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Study of Gap Junctions in Human Embryonic Stem Cells

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Abstract

Gap junctional intercellular communication (GJIC) has been described in different cell types including stem cells and has been involved in different biological events. GJIC is required for mouse embryonic stem cell maintenance and proliferation and various studies suggest that functional GJIC is a common characteristic of human embryonic stem cells (hESC) maintained in different culture conditions. This chapter introduces methods to study gap junctions in hESC, from expression of gap junction proteins to functional study of GJIC in hESC proliferation, apoptosis, colony growth, and pluripotency.

Keywords: Connexin, Gap junction, Gap junctional intercellular communication, Human embryonic stem cells

1 Introduction

Gap junctions are intercellular channels consisting of two connexons localized in the membrane of adjacent cells. Each connexon consists of six membrane proteins, termed connexins (Cx) (1, 2). Numerous molecules can diffuse through gap junction channels, including small ions, second messengers, amino acids, metabolites, short interfering RNA, and peptides involved in cross-presentation of major histocompatibility complex class I molecules (3–6). Such intercellular coupling is termed gap junctional intercellular communication (GJIC). GJIC is involved in various cellular mechanisms, including control of cell migration, proliferation, differentiation, metabolism, apoptosis, and carcinogenesis (4, 7–11). In human embryonic stem cells (hESC), various connexins have been found to be expressed: Cx25, Cx26, Cx30, Cx30.2, Cx30.3, Cx31, Cx31.1, Cx31.9, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx46, Cx47, Cx59, and Cx62 (12–14, 15). Among these connexins, Cx43 and Cx45 are highly enriched in undifferentiated hESC compared to its differentiated counterpart in many microarray studies, and the two connexins were identified as “undifferentiated hESC markers” (16). Moreover, Cx43 was identified as a downstream

target of key pluripotent transcription factors Oct4, Sox2, and Nanog (17). Undifferentiated hESC can communicate through functional GJIC, as determined by dye coupling (12–14) and ionic coupling (15). Finally, functional GJIC is a common characteristic of undifferentiated hESC maintained in different culture conditions, suggesting an understudied role of gap junctions in mediating hESC maintenance (13, 14). Studies of intercellular communication through gap junctions can potentially lead to novel methods to improve clonal survival and maintenance of hESC, which is fundamental to realize the therapeutic potential of these cells.

2 Materials

2.1 General (See Note 1)

1. hESC were cultivated in different formats depending on the experiments to perform. In all cases, we culture hESC with a feeder layer of mouse embryonic fibroblasts (MEF) supplemented with 20 % fetal calf serum or 20 % Knockout serum replacement (Invitrogen, #10828-028) plus 4 ng/ml bFGF (R&D, #233-FB-025/CF).
2. For reverse transcriptase-polymerase chain reaction (RT-PCR), western blot, cell proliferation assay, cell pluripotency assay, and colony growth assay, we culture hESC in center-well organ culture dishes, 60 $\times$ 15 mm style (35 mm culture dishes, Falcon, #353037).
3. Alternatively for western blot, we culture hESC with MEF in 6-well plates (Falcon, #353046).
4. For immunocytochemistry and SL/DT, we culture hESC in Lab-Tek Chamber slide w/cover, permanox slide sterile (8-well chamber slides, Lab-Tek, #177445).
5. TrypLE Express (Invitrogen, #12604).
6. Phosphate-buffered saline (PBS) 10×: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4.
7. Dispase (Invitrogen, #17105-041).
8. Ethanol and Methanol (Merck).
9. Tris base (Merck).

2.2 RT-PCR

1. RNase-free microtubes and pipette tips.
2. Dynabeads® Oligo $(dT)_{25}$ (Invitrogen, Dynal, #610.02, 610.05, 610.50) and Magnetic Particle Concentrator (Invitrogen, Dynal, #120.20D).
3. Lysis/binding buffer: 20 mM Tris–HCl (pH 7.5), 1 M LiCl, 2 mM EDTA.

4. Washing buffer A: 10 mM Tris–HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA, 0.1 % LiDS.
5. Washing buffer B: 10 mM Tris–HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA.
6. Superscript II Rnase H$^-$ Reverse Transcriptase (Invitrogen, #18064-014).
7. TAQ DNA polymerase (Biotech International, #TAQ-1).
8. dNTP mix.
9. Sense and antisense primers for Cx43 and Cx45: Cx43, sense 5′-ATGAGCAGTCTGCCTTTCGT-3′, antisense 5′-TCTGC TTCAAGTGCATGTCC-3′; Cx45, sense 5′-GGAAGATGGG CTCATGAAAA-3′, antisense 5′-GCAAAGGCCTGTAAC ACCAT-3′.
10. Agarose molecular biology grade (Scientifix, #9010B).
11. TAE buffer: 20 mM Tris–Cl, pH 7.8, 10 mM sodium acetate, and 0.5 mM EDTA.
12. Ethidium bromide (Sigma, #E1510).
13. MinElute Gel Extraction Kit (Qiagen, #28604).

2.3 Western Blot Analysis

1. Mini-protean 3 system (Biorad, #165-3301, 165-3302, 170-3930, 170-3935).
2. 30 % Acrylamide/Bisacrylamide solution 29:1 3.3 % C (w/v) (Biorad, #161-056): Toxic product that should be used with care under a fume hood.
3. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8.
4. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8.
5. Ammonium persulfate (APS, Biorad, #161-0700): Prepare fresh solution of APS 10 % in water.
6. TEMED (Biorad, #161-0800).
7. Laemmli sample buffer (Biorad, #161-0737) or reducing sample buffer: 0.1 M Tris–HCl (pH 6.8), 41.6 % (v/v) glycerol, 3.3 % (w/v) SDS, 0.02 % (w/v) bromophenol blue. Add 0.46 mM β-mercaptoethanol (Sigma, #M7154), 1 mM Sodium orthovanadate (Sigma, #S6508), and 1 mM Phenylmethylsulfonyl fluoride (PMSF, Sigma, #P7626) to the sample buffer prior to cell lysis.
8. Kaleidoscope prestained ladder (Biorad, #161-0324).
9. Running buffer 1×: 25 mM Tris base, 192 mM glycine, 0.1 % SDS (w/v), pH 8.3. Mix 3.03 g Tris base, 14.4 g glycine, and 1 g SDS, bring final volume to 1 l with distilled water, and check pH.

10. Transfer buffer 1×: 25 mM Tris base, 192 mM glycine, 20 % methanol, 0.1 % SDS (w/v), pH 8.3. Mix 3.03 g Tris base, 14.4 g glycine, 200 ml methanol, and 1 g SDS, bring final volume to 1 l with distilled water, and check pH.
11. Hybond-P PVDF membrane (Amersham Pharmacia Biotech #RPN 303F) or PVDF membrane (Biorad #162-0177) cut to the size of the gel or precut (ready gel Blotting Sandwiches, Biorad, #162-0219).
12. Bovine serum albumin (BSA) fraction 5, min 96 % (Sigma, #A4503).
13. Tris-buffered saline with Tween 20 (TBST) pH 7.6: 20 mM Tris base, 137 mM sodium chloride, 3.8 mM HCl, 0.05 % Tween-20 (Biorad, #170-6531). Mix 2.42 g Tris base, 3.8 ml HCl 1 M, and 0.5 ml Tween-20, bring final volume to 1 l with distilled water, and check pH.
14. Rabbit anti-mouse Connexin 43 affinity-purified polyclonal antibody (Chemicon, #AB1728).
15. Negative control Rabbit Immunoglobulin Fraction, Solid-Phase Absorbed (Dako, #X0936).
16. Mouse monoclonal anti-β-tubulin 1 (Sigma #T7816).
17. Polyclonal Goat Anti-Rabbit Immunoglobulins/Horseradish peroxidase (HRP) (Dako, #P0448).
18. ECL Plus (Amersham Pharmacia Biotech #RPN2132, RPN2133) or Pierce ECL western blot substrate (chemiluminescent detection reagent, Pierce #32209).
19. Hyperfilm (Amersham Biosciences) or Gel-Doc system (Biorad).
20. Western Blot Stripping Buffer (Pierce, #21059).
21. Ponceau S (Sigma, #P-3504).

2.4 Immunocytochemistry

1. Glass cover slips 22 × 60 mm.
2. Wax pen/liquid blocker.
3. Rabbit anti-mouse Connexin 43 affinity-purified polyclonal antibody (Chemicon, #AB1728).
4. Rabbit anti-Connexin 45 polyclonal antibody (Chemicon, #AB1745).
5. 6-Diamidino-2-phenylindole (DAPI, Sigma, #D9542) or Bisbenzimide H 33342 (Hoechst-33342, Sigma, #382065): Prepare a fresh solution at 1 μg/ml in water.
6. Mouse GCTM-2 antibody (gift from Prof M. Pera, University of Melbourne).
7. Mouse anti-Oct-3/4 C-10 antibody (Santa Cruz Biotechnology, Inc. #sc-5279).

8. Mouse TG-30 antibody, recognizing CD9 (gift from Prof M. Pera, University of Melbourne).
9. TRA-1-60 antibody (gift from Prof P. Andrews, University of Sheffield).
10. Negative control Rabbit Immunoglobulin Fraction, Solid-Phase Absorbed (Dako, #X0936).
11. Negative control Mouse IgM antibody (Dako, #X0942).
12. FITC-conjugated Swine Anti-Rabbit Immunoglobulins (Dako, #F0205) or Alexa Fluor® 488 goat anti-rabbit IgG (H + L) (Invitrogen, #A11008).
13. FITC-conjugated Rabbit Anti-Mouse Immunoglobulins (Dako, #F0261) or Alexa Fluor® 568 goat anti-mouse IgG (H + L) (Invitrogen, #A11004).
14. Vectashield (Vector Laboratories, # H-1000).
15. Nail varnish.

2.5 Scrape Loading/ Dye Transfer Assay

1. Ca^{2+}- and Mg^{2+}-PBS buffer (buffer 1): 140 mM NaCl, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, pH 7.35.
2. Lucifer Yellow CH dipotassium salt (Sigma, #L0144) to freshly dilute in buffer 1 (1 mg/ml).
3. Dextran, tetramethylrhodamine (Rhodamine dextran, Molecular Probes, #D1868) to freshly dilute in buffer 1 (1 mg/ml).
4. Surgical blades (Swann-Morton, #0201).

2.6 Chemical Closure of Gap Junctions

1. Phorbol 12-Myristate 13-acetate (PMA, Sigma, #P8139).
2. U0126 (Promega, #V112A).
3. α-Glycyrrhetinic acid (Sigma, #G8503).
4. Recombinant human BMP-4 (R&D, #314-BP-010).

2.7 Cell Apoptosis Assay

1. In situ Cell Death Detection Kit, Fluorescein (Roche, #11684795910), containing TUNEL reaction mixture.
2. 2 % (w/v) Paraformaldehyde (PFA) in PBS: Stock can be aliquoted and stored at −20 °C. Once thawed, aliquots should be used on the day and discarded.
3. 0.1 % Triton X-100 in PBS.

2.8 Cell Proliferation Assay

1. In situ Cell Proliferation Kit, FLUOS (Roche, #1810740), containing 10 mM BrdU, mouse anti-BrdU antibody conjugated to fluorescein.
2. 4 M HCl solution.
3. 70 % Ethanol.
4. Propidium iodide (Sigma, #70335).

2.9 Cell Pluripotency Assay

1. TRA-1-60 antibody (Santa Cruz, #sc-21705).
2. TRA-1-81 antibody (Santa Cruz, #sc-21706).
3. Goat serum (Invitrogen, #16210-046).
4. Goat anti-Mouse IgM antibody conjugated to Alexa fluor 488 (Invitrogen, #A21042).
5. Negative control Mouse IgM antibody (Dako, #X0942).

2.10 Image and Data Analysis

1. Inverted fluorescence microscope.
2. Western blot analysis: Scion image software (NIH) or Gel-Doc system (Biorad).
3. Statistical analysis: Graphpad Prism.
4. Cell proliferation, apoptosis, pluripotency assay: Flow cytometer (Moflo, DIVA, or FC500).
5. Colony growth assay: Analysis^B software (Olympus Software Imaging Solutions) or Adobe Photoshop.

3 Methods

3.1 Reverse Transcriptase-Polymerase Chain Reaction for Connexin mRNA Expression

1. Pre-warm dispase (10 mg/ml of culture medium) to 37 °C.
2. Harvest 10–12 day-8 hESC colonies by dispase treatment (10 min, 37 °C, *see* **Note 2**), and transfer cells to an Eppendorf tube.
3. Wash colonies four times with PBS. Centrifuge at 300 × *g* for 2 min to spin down cells.
4. Isolate poly-A+ mRNA using Dynabeads Oligo $(dT)_{25}$ adapted from the supplier's instructions (*see* **Note 3**). Using the magnetic particle concentrator, all Dynabead-binded mRNA are captured to one end of the tube, and the mRNA-free supernatant is discarded.
5. Add 300 μl of the lysis/binding buffer to hESC extracts in an Eppendorf tube and homogenize with a pipette until complete lysis.
6. Transfer 20 μl of homogenized Dynabeads into an Eppendorf tube, and place onto the magnetic particle concentrator for 2 min. Remove supernatant, resuspend the beads in 200 μl of lysis/binding buffer, place onto the magnetic particle concentrator for 2 min, and remove supernatant.
7. Mix lysed hESC with Dynabeads, incubate for 10 min at room temperature, then place onto the magnetic particle concentrator for 2 min, and remove supernatant.
8. Mix 400 μl of washing buffer A with the beads, place onto the magnetic particle concentrator for 2 min, and remove supernatant.

Table 1
Reverse transcription buffer for cDNA synthesis

	RT⁺	RT⁻	
Reagents	**Volume (μl)**	**Volume (μl)**	**Final concentration**
5× First Strand Buffer (Invitrogen)	4	4	1×
Dithiothreitol (DTT)	2	2	0.01 M
dNTP mix[a]	1	1	0.5 mM
Superscript II reverse transcriptase	1		200 Units
MilliQ	12	13	

[a]dNTP mix contained dATP, dCTP, dGTP, dTTP

9. To enhance the purity of the mRNA preparation, repeat **step 8** first with 400 μl of washing buffer A and then twice with 200 μl of washing buffer B.
10. Add 200 μl of washing buffer B to the beads, mix thoroughly, and separate into two Eppendorfs, each containing 100 μl of washing buffer B and beads. Place onto the magnetic particle concentrator for 2 min, and remove supernatant. Label one tube as +RT and the other tube as −RT.
11. Perform RT using Superscript II Rnase H⁻ Reverse Transcriptase, following the supplier's protocol. A negative control without the addition of reverse transcriptase (RT⁻) must be performed for each RNA sample to check the absence of contaminating genomic DNA. A 20 μl reaction volume is prepared as follows (Table 1) and added to the +RT and −RT tubes.
12. Incubate for 1 h at 42 °C, then place on ice, mix solution, place onto the magnetic particle concentrator for 2 min, and remove supernatant. Resuspend in 20 μl distilled/RNase-free water.
13. Perform PCR experiments using Taq DNA polymerase. A negative control (−RT) must be performed for each cDNA sample. A water control (no cDNA) should also be included. For each reaction a total volume of 25 μl is prepared as shown in Table 2. For the cDNA samples, homogenize the preparation (cDNA and beads) before pipetting. It is recommended to perform PCR immediately after RT.
14. PCR reaction is performed with the following conditions using the specific primers for Cx43 and Cx45: initial denaturation at 94 °C for 5 min, 35 cycles of "denaturation at 94 °C for 30 s, annealing at 55 °C for 2 min, and extension at 74 °C for 2 min," ending with a final incubation at 74 °C for 7 min.
15. Amplicons are sized by electrophoresis on 2 % (w/v) agarose gel stained with 0.001 % (v/v) ethidium bromide in TAE buffer.

Table 2
PCR reaction mix

	cDNA	−RT	Water control	
Reagents	Volume (μl)	Volume (μl)	Volume (μl)	Final concentration
10× Buffer	2.5	2.5	2.5	1×
dNTP mix	0.5	0.5	0.5	0.2 mM
Taq polymerase	0.25	0.25	0.25	0.25 Units
cDNA	3	0	0	
−RT	0	3	0	
Primer mix	2	2	2	2 μM
$MgCl_2$	1.5	1.5	1.5	1.5 μl
MilliQ	Make up to 25	Make up to 25	Make up to 25	

Table 3
SDS-polyacrylamide gel formulations

Percent of gel	MilliQ H_2O (ml)	30 % Acrylamide/Bis (ml)	Gel buffer (ml)	10 % w/v SDS (ml)
4 %	6.1	1.3	2.5[a]	0.1
10 %	4.1	3.3	2.5[a]	0.1

[a]Resolving gel buffer = 1.5 M Tris–HCl, pH 8.8
Stacking gel buffer = 0.5 M Tris–HCl, pH 6.8

16. Optional: Confirmation of the identity of amplicons: Excise DNA fragments of interest from the gels using a scalpel with the aid of a UV transilluminator; purify the amplified products using the QIAquick gel extraction kit following the supplier's instructions, and confirm the identity of the purified amplicons by DNA sequencing.

3.2 Western Blot Analysis of Connexin 43 Phosphorylation States: SDS-Polyacrylamide Gel Electrophoresis (See Note 4)

3.2.1 SDS-PAGE Gel Preparation

1. Western blot analysis is carried out using the Mini-protean 3 system, following the supplier's specifications.
2. Prepare a 10 % resolving gel and a 4 % stacking gel (Table 3). TEMED and freshly prepared 10 % (w/v) APS are to be added immediately prior to pouring the gel in order to catalyze polymerization.
   (a) Resolving gel: 50 μl 10 % (w/v) APS and 5 μl TEMED.
   (b) Stacking gel: 50 μl 10 % (w/v) APS and 10 μl TEMED.
3. Pour the 10 % resolving gel into a prepared gel cassette, leaving space for the stacking gel, overlay with water, and allow polymerization for ~40 min at room temperature.
4. Pour the 4 % stacking gel, add combs, and allow polymerization for ~40 min at room temperature.

3.2.2 Preparation of Cell Lysates

1. Harvest day-8 hESC colonies using dispase (10 min, 37 °C, *see* **Note 2**). Transfer cells to an Eppendorf tube.
2. Dilute 1:1 with commercially available Laemmli sample buffer or with a reducing sample buffer containing β-mercaptoethanol supplemented with 1 mM sodium orthovanadate and 1 mM PMSF.
3. Boil samples for 4 min and centrifuge for 10 min at 16,110 × *g*.
4. Samples should be kept at −80 °C for long-term storage. Avoid freeze-thawing samples, as this might impact on the dephosphorylation of the samples. Instead, aliquot samples.

3.2.3 Gel Electrophoresis and Transfer

1. Protein extracts (25 μl/well) are resolved on an SDS-polyacrylamide gel electrophoresis (PAGE) gel in running buffer at 200 V for ~35 min using a kaleidoscope prestained ladder (8 μl/well) to estimate the size of the resultant bands.
2. After separation, remove stacking gel with a surgical blade and transfer proteins in the resolving gel to a PVDF membrane. Do not touch the PVDF membranes with hands; use forceps.
3. Prior to use, the PVDF membrane must be activated with 100 % methanol for 30 s followed by a rinse in distilled water.
4. For optimum transfer, pre-equilibrate gels and the PVDF membrane in transfer buffer for at least 10 min prior to transfer.
5. Prepare a "gel sandwich" with pre-wet filter papers, the gel and the membrane in between, according to the manufacturer's instructions. Make sure that there is no air space (bubbles) between gel and membrane to ensure a good transfer.
6. Allow transfer for 1 h at 100 V in transfer buffer.
7. After transfer, remove the membrane and orientate it.

3.2.4 Immunoblotting and Protein Detection

1. All steps are performed on a rocking platform.
2. Block the PVDF membrane with 1 % BSA (*see* **Note 5**) in TBST either overnight at 4 °C or 1 h at room temperature.
3. Incubate the membranes with the primary rabbit polyclonal antibody against Cx43 (0.5 μg/ml in TBST) for 2 h or overnight. Negative control membranes should be incubated with the appropriate immunoglobulin-negative fraction at the same concentration.
4. Wash membranes three times in TBST (15 min each).
5. Incubate the membranes with the HRP-conjugated secondary antibody for 1 h at room temperature (0.15 μg/ml in TBST).
6. Wash membranes three times in TBST (15 min each).

7. Incubate the membranes with the chemiluminescent detection reagent for 5 min and expose to Hyperfilm. Hyperfilm exposure time must be optimized for the highest signal-to-noise ratio. Alternatively, chemiluminescence can be detected using the Gel-Doc system (Biorad). Cx43 appears as a triplet of band at approximately 43 kDa.

3.2.5 Membrane Stripping

1. Antibodies on the membrane can be stripped by incubation with the western blot stripping buffer (15–30 min at room temperature).
2. Following TBST washes, the membrane can be blocked and re-probe membranes with another antibody of interest, as described above.
3. Beta-tubulin antibody (1/10,000 in TBST for 1 h) followed by HRP-conjugated secondary antibody can be used as a lysate loading control, molecular weight 55 kDa (*see* **Note 6**).

3.3 Immunocytochemistry for the Expression of Cx43, Cx45, and Pluripotency Markers

1. Wash cells in PBS.
2. Fix cells in cold 100 % ethanol for 10 min at room temperature. Allow air-drying of the samples.
3. With a wax pen, delimit each individual well (to limit risks of "cross contamination" of reagents).
4. Wash cells three times in PBS.
5. Samples should be kept at −80 °C for long-term storage.
6. Block sample with 1 % serum for 1 h at room temperature (*see* **Note 7**).
7. Incubate cells with the following primary antibodies: Cx43 (20 μg/ml, 1/50–1/100 in PBS containing 0.1 % serum) and Cx45 (1/50–1/100 in PBS containing 0.1 % serum) for 30–60 min at room temperature. Negative control should be performed using sample incubated with the appropriate antibody isotype control.
8. Wash samples three times in PBS.
9. Incubate the samples with the appropriate secondary antibody conjugated with FITC (20 μg/ml, 1/40) or Alexa Fluor® 488 (6.67 μg/ml) for 30 min at room temperature.
10. Optional: To assess pluripotency, double staining can be performed using specific hESC markers such as the following antibodies: GCTM-2 (undiluted hybridoma supernatant), Oct-4 (4 μg/ml, 1/50 in PBS), TG-30/CD9 (undiluted hybridoma supernatant), and TRA-1-60 (undiluted) for 30 min at room temperature, followed by incubation with the appropriate secondary antibody conjugated with Alexa Fluor® 568 (6.67 μg/ml) for 30 min at room temperature.

11. Wash samples three times in PBS.
12. Counterstain nuclei with DAPI or Hoechst-33342 (1 μg/ml in water) for 5 min at room temperature.
13. After PBS washes, mount samples in Vectashield to enhance visualization of the immunostaining.
14. Cover the slide with a glass cover slip and seal with nail varnish.
15. Cx43 staining appears as a dotted staining at the membrane of cells, while we observed Cx45 staining to be at the membrane and the cytoplasm of hESC. Specificity is verified by the absence of immunostaining in the antibody isotype controls.

3.4 Scrape Loading/Dye Transfer Assay (See Notes 8 and 9)

1. GJIC in hESC is determined using the scrape loading/dye transfer (SL/DT) assay as described in (18, 19).
2. In all experiments, hESC must be kept moisturized in buffers at all time to prevent dehydration.
3. Although GJIC can be modulated by Ca^{2+} in different cell types, we previously demonstrated that Ca^{2+} does not modify GJIC in hESC. Thus, for an easier handling of the cells, we suggest to perform SL/DT in the presence of Ca^{2+}.
4. Wash hESC colonies three times in a pre-warmed Ca^{2+}- and Mg^{2+}-PBS buffer (buffer 1, *see* **Note 10**).
5. Remove the plastic chambers of the slide, and if necessary, individualize each well with a wax pen.
6. Cut hESC colonies with a surgical blade followed by 5 min of incubation with Lucifer yellow (1 mg/ml) and rhodamine–dextran (1 mg/ml) diluted in buffer 1 (*see* **Note 11**).
7. After further washes with buffer 1, live colonies are viewed under a fluorescence microscope.
8. Control colonies incubated with both biochemical dyes without scraping should demonstrate no uptake or dye transfer of Lucifer yellow or rhodamine–dextran, confirming that the Lucifer yellow transfer is solely due to gap junction coupling rather than a leaky membrane.
9. If experiments are performed in order to assess the effect of specific acute treatments, the drugs used must be incubated at each step of the experiments (i.e., in buffers and Lucifer yellow/rhodamine–dextran solutions).

3.5 Chemical Closure of Gap Junctions

We previously found that a number of specific inhibitors and ligands can induce chemical closure of gap junction in hESC, such as PMA (1 μM, 60 min to activate protein kinase C), U0126 (60 μM, 60 min to inhibit MEK phosphorylation), glycyrrhetinic acid (α-GA, 10 μM, 24 h), and BMP-4 (10 ng/ml, 30 min). Using these gap junction blockers, one can readily study the effect of gap

junction closure on cell apoptosis, proliferation, pluripotency, and colony growth of hESC (*see* **Note 12**).

3.6 Cell Apoptosis Assay

1. Cell apoptosis is quantified using the in situ cell death detection kit by flow cytometry analysis.
2. hESC were cultured with or without α-GA (10 μM) for 24 h.
3. Collect floating apoptotic bodies in the media. Wash cells with PBS, and centrifuge at 1,300 × *g* for 2 min to collect cells.
4. Harvest hESC colonies using dispase (10 min, 37 °C), and transfer hESC colonies to an Eppendorf tube.
5. Incubate with TrypLE Express (*see* **Note 13**) for 5 min at 37 °C. Wash cells with PBS, and centrifuge at 300 × *g* for 2 min to collect cells. Gently pipette the cells up and down to break clumps to achieve single-cell suspensions. Mix the apoptotic bodies to the hESC samples. Use no more than 2×10^7 cells/ml.
6. Fix cells with 2 % PFA for 1 h at room temperature.
7. Wash fixed cells twice with PBS. Centrifuge at 1,300 × *g* for 2 min to collect samples.
8. Permeabilize the samples with 0.1 % Triton X-100 in PBS for 2 min on ice.
9. Wash fixed cells twice with PBS. Centrifuge at 1,300 × *g* to collect samples.
10. Incubate cells with 50 μl of "TUNEL reaction mixture" for 60 min at 37 °C. Negative controls are performed by incubating the cells in "label solution" only for 60 min in an incubator at 37 °C. An unstained sample should be prepared as a control to determine autofluorescence background.
11. Wash samples twice with PBS. Centrifuge at 1,300 × *g* to collect samples.
12. Samples are analyzed by a flow cytometer. Negative control samples were used to set the gate and determine the background due to the secondary antibody; run the samples with voltages set so that the majority of the cells are in the left quadrant. All other subsequent samples should be run with the same voltage to ensure consistency. Collect at least 50,000 cells for analysis.

3.7 Cell Proliferation Assay

1. Cell proliferation in hESC can be quantified using the in situ cell proliferation kit by flow cytometry analysis.
2. Add BrdU (10 μM final) in the culture medium for 2 h in an incubator (37 °C) prior to harvesting the hESC. Negative control is performed with hESC without incubation with BrdU.
3. Harvest hESC by dispase treatment (10 min, 37 °C), and transfer cells to an Eppendorf tube.

4. Incubate with TrypLE Express (*see* **Note 13**) for 5 min at 37 °C. Wash cells with PBS, and centrifuge at 300 × *g* for 2 min to collect cells. Gently pipette the cells up and down to break clumps to achieve single-cell suspensions.
5. Rinse with PBS. Centrifuge at 300 × *g* for 2 min to collect samples.
6. Inject the cell suspension into 70 % ethanol and incubate for 30 min at −20 °C. Do not resuspend the cell pellet in 70 % ethanol to avoid cell aggregation.
7. Wash cells with PBS. Centrifuge at 300 × *g* for 2 min to collect samples.
8. Denature DNA with HCl 4 M for 10 min. Wash cells with PBS until pH >6.5 (~3–4 times). Centrifuge at 1,300 × *g* for 2 min to collect samples.
9. Incubate cells with 50 μl mouse anti-BrdU antibody conjugated with fluorescein for 45 min at 37 °C.
10. Rinse samples twice with PBS. Centrifuge at 1,300 × *g* for 2 min to collect samples.
11. Incubate with propidium iodide (20 μg/ml) for 15 min at room temperature. Do not wash away propidium iodide. Take samples directly to flow cytometry analysis.
12. Samples are analyzed by a flow cytometer. Negative control samples were used to set the gate and determine the background due to the antibody; run the samples with voltages set so that the majority of the cells are in the left lower quadrant. All other subsequent samples should be run with the same voltage to ensure consistency. Collect at least 50,000 cells for analysis.

3.8 Cell Pluripotency Assay

1. hESC pluripotency can be quantified by flow cytometry using stem cell markers TRA181 or TRA160.
2. Harvest hESC by dispase treatment (10 min, 37 °C), and transfer cells to an Eppendorf tube.
3. Incubate with TrypLE Express (*see* **Note 13**) for 5 min at 37 °C. Wash cells with PBS, and centrifuge at 300 × *g* for 2 min to collect cells. Gently pipette the cells up and down to break clumps to achieve single-cell suspensions.
4. Block with 1 % goat serum in hESC culture medium for 30 min on ice. *See* **Note 7**.
5. Incubate samples with TRA-1-60 or TRA-1-81 antibodies (1 μg/1 million cells, diluted in PBS with 1 % goat serum) for 30 min on ice. Negative control should be performed with hESC incubated with the appropriate concentration of mouse

IgM antibody. An unstained sample should be prepared as a control to determine autofluorescence background.

6. Wash samples twice with PBS. Centrifuge at 300 × *g* for 2 min to collect samples.
7. Incubate with goat anti-mouse IgM antibody conjugated with Alexa Fluor® 488 (4 μg/ml, diluted in PBS with 1 % goat serum) for 30 min on ice.
8. Wash samples twice with PBS. Centrifuge at 300 × *g* for 2 min to collect samples.
9. Samples were analyzed by a flow cytometer. Negative control samples were used to set the gate and determine the background due to the antibody; run the samples with voltages set so that the majority of the cells are in the left lower quadrant. All other subsequent samples should be run with the same voltage to ensure consistency. Collect at least 50,000 cells for analysis.

3.9 Colony Growth Assay

1. We previously found that the chemical inhibition of GJIC was accompanied by cell death and decrease of colony growth in serum-free medium, but not in culture medium containing serum.
2. Incubate hESC colonies for 5–7 days in serum-free medium in the presence or the absence of α-GA (10 μM), and change medium every 2 days.
3. Capture phase-contrast images of the morphology for at least 16 hESC colonies every day for 5–7 days.
4. Colony growth can be assessed by measuring hESC colony area using Analysis^B software. Alternatively, the colony diameter can be measured with reference to scale bar using Adobe Photoshop. The colony diameter is recorded as the average of the longest and shortest diameter of the colony.

3.10 Statistical Analysis

All experiments must be performed at least three times to ensure consistent results. Statistical analysis on raw data is performed using Graphpad Prism software. Different statistical tests may be used for the different experiments performed, such as the two-tailed *t*-test or one- and two-way ANOVA followed by Bonferroni or Tukey tests. Statistical significance is established at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

4 Notes

1. Formats of culture are reflective of what we found to be the easiest handling format to perform experimental work. These are only indicative and can be modified according to specific needs.

2. Dispase is a protease that cleaves adhesion molecules, thus allowing extraction of hESC from MEF and the plastic surface of the dish. hESC colonies can be readily sucked off using a pipette, leaving the MEF layer intact on the dish.
3. The system uses polyT magnetic beads to hybridize at high efficiency with the 3′ polyA tail of mRNA, thus yielding highly purified mRNA.
4. This technique allows for the study of the level of phosphorylation of Cx43 in hESC. Indeed, Cx43 can be present as unphosphorylated or phosphorylated once or twice. There is no strict correlation between the phosphorylation states of connexin proteins and the degree of functional coupling. Indeed, phosphorylation of Cx43 can influence GJIC both positively and negatively depending on the cell type (20, 21). The antibody used in this protocol recognizes three forms of Cx43 (unphosphorylated, phosphorylated once and twice).
5. Blocking the membrane with BSA rather than with dried milk gives a better resolution for the detection of phosphorylation.
6. If no detection is observed, the membrane can be incubated with 0.1 % (w/v) Ponceau S staining solution (0.1 % (w/v) Ponceau S, 5 % (v/v) acetic acid: mix 50 mg Ponceau S and 2.5 ml acetic acid, and bring to a final volume of 50 ml with distilled water) for 10–20 min with agitation to check for successful protein transfer. Membrane can then be washed in distilled water until clean.
7. The serum used for blocking should correspond to the species in which the secondary antibodies were raised. A combination of serum from different species can be used together.
8. hESC communicate through functional and opened gap junctions. The SL/DT assay allows for a quick, cheap, and reliable study of GJIC in cells. Controls: hESC in serum (GJIC are opened) and PMA (1 μM) for 60 min (GJIC are closed).
9. For an easier handling of cells during these experiments, it is advisable to cultivate hESC cells on 8-well chamber slides. Generally we use day-5 colonies as the colonies are large enough to handle and not yet started to spontaneously differentiate.
10. Ca^{2+}- and Mg^{2+}-free PBS buffer (buffer 2: 140 mM NaCl, 5.5 mM KCl, 10 mM glucose, 10 mM Hepes, 2 mM EGTA, pH 7.35) can be used to determine the effect of exogenous Ca^{2+} and Mg^{2+} in modulating gap junctions in hESC.
11. Due to its low molecular weight (522 Da), Lucifer yellow is able to diffuse from cell to cell through functional gap junctions. On the other hand, rhodamine–dextran (10 kDa) is too large to diffuse through gap junctions and thus serves as a

negative control. Time of incubation with Lucifer yellow can be modified depending on the size of the colonies.

12. Other techniques not used in the laboratory are available for the study of gap junctions in hESC. In particular, siRNA can be used to downregulate specific connexin proteins instead or in complement of a chemical inhibition of GJIC. Furthermore, other potent inhibitors of GJIC in different cell types, but not yet used in hESC, include heptanol, octanol, and halothane.
13. TrypLE Express is a trypsin-like enzyme used to dissociate cells into single cells. In our experience TrypLE Express is more gentle to hESC than trypsin.

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References

1. Kumar NM, Gilula NB (1996) The gap junction communication channel. Cell 84:381–388
2. Revel JP, Karnovsky MJ (1967) Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. J Cell Biol 33:C7–C12
3. Alexander DB, Goldberg GS (2003) Transfer of biologically important molecules between cells through gap junction channels. Curr Med Chem 10:2045–2058
4. Krysko DV, Leybaert L, Vandenabeele P, D'Herde K (2005) Gap junctions and the propagation of cell survival and cell death signals. Apoptosis 10:459–469
5. Valiunas V, Bukauskas FF, Weingart R (1997) Conductances and selective permeability of connexin43 gap junction channels examined in neonatal rat heart cells. Circ Res 80:708–719
6. Neijssen J, Herberts C, Drijfhout JW, Reits E, Janssen L, Neefjes J (2005) Cross-presentation by intercellular peptide transfer through gap junctions. Nature 434:83–88
7. Sheardown SA, Hooper ML (1992) A relationship between gap junction-mediated intercellular communication and the in vitro developmental capacity of murine embryonic stem cells. Exp Cell Res 198:276–282
8. De Maio A, Vega V, Contreras J (2002) Gap junctions, homeostasis, and injury. J Cell Physiol 191:269–282
9. Mesnil M, Crespin S, Avanzo JL, Zaidan-Dagli ML (2005) Defective gap junctional intercellular communication in the carcinogenic process. Biochim Biophys Acta 1719:125–145
10. Trosko JE, Chang CC (2003) Isolation and characterization of normal adult human epithelial pluripotent stem cells. Oncol Res 13:353–357
11. Yamasaki H, Krutovskikh V, Mesnil M, Tanaka T, Zaidan-Dagli ML, Omori Y (1999) Role of connexin (gap junction) genes in cell growth control and carcinogenesis. C R Acad Sci III 322:151–159
12. Carpenter MK, Rosler ES, Fisk GJ et al (2004) Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. Dev Dyn 229:243–258
13. Wong RC, Dottori M, Koh KL, Nguyen LT, Pera MF, Pebay A (2006) Gap junctions modulate apoptosis and colony growth of human embryonic stem cells maintained in a

serum-free system. Biochem Biophys Res Commun 344:181–188

14. Wong RC, Pebay A, Nguyen LT, Koh KL, Pera MF (2004) Presence of functional gap junctions in human embryonic stem cells. Stem Cells 22:883–889
15. Huettner JE, Lu A, Qu Y, Wu Y, Kim M, McDonald JW (2006) Gap junctions and connexon hemichannels in human embryonic stem cells. Stem Cells 24:1654–1667
16. Assou S, Lecarrour T, Tondeur S et al (2007) A meta-analysis of human embryonic stem cells transcriptome integrated into a web-based expression atlas. Stem Cells 25:961–973
17. Boyer LA, Lee TI, Cole MF et al (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122:947–956
18. el-Fouly MH, Trosko JE, Chang CC (1987) Scrape-loading and dye transfer. A rapid and simple technique to study gap junctional intercellular communication. Exp Cell Res 168:422–430
19. Venance L, Piomelli D, Glowinski J, Giaume C (1995) Inhibition by anandamide of gap junctions and intercellular calcium signalling in striatal astrocytes. Nature 376: 590–594
20. Lampe PD, Lau AF (2000) Regulation of gap junctions by phosphorylation of connexins. Arch Biochem Biophys 384:205–215
21. Lampe PD, Lau AF (2004) The effects of connexin phosphorylation on gap junctional communication. Int J Biochem Cell Biol 36:1171–1186