C post shipping. The immunostaining was carried out at room temperature unless specified otherwise²⁷. For claudin-5, cells were fixed and permeabilized in ice-cold methanol, blocked in DPBS containing 40% goat serum (40% DPBS-G, Sigma) and 0.1% Triton X-100 for 30 min, and

incubated with mouse anti-claudin-5 monoclonal antibody (1:50, 10 µg/mL) in 40% DPBS-G at 4°C overnight. The samples were then washed and incubated with Alexa Fluor 488-labeled goat anti-mouse secondary antibody (1:200, 10 µg/mL) in 40% DPBS-G for 1 hour. For ZO-1, the cells were fixed for 10 min in 4% paraformaldehyde (Boston Bioproducts, Ashland, MA), washed with DPBS, permeabilized with 0.1% Triton X-100 in DPBS for 10 min, and blocked with DPBS containing 5% bovine serum albumin (BSA, Sigma) and 0.1% Triton X-100 for 1 hour, and then incubated with fluorescein isothiocyanate (FITC)-conjugated ZO-1 monoclonal antibody (10 µg/mL) in DPBS containing 1% BSA for 1 hour. All samples were washed in DPBS three times for 5 minutes each, and mounted on slides with Fluoroshield mounting medium with DAPI (Sigma) to stain the nuclei. Images were acquired with a Zeiss LSM 710 confocal microscope and analyzed in ImageJ. All reagents not specified were from Thermo Fisher Scientific (Waltham, MA).

Statistical analysis

Data are presented as mean $\pm$ SEM. Multiple groups were analyzed by ANOVA or two groups compared using Student's t-test (GraphPad Prism). $P < 0.05$ was considered significant.

Results

Cell Line Viability

Experiments ($n = 3$) were done to determine if three different cell lines (Caco2, A549, and HepG2 C3A) could survive in the transporting device for 48 h. The viability of cells grown on transwell inserts incubated at 5% CO₂ and 37°C (Control inserts) was compared to the viability of cells grown on transwell inserts that were placed into the transporting device for 48 h under a simulated shipping condition (Shipped inserts). Fluorescent micrographs (100 $\times$ magnification) of the Control inserts and the Shipped inserts were compared using the fluorescent microscope, see Figure 3. The percent viable for the three cell lines was determined by evaluating the micrographs in ImageJ (Table I). Values were recorded as means $\pm$ SEM ($n = 3$). Standard errors were calculated and there were no significant differences between the insert controls of a given cell line vs. the inserts that experienced our 48 h simulated shipping procedure. These results demonstrated that the transporting device when packaged in Styrofoam could maintain the cellular viability of representative cell lines over a 48 h period. The micrographs showed that the cellular integrity of the controls and transported groups were similar.

The next set of experiments ($n = 4$) were done to determine if the three different cell lines could survive being transported at a subconfluent stage, support cell division and to see if the transporting device was really necessary. The viability of cells grown on transwell inserts incubated at 5% CO₂ and 37°C (Control inserts) was compared to the viability of cells grown on transwell inserts that were placed into the transporting device under a simulated shipping condition (Shipped inserts) and inserts that were incubated at room temperature in the dark (RT inserts). Fluorescent micrographs (100 $\times$ magnification) of these inserts were compared using the fluorescent microscope, see Supplemental Materials. The percent viable for the three cell lines was determined by evaluating the micrographs in ImageJ,

Supplemental Materials. Values were recorded as means $\pm$ SEM ($n = 4$). The viability for the three cell lines was also determined by using trypan blue and a hemocytometer to obtain cell numbers, see Table II. Cell numbers were recorded as means $\pm$ SEM ($n = 4$) and presented as a percent of Incubated Control.

Barrier integrity of the Blood Brain Barrier post shipping

Experiments were done to determine if a blood brain barrier construct could survive an actual 24 hour shipping procedure in the transporting device. These experiments were carried out between November 2015 and July 2016 resulting in exposure to a wide variety of ambient temperature during the shipping, especially between Ithaca, New York and Orlando, Florida. To evaluate the integrity of our blood brain barrier in the shipping device, we monitored TEER, determined viability and stained for tight junctions.

The TEER of the blood brain barrier constructs grown on inserts that were incubated at 5% CO₂ and 37°C (Control inserts) was compared to the TEER of the cells grown on inserts that were placed into the transporting device for 24 h and shipped (Shipped inserts). TEER readings were done on Day 3 in culture before being placed into the device, immediately after receiving the shipped transwells, 1 h after re-incubating (5% CO₂ and 37°C), and then 24 h after re-incubating. Figure 4 shows TEER of the blood brain barrier constructs on the transwells on Day 3 in culture, and at various times post shipping (0 h, 1 h, and 24 h). A significant decrease in the TEER was observed in the shipped group immediately after being received when compared to the control. It appeared that TEER starts to recover after 1 h of incubation but is still significantly lower than the control. After 24 h of incubation, TEER results of the shipped group increased and recovered enough so that the control and the shipped groups were not significantly different ($P < 0.05$). Values were recorded as means $\pm$ SEM ($n = 6 - 9$).

The viability of BBB constructs grown on transwell inserts incubated at 5% CO₂ and 37°C (Control inserts) was compared to the viability of cells grown on transwell inserts that were placed into the transporting device for 24 h after being shipped (Shipped inserts). Fluorescent micrographs (100 $\times$ magnification) of the Control inserts and the Shipped inserts were compared using the fluorescent microscope, see Figure 5. The viability of the BBB constructs was determined by evaluating the micrographs in ImageJ (Table III). Values were recorded as means $\pm$ SEM ($n = 3$).

To further investigate the effects of the shipping procedure on barrier integrity of the BBB constructs, we visualized the expression of two major tight junction (TJ) proteins by immunostaining: 1) claudin-5, a transmembrane protein that forms the backbone of the BBB TJs²⁷; and 2) ZO-1, a membrane associated cytoplasmic protein that plays a key role in maintaining TJ stability and functionality²⁸. The fluorescent images show high levels of protein expression of ZO-1 and claudin-5 in both the control and the shipped groups (Figure 6). For the ZO-1 stained inserts, a monoclonal antibody conjugated with FITC (green) was used but to show contrast in the Figure we substituted the red color. Continuous networks of these TJ proteins outlining the endothelial cell contour were formed in both groups of BMEC astrocyte cocultures.

These results demonstrate that the transporting device, when packaged in Styrofoam, could maintain the cellular viability of the blood brain barrier constructs over a 24 h period. The fluorescent micrographs for viability showed that cells of the controls and transported groups were not significantly different. The barrier integrity of the shipped inserts was affected by the shipping procedure when compared to the control inserts. This barrier integrity of the shipped inserts was capable of recovery and after incubating for 24 h the TEER was not significantly different from the control inserts.

Discussion

We developed a transwell transporting device that can be used to ship intact cellular constructs from one facility to another via an express mail carrier. This device was designed to enable an investigator to obtain a special tissue construct from a facility or collaborator who has expertise in creating that construct. The transporting device suspended the transwell in medium to sustain the viability of the cells, provide physical support and keep the transwell secure within the container to prevent damage to the cellular/tissue layer or construct.

Initially, three cell culture lines were used to test whether that we could obtain comparable viability of cells (heavy density) on inserts in our transporting device for 48 h (Shipped inserts) vs. the viability of cells (heavy density) on inserts that were maintained at 5% CO₂ and 37°C (Control inserts). The heavy density was used to simulate fully developed cellular/tissue constructs which was the primary motivation for this project. Conservatively, we chose a 48 hour simulated shipping procedure as our overnight shipping process for the cell lines. We used Caco2, A549 and HepG2 C3A cell lines for our demonstration because they represent different tissues; Caco2 represents GI tract cells, A549 represents lung cells and HepG2 C3A represents liver cells. Cell viability was above 97% for all three cell lines whether they were exposed to the simulated shipped procedure or in the control group (normal incubation). There was no significant difference observed between the viability of the two groups for any of the cell lines. This experiment demonstrated that the transporting device was capable of sustaining cell lines over a 48 hour period without an incubator.

The second series of experiments used Caco2, A549 and HepG2 C3A cell lines to test whether we could use the transporting device to ship sub-confluent cultures, support cell division and see how the device compared to a room temperature control. The Day 2 or Day 3 viability for the shipped and the room temperature groups was significantly lower than the incubated controls. There was only one RT group (A549) that was recorded as having a cell viability that was less than the initial plating concentration. The cell lines at sub-confluent states appeared to handle the experimental conditions differently suggesting that some cell lines are more sensitive to conditions, such as pH and temperature changes. Also, as expected the experimental conditions do not support proliferation as well as the incubated controls. This experiment demonstrated that the transporting device was capable of sustaining cell lines without an incubator. The transporting device was also better than the RT control and does have the capability of being shipped without leaking.

Next, a blood brain barrier construct was tested to determine if the transporting device could maintain barrier integrity in a more biologically complex tissue construct. The criteria that we used to determine our success was viability, TEER and the presence of tight junctions. The 24 hour viability of the blood brain barrier cells was above 97% whether they were shipped or maintained in the incubator. There was no significant difference observed between the viability of the two groups. The TEER comparison showed some group differences. Immediately after shipping, the initial TEER readings of the shipped inserts were significantly lower than the TEER of the control inserts. The TEER of the shipped inserts remained significantly lower than the control after 1 hour of incubation but increased thereafter. After 24 h in the incubator the TEER of the shipped inserts recovered enough so that it was not significantly different than the controls. This observation was encouraging because it meant that the barrier was capable of recovering its integrity after the shipping process and equilibration in the incubator. The immunostaining of the tight junctions (ZO-1 and claudin-5) after 24 h in the incubator showed continuous networks outlining the contours of the brain microvascular endothelial cells in both the shipped and the control groups. This indicated that well-organized tight junctions were present and confirmed the capability of the transporting device.

After the successful results from shipping the complete cell line constructs and blood brain barrier constructs, we used our shipping device to receive skin constructs at Cornell from Columbia University²⁹. This method was very beneficial because we did not have to endure the complications of physical transporting the skin constructs which we had done previously. This observation suggests that the process can be generalized.

Conclusion

In conclusion, this transporting device has made it possible to ship intact cell/tissue constructs on transwell inserts from one facility to another. Subconfluent transwell cultures could be shipped as well however a decrease in proliferation rates and some death may be observed depending on the type of cultures that are being used and the culture's sensitivity. Since many labs are currently constructing special tissue engineered constructs, the ability to successfully ship transwell inserts with complete cell/tissue constructs can promote and support collaborations between these labs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A Transwell insert for
6 well plate

B Transwell transporting device for a
transwell from a 6 well plate

C Comparison of the transwell transporting
device for the inserts of a 24 well plate vs. a
6 well plate

D

Description	Location of Measurement	Transporting Device for 24 well plate (mm)	Transporting Device for 6 well plate (mm)
Base Inner Diameter		15 mm	35 mm
Base Inner Height		30 mm	30 mm
Supporting Lip Inner Diameter		20 mm	40 mm
Supporting Lip Inner Height		10 mm	10 mm

Figure 1.

Design of the Transporting Devices. (A) Photograph of a transwell insert for a 6 well plate. (B) Photograph of a transporting device for a transwell for a 6 well plate. The device consists of (i) a threaded polycarbonate lid with a vitron o-ring to prevent leaking and secure the transwell and (ii) a polycarbonate container (base) that supports and suspends the insert in medium. (C) Photograph comparing transporting devices. A comparison of the transwell transporting device for the inserts of (i) a 24 well plate vs. (ii) a 6 well plate. (D) Important dimensions of the transporting device for the inserts of a 24 well plate vs. a 6 well plate.

Figure 2. Schematic view (Solid Work's diagram) of the assembly of the transporting device for the transwell inserts. (A) Medium is pipetted into the bottom of the base of the transporting device. The transwell insert is placed into the base containing medium. The transwell insert is suspended in the base of the transporting device. More medium is added into the insert and to fill the base. (B) A threaded polycarbonate lid with a vitron o-ring is screwed down to secure the transwell in place and (C) Diagram shows the secured transwell within the transporting device.

Figure 3.

Forty eight hour viability of cells grown on inserts in the transporting device (shipped) vs. incubated controls. Three cell lines (Caco2, A549, and HepG2-C3A) were grown on transwell inserts for 5 days, transported in the transporting device or maintained in the humidified incubator (at 5% CO₂ and 37°C) for 48 hours, and then stained with Invitrogen's Live/Dead Stain. Fluorescent micrographs of 48 hour viability of cells grown on transwell inserts or in the transporting device. Scale bar = 100μ.

Figure 4.

TEER values from BBB constructs grown on transwell inserts before and after shipping vs. controls to show barrier tightness. Cocultures of hiPSC-derived BMECs and astrocytes were shipped in the transporting devices with overnight delivery or remained in a cell culture incubator. All samples were equilibrated in the incubator for 24 hr after shipping.

Comparison of TEER values of BBB constructs that were transported to the University of Central Florida from Cornell University vs. control BBB constructs that were maintained in a humidified incubator (at 5% CO₂ and 37°C) at Cornell University. Values are means + SEM. (n = 6 – 9). Significant differences (p<0.05) using a two tailed Student t-test between the shipped group versus the incubated control are indicated with an asterisk (*).

BBB Control - Bottom

BBB Shipped - Bottom

BBB Control – Top

BBB Shipped - Top

Figure 5.

Twenty-four hour viability of the BBB constructs grown on snapwell inserts in the transporting device (shipped) vs. controls. The BBB constructs were grown on snapwell inserts, shipped in the transporting device or maintained in the humidified incubator (at 5% CO₂ and 37°C) for 24 hours, and then stained with Invitrogen's Live/Dead Stain. Fluorescent micrographs of 24 hour viability of cells grown on snapwell inserts (top and bottom surfaces of the membrane) in the incubator or in the transporting device are shown. Scale bars = 100μ.

Figure 6. Barrier integrity of the BBB constructs post shipping. Cocultures of hiPSC-derived BMECs and astrocytes were shipped in the transporting devices with overnight delivery or remained in a cell culture incubator. All samples were equilibrated in the incubator for 24 hr after shipping. Representative fluorescent images reveal continuous networks of tight junction proteins in both the shipped and control samples. Red, ZO-1; Green, claudin-5; Blue, DAPI nuclear stain. Scale bar = 50 μ m.

Table I

Viability of Fully Developed Cellular Constructs

Cell Line Tested	% Viable + SEM
Caco2	
Control	99.28 ±0.02
Shipped	98.3 ±0.76
A549	
Control	97.65 ± 1.02
Shipped	97.49 ± 1.19
HepG2 C3A	
Control	98.75 ±0.51
Shipped	97.47 ±0.68

Table II

Viability of Sub-Confluent Constructs

Cell Line Tested	Total Viable Cells Initial vs. Final		% of Control
Caco2	Control	2×10 ⁴	(Day 2) 5.1±0.4×10 ⁴ 100%
	Shipped		*3.6±0. 1×10 ⁴ 70%
	Room Temperature		*3.1±0.2×10 ⁴ 61%
A549	Control	2×10 ⁴	(Day 3) 2.4±0.4×10 ⁵ 100%
	Shipped		*5.3±0.2×10 ⁴ 22%
	Room Temperature		• *6.7±0.5×10 ³ 2.8%
HepG2 C3A	Control	2×10 ⁴	(Day 3) 6.6±0.2×10 ⁴ 100%
	Shipped		*4.6±0.3×10 ⁴ 69%
	Room Temperature		*3.2±0.3×10 ⁴ 48%

Table III

Viability of BBB Constructs

BBB Tested	% Viable + SEM
Control	
Bottom	98.037 ±.003
Top	97.519 ±.007
Shipped	
Bottom	97.425 ±.002
Top	97.193 ±.007