Technique of Human embryonic stem cell (hESC) Research

Ma Hongbao *, Yang Yan *, Sun Yiwu *, Magaret Ma **

* Brookdale Hospital, Brooklyn, New York 11212, USA, ma8080@gmail.com
** Boston, Massachusetts 02138, USA

Abstract: The definition of stem cell is "an unspecialized cell that gives rise to a specific specialized cell, such as a blood cell". Embryonic stem cells are derived from the inner cell mass of blastocyst stage embryos. Somatic stem cells differentiate into only the cells the specific tissue wherein they reside. Stem Cell is the original of life. All cells come from stem cells.
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Abbreviations:
ESCROs - Embryonic Stem Cell Research Oversight Committees
HCT/P - human cells, tissues, organs, and cellular and tissue-based products
hESC - human embryonic stem cell
MEF - mouse embryonic fibroblasts
MEF-CM - MEF - conditioned medium
NAS - National Academy of Sciences
NIH - National Institutes of Health

Introduction

Scientific researches in human embryonic stem cell (hESC) and social concerning make it imperative to look the expected use of hESC for transplantation. According to the social concerns, donation is important. The special ethical concerns in clinical trials of hESC transplantation and their implications are related to stem cell therapies and social affair.

hESC researches are increasing very fast, but create the ethical debate, which has focused on the moral status of the embryo and the acceptability of using embryos for research purposes. This is a complex philosophical question. Is an embryo an alive baby? This has not been resolved, and the issue is problem for the hESC research.

The hESC transplantation is still a critical question, and should be problem in a certain future. Crucial issues concerning safety of hESC transplantation are serious. The donors of materials used to derive new hESC lines are concerned. Failure to address the ethical issues in stem cell application may create biological questions, social problems, even the serious legal affairs.

1. Scientific, ethical, and policy context of hESC studies

Scientific discoveries in stem cells are come our every day. New hESC lines are needed if hESCs or their products are to be used for transplantation into humans. The twenty or so hESC lines approved for federally funded studies in 2001 in USA by US President George W Bush were derived using nonhuman feeder cells and serum and express the nonhuman antigen Neu5Gc. Thus, they could be immunologically rejected by the recipients unless this problem is remedied. Derivation of new hESC lines will be stimulated by $3 billion in funding for stem cell research authorized by California State of the United States in 2004. This measure will give priority to funding research that cannot be funded by US National Institutes of Health (NIH), which is currently the case for derivation of new hESC lines. Other states of America and private funders have followed suit in providing nonfederal support for hESC research. Outside of the US, hESC research is advancing vigorously. In May of 2005, researchers from South Korea reported the derivation of 11 hESC lines using somatic cell nuclear transfer, demonstrating that technical obstacles to developing such stem cell lines can be overcome more readily than expected. In turn, such findings will stimulate further studies.

hESC lines are important in stem cell application. Current ethical guidelines for hESC studies focus on the derivation of new hESC lines. There are many guidelines approved in the world, such as, in 2005, the US National Academy of Sciences (NAS) panel called for voluntary adoption of ethical guidelines in hESC studies. Their recommendations included institutional oversight of hESC studies protocols through Embryonic Stem Cell Research Oversight Committees (ESCROs), informed consent from donors of materials for new hESC lines, restrictions on payment

to gamete donors, and guidelines for banking stem cells and documentation. A lot of news on stem cells is released every day. An event is that 23 NRC recommendations have been endorsed by scientific organizations and adopted as interim regulations for research funded by the state of California State. In the year 2005, the US FDA issued regulations on screening and testing donors of human cells, tissues, organs, and cellular and tissue-based products (HCT/P). These initial efforts do not address crucial ethical issues in clinical applications of hESC transplantation, which have important upstream implications for how hESC lines should be derived, as well as for the conduct of the trials themselves. The ethic concerns and legal questions are existed in the entire world, not only America. We need to be very carefully in the stem cell research, which not only for science concerning but also for social concerning.

2. Protection of participants in donors

The safety of the stem cell studies is seriously concerned. Especially, how to protect the donor participants is important. It must be not harmful. In the transplant, no harmful genetic change must be paid attention to. Transmission of infectious agents or serious genetic conditions should be avoided. Potential cancer problem should be paid attention to. Any research project must concern the protections against diseases transmitted through hESC transplantation.

As the serious genetic diseases could be transmitted in the transplantation, the careful diagnosis must be done before the transplantation.

Stem cell is a kind of cells that has many similar properties to the cancer cells. In a certain case, the cancer may be developed in the transplantation. The cancer problem must be carefully concerned. The responsibility to protect hESC transplant recipients from harm must be balanced against a responsibility to respect donors and protect their confidentiality.

Because of the public interest in hESC research, it would be important for researchers to develop stringent mechanisms, extending beyond those employed in routine clinical care, in order to assure donors that their identity and contact information remain protected. Human factors in breaches of confidentiality should be considered.

The donors' health condition and genetic information should be paid attention to when the stem cells collection is scheduled.

3. Agreement for recipients of hESC transplantation

Problems could be come out with informed consent commonly that occurs in clinical trials. It is important to offer the clear information to the patients in any clinical trials. Participants receiving hESC transplantation could overestimate the benefits and underestimate the risks. Some measurements may reduce the therapeutic misconception in recipients of hESC transplantation. Researchers need to communicate the distinction between the long-term hope for such effective treatments and the uncertainty inherent (Bernard, 2005).

4. Examples of the Human Embryonic Stem Cell experimental Methods (experiment techniques) (http://stemcells.nih.gov/research/training)

There are different types of stem cells. hESCs are pluripotent cells that can differentiate all three germ layers. hESCs are important in human body development. The maintenance and differentiation of hESCs will form the basis for significant research in human cell and developmental biology, and in the potential clinical application of cell replacement therapies. Because of the inherent capacity for differentiation, the maintenance of undifferentiated cultures of hESCs is not as simple as growing other types of mammalian cells. It is a difficult task to keep stem cells from differentiation.

To offer a reference to the readers in the stem cell research, the following collects and describes some technique examples on hESC studies. Thank all who did the original contributions in the techniques and methods in stem cell researches. All the tools and reagents in this experiment are sterile.

4.1 BresaGen's hESC lines

BresaGen is important in stem cells researches. As the important event for stem cell researches, in 2001, BresaGen isolated four hESC lines that qualified for US NIH funding under the criteria outlined by US President George W Bush (Mitalipova et al. 2003). The three cell lines BG01, BG02 and BG03 have been successfully recovered, expanded and are listed on the NIH registry (http://stemcells.nih.gov/index.asp). However, the BG04 cell line is not successfully recoverable and this cell line is removed from the NIH registry (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi).

4.2 hESC culture protocol

hESCs can be maintained on a layer of mouse embryonic fibroblasts (MEF) for 24 h. In the media it consists either 20% KSR hESC medium (DMEM:F12, 20% KSR, 4 ng/ml bFGF, 2 mM glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin and 50 µg/ml streptomycin, 0.1 mM ß-Mercaptoethanol (ß-ME)) or 20% KSR hESC medium conditioned by MEFs. This medium is called MEF - conditioned medium (MEF-CM).

- MEF-CM is used as small amount for a minimum requirement, but never eliminates the MEF-CM.
- Always pay attention to the spontaneous differentiation of pluripotent stem cells carefully.
- hESC cultures are split primarily under sterile condition using microdissection passage.
- Sterile condition is always important in stem cell processing.
- Using a dissecting microscope to observe the cells within a laminar flow cabinet treated by UV light. UV light is a good method to kill bacteria.
- Cultures are kept on MEFs (either high or low density), which results in different colony morphologies. hESCs grow as domed colonies on high density MEFs, and colonies are flat and individual cells exhibit prominent nucleoli on low density MEFs.
- Both these colony types express pluripotent markers and differentiate effectively as embryoid bodies.
- Check cell condition routinely.
- hESC is expanded with EDTA-free trypsin solution.
- Differentiated cell colonies are removed prior to each passage to make sure that all the cells in the experiment are the general undifferentiated state cells.
- After then, the MEF feeder layer is removed by peeling it away from the hESC colonies.
- MEFs are plated onto dishes that have not been coated with gelatin.
- In the experiment, the MEFs and MEF-CM are prepared several days before it is used.
- MEFs are prepared using standard procedures, or purchased from commercial companies such as Specialty Media (www.specialtymedia.com), or Stem Cell Technologies (http://www.stemcell.com/default.asp).
- To culture hESCs at high density, with ~200-250 colonies per 35 mm dish and ~500 colonies per 60 mm dish (when high density MEFs are used).

4.3 Isolation of Primary Mice Embryo Fibroblasts

Mouse embryo fibroblast is important in hESC culture. Here it gives a description of the isolation of primary mice embryo fibroblasts:

- Pregnant mice are anesthesia.
- Place the mice body on a bench and spray with 70% ethanol on the body skin for sterile purpose.
- Use scissors to make a cut through the belly skin.
- Carefully dissect out the uterus with sterile scissors and forceps and place it into a Petri dish containing sterile PBS.
- Be careful to keep the mouse body in a no damaged condition.
- Isolate the mouse embryos from the uterus, release the embryos from the embryonic sacs, and transfer the embryos to a second Petri dish containing sterile PBS.
- Remove the mouse embryo heads, livers, kidneys, lung, stomach, intestines, heart, etc., and all viscera organs.
- Transfer the mouse embryo to a fresh Petri dish. Mince the embryos with a sterile scalpel blade or scissors.
- Add 5.0 ml of 0.25% trypsin/EDTA per 10 fetuses, and triturate through a 10 ml pipette.
- Transfer the trypsin treated tissue solution with a 10 ml pipette from the Petri dish to the barrel of a 10 ml syringe with an attached 16-20 g needle.
- Replace the syringe plunger, invert and expel air, and then slowly, gently push the trypsin treated tissue solution through the needle, and collect in a 50 ml centrifuge tube.
- Gently pass the trypsin treated tissue suspension through the needle again.
- Incubate the tissue suspension solution for 15 min at 37°C. Add an equal volume of complete MEF medium to inactivate the trypsin activity.
- Triturate the suspension vigorously with a 10 ml sterile pipette.
- Add the complete MEF medium to make up a final volume of 30 ml per flask.
- This density allows the cells to adhere but not become overly confluent before harvest at day 3.
- Incubate at 37°C with 5% CO_2.
- The dissociated MEFs will attach to the flask and begin to divide overnight.
- Replace the medium the next day with an equal volume of fresh complete MEF medium.
- After 3 days, when the cells are nearly confluent, the MEF p0 cells are ready to be frozen.
- Rinse the cells once with Ca^{2+}/Mg^{2+}-free PBS. Detach the cells by 3 ml 0.05% trypsin/EDTA solution per flask.

- Inactivate the trypsin solution by adding 5 ml complete MEF medium.
- Pool cells from the various flasks and count the number of viable cells using trypan blue exclusion and a hemocytometer immediately.
- Centrifuge the cell suspension solution at 23°C for 4 min at 200g.
- Aspirate medium and resuspend the cells in complete MEF medium at 2x the desired final freezing concentration (about $2.4x10^7$/ml).
- Add an equal volume of fetal bovine serum with 20% DMSO to get 1x final stocks. Dispense 1 ml per cryovial and freeze.
- Each vial contains about cell 1.2 x 10^7/ml in MEFs.
- Freeze cells using the NALGENE™ Cryo 1°C Freezing Container.
- The container is stored at room temperature and filled with 250 ml of isopropanol.
- Cryovials are placed in the freezing container, and the container is then placed in a -70°C freezer overnight. This procedure ensures that a -1°C/min rate of cooling is achieved, a step critical for retaining cell viability.
- After an overnight incubation, cryovials are removed from the freezing container and placed in liquid nitrogen (-196°C) for the long-term storage.

4.4 Thawing and preparing p1 MEF feeder plates

- Work in a laminar flow cabinet.
- The cabinet is steriled by UV light before the experiment.
- Off the UV light when start the work in the cabinet, as UV light is harmful for human, especially skin.
- Thaw the cultured cells as a suitable number.
- Using gelatin coated plates for plating MEFs.
- Store the frozen stocks of MEF p0 in -70°C freezer fort the storage.
- Thaw cells quickly in a 37°C water bath, with gently shaking.
- Transfer the cells gently to a 50 ml sterile centrifuge tube.
- Add 10 ml of complete MEF medium dropwise, slowly with swirling.
- Add another 10 ml of medium and centrifuge at 200g for 4 min.
- Resuspend the cells in 50 ml complete MEF medium.
- Count viable cells using trypan blue exclusion. Viability is usually >95%.
- Plate cells into flasks at $3x10^6$ cells per flask with 30 ml medium per flask.
- Flasks are incubated at 37°C, 5% CO_2.
- Three days after thawing, put the cells in 95% confluent in flasks.
- Check each flask microscopically to assure cell growth and sterility (no bacteria growth in the flask).
- Aspirate the medium and treat the cells with 10 µg/ml mitomycin C, 16 ml per T175 in complete MEF medium for 3 h at 37°C, 5% CO_2 incubator.
- After the treatment, aspirate the mitomycin C.
- The cells receive a total of 5 washes after mitomycin C treatment.
- WASH 1: Wash the cells with 20 ml Ca^{2+}/Mg^{2+}-free PBS. Lay flask down and rock gently to wash the cells. Aspirate the PBS.
- Add 3 ml of 0.05% trypsin/EDTA.
- Disperse the trypsin by tilting and tapping the flask.
- When the cells detach from the flask (normally after 2 min), add 5 ml MEF medium to each flask to neutralize the trypsin.
- Pipette the medium 3 times across the flask to wash off remaining adherent cells.
- Pool cell suspensions into a 50 ml tube (up to 6 flasks can be pooled into one 50 ml centrifuge tube).
- Use 10 ml complete MEF medium to rinse all the flasks, and use this rinse to bring the pooled cell suspensions to 50 ml per tube.
- WASH 2: Centrifuge cells at 200g for 4 min.
- WASH 3: Aspirate and resuspend the cell pellets with 10 ml complete MEF medium. Cell pellets can be pooled at this time.
- After pooling, rinse the empty tubes with 5 ml complete MEF medium and add to the pooled tubes.
- Bring each tube to 50 ml with complete MEF medium. Centrifuge cells at 200g for 4 min.
- WASH 4: Aspirate and resuspend the cell pellets with 10 ml, triturate to resuspend cells, then bring each tube to 50 ml with complete MEF medium. Centrifuge cells at 200g for 4 min.
- Resuspend in complete MEF medium. Count with trypan blue exclusion using a hemocytometer.
- If the cell suspension is too concentrated to count accurately, dilute a sample appropriately and recount.
- Adjust the cell suspension to the desired concentration with complete MEF medium and plate out to generate inactivated MEF feeder layers.

- The MEF plates are cultured at 37°C, 5% CO_2 at least 24 h prior to use.
- The cell density for hESC cultures could be $2x10^5$ - $2x10^6$ cells per 35 mm tissue culture dish.

4.5 MEF- Conditioned Medium (MEF-CM)

- MEF-CM is the conditioned medium of MEF.
- First, plate $4x10^6$ mitomycin C treated MEFs cells in a T75 Flask coated with 0.5% gelatin, in complete MEF medium.
- Incubate for 24 h at 37°C, 5% CO_2.
- 24 h after the above, replace the MEF medium with 37.5 ml of 20% KSR hESC medium containing 4 ng/ml bFGF, and incubate for 24 h at 37°C, 5% CO_2 Again.
- Collect MEF-CM from the flasks after 24 h and use the 0.22 μM filter to sterilize the cells.
- Add fresh 20% KSR hESC medium containing 4 ng/ml bFGF to the flasks.
- Collect MEF-CM for up to 7 days using.
- L-Glutamine (2 mM as the final concentrition) and bFGF (4 ng/ml as the final concentrition) are added back prior to using MEF-CM with hESCs.
- ß-Mercaptoethanol (ß-Me) is added (to 0.1 mM as the final concentration) each day to keep the reduction environment.
- All are under sterile condition.

4.6 Cell Microdissection Passaging

- Use Microdissection passaging to maintain undifferentiated hESC cultures.
- The undifferentiated cells are identified morphologically and specifically excised for passaging.
- The culture can be viewed and manipulated.
- The choice of equipment and environment will be site specific.
- Biosafety cabinets with incorporated dissecting microscopes are available, but the use of open front laminar flow cabinets, or a designated clean room with dissecting microscopes may be appropriate.
- hESCs grow in the best condition when plated at high density.
- 200-250 colonies per 35 mm dish and ~500 colonies per 60 mm dish will be good.
- Fewer colony pieces can be plated when using low density MEFs (~150 colonies per 35 mm dish), as hESC generate flatter, larger colonies under these conditions.
- After 4-6 days of culture the colonies should be ~1 to 2 mm in diameter when grown on high density MEFs.
- When grown on low density MEFs, colonies should be ~3 to 5 mm in diameter after 5-7 days.
- Ideally, colonies should be spread evenly over the dish, with minimal overlap.
- Microdissection passaging requires (a) the identification and discrimination of undifferentiated and differentiated hESC colonies and (b) the capacity to excise the undifferentiated cells and transfer them to a new plate.
- Undifferentiated colonies exhibit the following morphological characteristics.
- On high density MEFs: hESCs grow as uniform domed colonies.
- Individual cells have phase bright edges.
- As the colonies get larger, a central depression can form, giving the colony a saucer-like shape.
- On low density MEFs: hESCs grow as uniform flat colonies.
- The cells exhibit prominent nucleoli and cell-cell edges are not phase bright.
- Differentiation is often observed as visible structure or organization, or it exhibits a different shade, within a hESC colony.
- Colonies or regions of colonies with overt differentiation are avoided upon passaging.

4.7 Morphology of hESC Cultures.

- Warm an appropriate amount of MEF-CM (or 20% KSR hESC medium) to 37°C.
- Add freshly thawed 1 M ß-Me to a final concentration of 0.1 mM.
- Set the hESC plate on a dissecting microscope in a biosafety cabinet or laminar flow.
- Assess the culture to identify undifferentiated colonies or colony regions from differentiated colonies.
- Use a fine pointed tool to grid the undifferentiated colonies.
- hESC colonies are typically ~1-2 mm diameter when grown on high density MEFs, and 3-5 mm when grown on low density MEFs.
- Use the needle or pipette tip to lift the colony pieces from the dish or feeder layer.
- Repeat this for all the undifferentiated colonies in the dish.
- Use a pipette to transfer the pieces to another MEF plate, or split between plates as appropriate.

4.8 Cell Passaging

- hESC cultures can also be expanded using several different bulk passaging methods.
- It is important to be careful to avoid disaggregation to single cells during passaging, as hESC have a low viability when split to single cells.
- All tools are sterile.
- Generating clumps of ~10-100 cells appears to be effective when do the passaging.
- Use hESC maintained on high density feeders.
- The MEFs are removed during passaging by peeling them away from the hESC colonies.
- hESCs grow best when plated at high density and we typically aim for 200-250 colonies per 35 mm dish and ~500 colonies per 60 mm dish.
- After around 5 days of culture the colonies should be about 0.5 - 2 mm in diameter when grown on high density MEFs.
- Colonies should be spread evenly over the dish, with minimal overlap.
- Warm appropriate amount of MEF-CM to 37°C.
- Once media is warm, add freshly thawed 1M ß-Me to a final concentration of 0.1 mM.
- Unused media is discarded at the end of the day, so only add ß-Me to the amount required for that day.
- Set the hESC plate on a dissecting microscope in a biosafety cabinet or laminar flow so that it is comfortable to see the colonies.
- Assess the culture to identify overtly differentiated colonies within the plate of undifferentiated cells.
- Cut out any overtly differentiated colonies with a fire drawn pipette needle, or a 21G 1½ needle, and remove them with a P1000 pipette.
- Aspirate the plate and add 0.05% EDTA-free trypsin (1 ml for a 35 mm dish, 2 ml for a 60 mm dish) and leave for 30 seconds.
- Remove the MEF layer using flamed watchmakers forceps.
- Take hold of the side of the MEF layer and gradually peel the MEF layer from the dish.
- The MEFs will pull off as a mesh, taking some of the hESC colonies.
- Discard this layer, most of the hESC colonies are left attached to the dish.
- Large colonies should be scored with a needle as per microdissection passaging. Break up the undifferentiated hESC colonies using a tip.
- Pass the tip back and forth across the dish, scraping the entire surface. This will break the colonies into pieces.
- While viewing under a dissection microscope, gently triturate the clumps (3-4 strokes) using a pipetteman and a 1000 µl tip until clumps consist of ~10-100 cells.
- Transfer the cell suspension into a 15 ml centrifuge tube containing 9 ml 10% FCS in DMEM/F12.
- Spin cells at 1000 rpm (200g) for 4 min at room temperature.
- Gently aspirate media and flick tube to loosen cells from the bottom.
- Gently resuspend the cells in the appropriate amount of MEF-CM for as many plates as you are preparing, with a 5 ml or 10 ml serological pipette.
- Aspirate the medium from the new plates of mitomycin C treated MEFs and dispense the hESC suspension.
- Place the plate into an incubator set at 37°C with 5% CO_2, and mix gently to spread the clumps out evenly.
- Feed cells the next day and every second day there after.
- Observe cells every day and passage by the above protocol whenever required (about 5 days).
- Overgrowth of the culture will result in increased differentiation.

4.9 Cell Cryopreservation

- hESCs do not survive well when passaged as single cells and are therefore routinely passaged as small clusters or clumps of cells.
- It is not uncommon to only have 5-20 colonies recover from a hESC thaw.
- Collect cell suspension as per routine hESC passaging
- Pellet the cells at 200g (1000 rpm) for 4 min at 23°C and aspirate the supernatant. Pre-label the appropriate number of cryovials.
- During the spin, prepare the freezing solutions: Solution I: 50% MEF-CM, 50% FCS Solution II: 80% MEF-CM, 20% DMSO.
- Be as gentle as possible after this point. Resuspend the pellet in 250 µl Solution I per vial to be frozen. Transfer the cell solution to a 50 ml tube so that the cells can be mixed by swirling during the procedure.
- Add 250 µl Solution II per vial to be frozen, dropwise, over 2 min with gentle swirling.
- Dispense 500 µl aliquots into pre-labeled cryovials.

- We freeze cells using the NALGENE™ Cryo 1°C freezing container.
- The container is stored at room temperature and filled with 250 ml of isopropanol.
- Cryovials prepared as described above are placed in the freezing container, and the container is placed in a -70°C freezer overnight. This procedure ensures that a -1°C/minute rate of cooling is achieved, a step critical for cell viability**.**
- After an overnight incubation, cryovials are removed from the freezing container and placed in liquid nitrogen (-196°C) for long-term storage.

4.10 Cell Thawing

- Perform the following steps as gently as possible.
- Remove the vial of hESCs from the liquid nitrogen storage tank.
- Thaw the cells by swirling gently in a 37°C water bath, wipe the vial with 70% Ethanol.
- Pipette the cells gently to a 50 ml tube, and add 10 ml MEF-CM dropwise over 2 minutes with gentle swirling.
- Transfer the cells to a 15 ml tube and pellet the cells at 200g for 4 min at room temperature.
- Aspirate the supernatant, resuspend the cells in 2 ml MEF-CM.
- Aspirate the medium on a 35 mm plate of $2x10^6$ MEF feeders, and plate the 2 ml of hESCs.
- Feed the cells the following day, and every 2 days after that. Visible colonies can often be observed within several days, but may appear later and only be ready for passaging up to two weeks after thawing.

4.11 Cell Karyotyping

- While the early passage BG01, BG02 and BG03 cells are determined, the karyotypic instability can sometimes be observed with long term passage of hESCs (Draper et al, 2004).
- Karyotype from 2x60 cm plates of hESCs grown on high density MEFs, although using fewer hESCs is possible.
- Cell cultures could be fed with 5 ml of MEF-CM the day before the procedure.
- The following day, add 10 µl KaryoMAX® Colcemid Solution directly to the plate (to make a final concentration of 0.02 µg/ml). Colcemid is toxic, wear gloves.
- Incubate 2 h at 37°C, 5% CO_2.
- Remove the culture medium containing colcemid.
- Add 1 ml 0.05% trypsin (EDTA free) to the plate.
- After 30 sec, use watchmakers forceps to peel off the MEFs.
- Use a tip to scrape all the hESCs off the plate, by passing it back and forth across the dish. Triturate the cell suspension and ensure cells have been split to primarily single cells.
- Transfer the cells to a 15 ml tube and add 10 ml 10% FCS in DMEM:F12.
- Spin cells at 200g (1000 rpm) for 4 min, at room temperature.
- Resuspend the pellet in 2 ml of warm (37°C) Hypotonic Solution and vortex for 10 seconds.
- Incubate in a water bath at 37°C for 30 min.
- Add 0.5 ml of freshly prepared Fixative**,** dropwise with swirling.
- Centrifuge cells at 200g for 4 min, at room temperature.
- Carefully aspirate the supernatant.
- Add 1 ml fixative dropwise, swirling the cells.
- Repeat steps 14-16 two more times.
- Store at 4°C until cells are sent for analysis.

4.12 Cell Shipments

- BresaGen Inc. routinely ships frozen vials of hESC in N2-cooled (-190°C) Dry shippers.
- There are different initial handling requirements for each format, but the receiving lab must prepare MEFs and MEF-CM in advance.
- The receiving laboratory should be prepared to place a high priority on the careful expansion of the hESCs and cryopreservation of stocks.
- To expect to succeed in maintenance of hESC cultures, the receiving laboratory should at a minimum have a member attend one of the hESC culture workshops (http://stemcells.nih.gov/research/training).

4.13. Cryopreserved vials

Cryopreserved vials are prepared according to section 2.6 and can be thawed according to section 2.7. A minimum of 2 vials are supplied per shipment.

4.14. T25 flask

- The flask will contain hESCs within 3 or 4 days of plating, and it is shipped overnight within the USA, in a warmed foam box.
- When the flask arrives, it should be aspirated and the cells fed with 5 ml fresh medium.

- The hESC should be incubated at 37°C until ready for passaging.
- To passage from the T25 flask, place the flask in a laminar flow cabinet and use a heated razor blade to cut a window in the top surface of the flask.
- Use microdissection passaging to split the hESC to new dishes.

4.15 Media Recipes and Solutions

- Complete MEF medium. DMEM with high glucose, 10% FBS, 2 mM glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin and 50 µg/ml streptomycin.
- 20% KSR hESC Medium. DMEM:F12, 20% KSR, 4 ng/ml bFGF, 2 mM glutamine, 0.1 mM nonessential amino acids, 50 units/ml penicillin and 50 µg/ml streptomycin.
- ß-ME. Dilute ß-Mercaptoethanol to a 1 M solution in water. Aliquot into small (10-50µl) aliquots and freeze at –20°C.
- Thaw a fresh vial each day and add to warmed medium at 1/10000 dilution.
- Hypotonic Solution (0.075M KCl). Add 5.6 gm of KCl to 1 L of distilled MQ water and stir until dissolved.
- Fixative: Methanol:Acetic Acid 3:1. Use fresh.
- Mitomycin C

4.16. Mitomycin C is highly toxic. Handle accordingly.

Dissolve 2 mg Mitomycin C in 200 ml complete MEF medium in a vial to generate a 10 µg/ml working stock. Store protected from light at 4°C, for up to 6 weeks or frozen at -20°C for longer storage. After inactivating MEFs, collect the used Mitomycin C solution add 15 ml bleach per 500 ml to neutralize it.

4.17 The Human Colony Forming Cell (CFC) Assay using Methylcellulose-based Media

The colony forming cell (CFC) assay, also referred to as the methylcellulose assay, is an *in vitro* assay used in the study of hematopoietic stem cells. The assay is based on the ability of hematopoietic progenitors to proliferate and differentiate into colonies in a semi-solid media in response to cytokine stimulation. The colonies formed can be enumerated and characterized according to their unique morphology.

5. Methylcellulose Assay

- Thaw aliquots of Methylcellulose-based Media and Cell Resuspension Solution at room temperature for approximately 30 minutes without disturbance.
- Cell resuspension solution is included in Catalog # HSC002, HSC003, HSC004, and HSC005 only.
- While the aliquots are thawing, resuspend the mononuclear cells in 10 ml of IMDM and count.
- Calculate the total number of cells by a cell count meter.
- Remove the supernatant and resuspend the cells in cell resuspension Solution to the desired stock cell number.
- The stock cell number is approximately 10x the final number needed for the experiment.
- Vigorously vortex the vial to thoroughly mix cells with the media.
- Wait for approximately 20 min before continuing to allow air bubbles to escape.
- Add 1.1 ml of the final cell mixture to a 35 mm culture plate using a 3 ml syringe fitted with a 16 gauge needle.
- Spread the media evenly by gently rotating the plate.
- Place two sample plates and an uncovered plate containing 3 - 4 ml sterile water in a 100 mm culture plate and cover.
- The sterile water plate serves to maintain the humidity necessary for colony development.
- Incubate the cells for 14 - 16 days at 37°C and 5% CO_2.
- Avoid disturbing the plate during the incubation period to prevent shifting of the colonies.

6. Colony Scoring

Score the colonies at the end of the incubation period. Identify and count the individual colonies using an inverted microscope and a scoring grid.

- Prepare the scoring grid as described in the Scoring Grid section.
- The diagram provided below can be used as a template to reproduce the scoring grid on a 100 mm culture plate.
- Mark the grid on a new 100 mm culture plate by placing the culture plate on the template and tracing the grid with a marker.
- Refer to the counting criteria section for guidance on how to identify and count colonies.

7. Counting Criteria

Colonies consisting of at least 40 cells are counted.

8. Culturing BG01V Human Embryonic Stem Cells on Irradiated Mouse Embryonic Fibroblasts (iMEF)

hESCs can be maintained on a layer of mitotically inactivated feeder cells such as irradiated mouse embryonic fibroblasts (iMEF, Catalog # PSC001). The protocol below has been used with the BG01V line of hES cells. Please note that other hES cell lines may require modifications of this protocol. Optimal culture conditions must be determined by the investigator for each hES line. Please read the protocol in its entirety before starting.

9. Reagent & Media Preparation

- **MEF Media** - MEF media consists of high glucose DMEM, 10% fetal bovine serum, 2 mM L-glutamine, and if desired, add a 1:100 dilution of penicillin/streptomycin (100x) stock.
- Filter sterilize the media using 0.2 µm sterile filter unit.
- **hES Media** - hES media consists of DMEM/F12, 15% fetal bovine serum, 5% Knockout serum replacer, 1:100 dilution of non-essential amino acids stock (100x), 1:100 dilution of penicillin/streptomycin (100x) stock, and 0.1 µM ß-Me.
- Filter sterilize the media using 0.2 µm sterile filter unit. Just prior to use, the media should be supplemented with recombinant human FGF basic at 4 ng/ml.

9.1 Reagent & Media Preparation Procedure

Note: When handling biohazard materials such as human cells, safe laboratory procedures should be followed and protective clothing should be worn.

I. Thawing and Plating of iMEF Feeder Cells (plate feeder cells one day prior to stem cell seeding)

- Coat the appropriate sized plate(s) for the desired number of cells by covering the surface of the dish with 0.1% sterile gelatin for 15 minutes.
- For example, one vial of 6 x 10^6 iMEF can be plated on two 100 mm dishes, six 60 mm dishes, or two 6 well plates.
- Warm MEF media to 37° C.
- Thaw the desired number of vials of iMEF cells by quickly warming the cryotube (s) in a 37°C water bath until the cells are just thawed and then immediately transferring the contents of one vial to a 15 ml conical tube containing at least 5 ml of pre-warmed MEF media.
- Rinse the cryotube with an additional 1 ml of media to ensure the removal of all the cells.
- Spin at 200g in a clinical centrifuge for 5 min.
- Remove the supernatant and gently flick the pellet.
- Aspirate the 0.1% gelatin from the plate(s).
- Resuspend the iMEF cells in MEF media and transfer to the gelatin-coated plates at a density of approximately 1 x 10^6 cells/60 mm plate.
- Incubate overnight in a 37°C, 5% CO_2 incubator before seeding with stem cells.

9.2 Thawing the BG01V hES cells

- Warm the hES media to 37°C.
- Place the cryovial of hES cells in a 37°C water bath until just thawed and then transfer the cells immediately to a 15 ml centrifuge tube containing at least 5 ml of pre-warmed hES media.
- Rinse the cryovial with an additional 1 ml of media to ensure the removal of all the cells.
- Spin at 200 x g in a clinical centrifuge for 4 minutes.
- Remove the supernatant and gently flick the pellet.
- Resuspend the pellet in an appropriate amount of hES media freshly supplemented with 4 ng/ml of recombinant human FGF basic (typical volume is 5 ml per 60 mm plate).
- Remove the MEF media from a plate containing iMEF cells and add the hES cell suspension. Typically 1 x 10^6 hES cells are thawed for a 60 mm plate.
- Place the cells in a 37° C, 5% CO_2 incubator.
- Cells should be fed daily with hES media freshly supplemented with recombinant human FGF basic.
- Passage the cells before the hES colonies touch at their edges.

9.3 Passaging of the BG01V hES cells

Note: Plate iMEF feeder cells on the desired number of plates 1 day prior to passaging. Warm the hES media to 37° C.

- Remove the hES media from BG01V cells. Add 1 ml of Accutase solution to each 60 mm plate.
- Incubate at room temperature for 5 - 10 min or until cells begin to slough off the plate.
- Pipette Accutase gently over the plate until all the cells have been detached.
- Gently pipette cell suspension up and down to break up large cell clumps.

- Transfer the cell suspension to a 15 ml centrifuge tube containing 5 ml of hES media and spin at 200 x g for 4 min.
- Remove the supernatant and gently flick the pellet.
- Resuspend the pellet in pre-warmed hES media and count the cells using a hemocytometer.
- Plate the desired number of cells (approximately 0.5 - 1.0 x 10^6 cells/60 mm plate) on the iMEF monolayer in hES media supplemented with 4 ng/ml of recombinant human FGF basic.
- Feed the cells daily with hES media freshly supplemented with recombinant human FGF basic.

Correspondence to:
Ma Hongbao, PhD
Brookdale Hospital,
Brooklyn, New York 11212, USA,
ma8080@gmail.com

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