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Using Electric Pulses to Prepare Feeder Cells for Sustaining and Culturing of Undifferentiated Embryonic Stem Cells

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Abstract

Current challenges in embryonic-stem-cell (ESC) research include inability of sustaining and culturing of undifferentiated ESCs over time. Growth-arrested feeder cells are essential to the culture and sustaining of undifferentiated ESCs, and they are currently prepared using gamma-radiation and chemical inactivation. Both techniques have severe limitations. In this study, we developed a new, simple and effective technique (pulsed-electric-fields, PEFs) to produce viable growth-arrested cells (RTS34st) and used them as high-quality feeder cells to culture and sustain undifferentiated zebrafish ESCs over time. The cells were exposed to 25 sequential 10-nanosecond-electric-pulses (10nsEPs) of 25, 40 and 150 kV/cm with 1s pulse interval, or 2 sequential 50-microsecond-electric-pulses (50μsEPs) of 2.83, 1.78 and 0.7 kV/cm with 5s pulse interval, respectively. We found that cellular effects of PEFs depended directly upon the duration, number and electric-field-strength (E) of the pulses, showing the feasibility of tuning them to produce various types of growth-arrested cells for culturing undifferentiated ESCs. Either 10nsEPs of 40 kV/cm or 50μsEPs of 1.78 kV/cm provided by inexpensive and widely available conventional electroporators, generated high-quality growth-arrested feeder cells for proliferation of undifferentiated ESCs over time. One can now use PEFs to replace radiation methods for preparation of growth-arrested feeder cells for advancing ESC research.

Keywords

Embryonic stem cells; culturing of undifferentiated embryonic stem cells; feeder cells; pulsed electric fields; electric pulses; single living cell imaging

1. Introduction

Embryonic stem cells (ESCs) are pluripotent cells that can self-renew indefinitely, differentiate and develop into various specialized cell types with specific functions, offering possibilities of using ESC-based therapies to treat an array of diseases and disorders [1–5]. However, enormous challenges must be overcome in order to realize such incredible therapeutic potentials [1,4–7]. For example, it remains essentially unknown how ESCs differentiate, how one can effectively and specifically guide their differentiation, and how one can design simple and effective techniques to culture and sustain undifferentiated ESCs over time [1,4–7]. The effective means to culture and maintain ESCs in undifferentiated states will lay the foundation to address the critical questions about the differentiation of ESCs.

Currently, ESCs are cultured on a layer of feeder cells, which are viable and growth-arrested (or mitotically inactive) and provide required growth factors to sustain and support the self-

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renewal of the ESCs [4–6,8]. The types of feeder cells vary with the species of ESCs [4,6–8]. For instance, human ESCs (e.g., BG01V) typically use either human (e.g., fibroblasts) or mouse cells (e.g., CF-1) as feeder cells [6,9,10], and mouse ESCs (e.g., C57Bl/6) use mouse cells as feeder cells (fibroblasts) [8,10]. Zebrafish ESCs use rainbow trout cells as feeder cells [11]. Therefore, specific feeder cells are essential for the proliferation of undifferentiated ESCs.

To date, only two methods, gamma (γ)-radiation or chemical inactivation using mitomycin-C, have been used to prepare growth-arrested feeder cells [5,9–15]. Gamma-radiation requires expensive equipment with specific radiation sources and the sources must be appropriately calibrated in order to effectively prepare the feeder cells. Notably, distance and shielding factors can dramatically affect radiation doses, leading to irreproducible results [8,13,14]. Furthermore, the feeder cells prepared using gamma-radiation often become apoptotic with time and detach from the surface after weeks of culture, which affects the growth of ESCs [13–15]. Mitomycin-C (a chemotherapeutic agent) arrests cell proliferation by inhibiting the separation of ds-DNA during cell replication [13,14]. Mitomycin-C treatment is cheaper and faster than gamma-radiation. Unfortunately, it can metabolically alter the feeder cells and inhibit their expression of specific ligands or cytokines required to culture and sustain undifferentiated ESCs [13,15]. The deficiencies of both methods demand the development of new and effective means to prepare high-quality feeder cells for the culture of ESCs.

Recent studies show that ultrashort electric pulses (e.g., 10 ns) can penetrate inside the cells and induce intracellular responses while maintaining viability of cells [16,17]. The intracellular structures and viability of cells depend on the number and electric-field-strength (E) of 10-nanosecond-electric-pulses (10nsEPs), showing the possibility of tuning them to prepare a wide variety of viable growth-arrested cells. Conventional electroporators offering 50-microsecond-electric-pulses (50μsEPs) have been widely used to deliver genes into living cells. They are inexpensive and widely available in various research laboratories. In this study, we used 10nsEPs or 50μsEPs to successfully prepare viable and growth-arrested feeder cells to culture and sustain undifferentiated zebrafish ESCs over time. To our knowledge, this is the first time that the pulsed electric fields (PEFs) have been used to prepare the growth-arrested feeder cells for culturing ESCs.

2. Materials and Methods

2.1. Culture and Assay of the Cells (RTS34st)

We received four 100% confluent flasks (25-T) of rainbow trout spleen cells (RTS34st), generously provided by Dr. Paul Collodi, and harvested the cells by trypsinization (2 mg/mL trypsin, 1 mM EDTA) and centrifugation (1915 rpm for 10 min) [11,18]. We resuspended the cells in Leibovitz's L-15 medium, and determined their number and viability using a hemacytometer and trypan blue assay, respectively. All reagents in this study, including those described below, were purchased from Sigma-Aldrich, except those indicated.

2.2. Utilizing Pulsed-Electric-Fields to Prepare Growth-Arrested Feeder Cells

We added 500 μL of the cells (1.0×10^6 cells/mL, RTS34st) into each electroporation cuvette (Biosmith) (distance between two electrodes in the cuvette = 0.4 cm). The same amount of the cells was used for control experiments. We exposed the cells in the cuvettes to the 25 sequential 10nsEPs of 150, 40 and 25 kV/cm (1-s pulse interval), provided by a 10 ns pulse generator (ARC Technology), as we described previously [16]. The 10nsEPs were measured in real-time using an oscilloscope (TDS 3052B, Tektronix) [16]. The cells in each cuvette were centrifuged at 1915 RPM for 5 min and rinsed with the L-15 medium twice via

centrifugation. The cells from each cuvette were finally seeded in each flask (T-25), and incubated at 19°C. After 30 min, 30% (v/v) heat-inactivated fetal bovine serum (FBS, GIBCO-BRL) was added into each flask.

Using the same approaches, we exposed the cells in each cuvette to two sequential 50 μs electric pulses (50μsEPs) of 2.83, 1.78 and 0.75 kV/cm (5-s pulse interval), generated by a conventional electroporator (Bio-Rad, Gene Pulser Xcell). The cells were then seeded and cultured in the flasks, as described above. The cells (500 μL per well) were also seeded in each well (diameter = 3.5 cm) of the plate (Greiner Bio-One). An additional 3 mL of L-15 medium was added to each well. The 6-well plate was sealed and incubated at 19°C for 30 min, then 30% FBS was added to each well.

We imaged the cells in the flasks and in the wells daily using optical microscopy with 10× objective (bright-field upright Nikon E-400 or phase-contrast inverted Zeiss Axiovert), respectively. The microscopes were equipped with high-resolution CCD camera (Micromax and CoolSNAP, Roper Scientific) and high-definition color digital camera to acquire the images of cells over time [19,20]. The twenty identical locations on the surface of each flask and well were marked, allowing us to image and monitor the cell growth in given areas (1.2 mm^2) over time. Note that the cells (RTS34st) are adherent cells and only the cells attached on the surface can grow over time. Once the feeder cells in each well became a confluent monolayer, the ESCs were transferred onto the wells and cultured over time.

We determined the cellular viability at the end of cell culture in the flasks and in the wells using alamar-blue viability assay (Invitrogen) [21]. We replaced the medium in each flask with fresh L-15 medium, and incubated them for 2 h. We then added alamar-blue dye (2% v/v) into the flasks and incubated them over night. Photos of the flasks were taken over time.

2.3. Isolation, Culture and Assay of Undifferentiated Zebrafish Embryonic Stem Cells

We placed 10 wild-type mature females and 5 wild-type mature males (Aquatic Ecosystems) in a clean 5-gallon breeding tank and used a light (14 hours)-dark (10 hours) cycle to trigger breeding [22–25]. For each experiment, we collected ~300 embryos at the blastula stage (approximately 4 hours post-fertilization), transferred them to a petri dish containing egg water (1.0 mM NaCl in DI water), and rinsed them thoroughly with egg water to remove any debris.

We isolated the ESCs from the embryos, as described following [11,18]. The embryos were soaked in 70% ethanol for 5 s, thoroughly rinsed with egg water and LDF medium. LDF medium was prepared by well mixing L-15, Ham's F12, and Dulbecco's modified Eagle's media at the ratio of 50:35:15 in 15 mM HEPES buffer containing 120 μg/mL penicillin G, 25 μg/mL ampicillin and 200 μg/mL streptomycin sulfate, with the addition of sodium bicarbonate (0.180 g/L) and sodium selenite (10^{-8} M) [11,18]. We removed the chorions from embryos by repeatedly incubating the embryos with bleach solution (0.5% v/v) for 2 min and rinsing them with LDF medium immediately after each incubation for 4 times, and finally incubating the embryos in the pronase-E solution (0.5 mg/mL in Hank's Balanced Salt Solution) until the chorions started to break apart. We well rinsed the embryos using LDF medium and dissociated ESCs in the inner mass of embryos by incubating them with trypsin/EDTA (2 mg/mL trypsin, 1 mM EDTA) for 2 min. We removed the trypsin/EDTA via centrifugation (1514 rpm for 5 min) and resuspended the ESCs in LDF medium.

We seeded the ESCs on the surface of the wells with and without monolayer of the feeder cells and incubated the cells at 22°C over time. Each well contains 3.015 mL of 0.4 mL of freshly prepared ESCs (3.0×10^5 cells/mL), 1.4 mL LDF medium, 5% FBS, 1% trout plasma (SeaGrow, East Coast Biologics), 1% bovine insulin, 1% EGF (human epidermal growth

factor), 0.5% bFGF (human basic fibroblast growth factor), 0.5% zebrafish embryo extract (10 mg/mL of proteins), and 31% RTS34st cell-conditioned medium, which was prepared by incubation of L-15 medium containing 30% FBS with a confluent of cells (RTS34st) for 3 days at 19°C and then filtered using 0.2 μm filters. The embryo extra was prepared as followed. Approximately 500 embryos were homogenized in 0.5 mL LDF medium, followed by centrifugation at 10,000 rpm for 10 min. The supernatant was collected and filtered using 0.2 μm filters. The concentration of proteins in embryo extra was determined using a biophotometer (Eppendorf). The extra was prepared freshly and stored at −20°C until use.

We imaged the ESCs in the wells daily using a phase-contrast inverted microscope (Zeiss Axiovert) equipped with the CCD camera and color digital camera. We used alkaline phosphatase (ALP) staining assay (Cell BioLabs Inc) to characterize the undifferentiated ESCs over time. We first removed the medium from the wells cultured with the ESCs and feeder cells alone, and washed the cells with PBST (1× PBS containing 0.05% Tween-20). The fixing solution (400 μL) of assay kit was added into each well and incubated at room temperature for 3 min. The cells were then washed twice with PBST. The staining solution (400 μL) freshly prepared by mixing equal volume of staining solution A and staining solution B of the assay kit was added into each well of the culture plates, which were incubated in the dark at room temperature for 30 min. The cells were washed twice with PBS and imaged using the phase-contrast optical microscopy.

2.4. Data Analysis and Statistics

At each given time, the cells (RTS34st or ESCs) on given areas (20 selected surface locations) of the flasks or the wells (1.2 mm^2) were imaged using the bright-field (10× objective) or phase-contrast (10× objective) optical microscopy. The viability assays in Figures 1D and 2D were carried out accordingly. Each experiment was repeated at least three times. For alkaline phosphatase staining assay, the cells (ESC or growth-arrested feeder cells) on given areas (20 selected surface locations) of the wells (1.2 mm^2) were imaged using the bright-field (10× objective) optical microscopy (Figure 5). This experiment has been repeated at least six times.

3. Results and Discussion

3.1. Using Pulsed Electric Fields to Generate Growth-Arrested Feeder Cells

We exposed the cell (RTS34st) suspension in the cuvettes to 25 sequential 10nsEPs at 40 kV/cm (1-s pulse interval) and cultured the exposed cells in the medium over time. The growth curve of the cells in Figure 1A–a shows that their number decreases slightly and then remains unchanged over time after the third day of culture. In contrast, the number of the unexposed cells (without being exposed to 10nsEPs) cultured in the medium continues to increase with time (Figure 1A–b). Representative optical images of the cells on the surface of the flasks containing the medium illustrate that the exposed cells elongate, but do not replicate and divide over time (Figure 1B), while the unexposed cells grow and divide over time (Figure 1C).

We determined the viability of the cells cultured in the medium for 14 days using an alamar-blue assay. AlamarBlue (resazurin, λ_{max} of absorption spectrum at 600 nm) is highly permeable to live cells and is non-cytotoxic [21]. It can be reduced to resorufin (pink, λ_{max} = 570 nm) by reductases in living cells, while it remains blue in dead cells. Therefore, alamar-blue can serve as a colorimetric assay to determine cellular viability in real time [21]. We assayed the suspensions in the flasks (Figure 1D: i–iii) containing the medium alone (control), unexposed or exposed cells cultured for 14 days using alamar-blue, respectively.

The medium alone remained the blue, while the unexposed or exposed cells cultured for 14 days turned from blue to pink, showing that both the unexposed and exposed cells were alive. These results demonstrate that 10nsEPs can be used to prepare viable growth-arrested cells, suggesting that 10nsEPs penetrate into the cells and inactivate mitotic processes by mechanisms, such as disruption of DNA replication or cell cycle check-points, but do not disrupt cellular viability.

The growth curves of the cells exposed to 25 sequential 10nsEPs of 150 or 25 kV/cm with 1s pulse interval in Figure 2a show that the number of cells decreases or increases with time, respectively. Their representative optical images in Figures 2b–d illustrate that the cells exposed to the 25 sequential 10nsEPs of 150 kV/cm show abnormal spherical morphologies and do not elongate over time. Note that the cells with spherical shape are generally apoptotic, due to the loss of membrane integrity [26]. In contrast, the cells exposed to the 25 sequential 10nsEPs of 25 kV/cm elongate, grow and divide over time. The results show that the effects of 10nsEPs on the cellular functions are highly dependent upon their electric-field strength (E). In this study, we selected an approximate pulse number and E of 10nsEPs based upon calculated energy levels, and used experimental results (Figures 1 and 2) to determine optimum pulse number and E for the preparation of high-quality growth-arrested feeder cells.

Using the same approaches, we explored the possibility of using 50μsEPs provided by conventional electroporators that are widely available in research labs and much more user-friendly than 10nsEPs, to prepare the viable growth-arrested cells. The cells exposed to two sequential 50μsEPs of 2.82, 1.78 and 0.78 kV/cm with 5-s pulse interval, were cultured in the medium and imaged over time. The growth curves in Figures 3A–C:a show that the number of cells decreases with time, remains constant and increases with time for the cells that were exposed to two sequential 50μsEPs of 2.82, 1.78 and 0.78 kV/cm, respectively. Their representative optical images in Figures 3A–C:b–d illustrate the growth-arrested cells with abnormal cellular morphology (spherical shape) in Figure 3A, elongated and undivided cells with normal morphologies in Figure 3B, and growth and division of cells with normal morphology in Figure 3C. As noted above, the spherical cells are apoptotic, due to the loss of membrane integrity [26].

The exposed cells that were cultured for 5 days (Figure 3A–C) are viable, as determined by alamar-blue assay and shown in Figure 3D. Notably, the flasks (i–v) in Figure 3D containing alamar-blue with the medium alone (blank control), the unexposed cells (positive control), and the cells exposed to the 50μsEPs of 2.82, 1.78 and 0.78 kV/cm, show blue, bright pink, dark blue-pink, light pink, and bright pink, respectively. The results show that the 50μsEPs of 2.82 kV/cm affect the cellular viability, while the pulses of 1.78 and 0.78 kV/cm do not. Taken together, these results demonstrate that the two sequential 50μsEPs of 1.78 kV/cm produce viable growth-arrested cells, which can serve as feeder cells for culture of undifferentiated ESCs. The effects of 50μsEPs on the cellular functions highly depend upon their E as shown in Figure 3, which is similar to those observed using 10nsEPs in Figure 2.

3.2. Culture and Sustainability of Undifferentiated ESCs on the Feeder Cells

We used the viable growth-arrested cells prepared using pulsed electric fields (PEFs) as described above, as a monolayer of the feeder cells, and cultured the zebrafish ESCs on them. We freshly isolated the ESCs from zebrafish embryos as described in Methods, and directly cultured them on the monolayer of feeder cells (Figure 4A) and on the bare surface of wells (Figure 4B) for 5 days. The cells (RTS34st) exposed to two sequential 50μsEPs of 1.78 kV/cm were cultured on the wells of cell plates for 5 days, producing a monolayer of the growth-arrested cells. Notably, the cell number remained unchanged for additional 6 days of cultures during which ESCs were cultured on them, as shown in Figure 4C.

The ESCs cultured on the feeder cells (Figure 4A) display small cellular aggregates that increase in size with time and remain attached to the layer of feeder cells over time, exhibiting unique morphologies and growth characteristics of ESCs [1, 11]. In contrast, the ESCs cultured on the bare surface without the feeder cells (Figure 4B) show tiny spherical and isolated aggregates, which deteriorate over time. The results illustrate the significance of feeder cells that provide required proteins and scaffolds to sustain the proliferation of undifferentiated ESCs over time, and demonstrate that given pulse electric fields are well suited for the production of viable growth-arrested feeder cells for culture and sustaining of undifferentiated ESCs over time.

We further characterized the ESCs cultured on the feeder cells by detecting a widely-used molecular marker, membrane alkaline phosphatase (ALP), of undifferentiated ESCs, using an ALP staining assay [4,7,11]. The ALP assay determines the presence of ALP using immunocytochemistry staining [3]. The dye-tagged antibody specifically binds with ALP on the cells, leading to a red-orange color. In contrast, no color is observed in the absence of ALP. As reported previously, ESCs express high levels of membrane ALP, which is down regulated upon the differentiation.

The ESCs cultured on the feeder cells and the bare surface without the feeder cells for 3 days, and the feeder cells alone were assayed using the ALP staining kits, as shown in Figures 5A–C, respectively. We observed the red-orange stains on ESC aggregates cultured on the growth-arrested feeder cells in Figure 5A, and colorless for the cells cultured in the absence of the feeder cells (Figure 5B) and feeder cells lone (Figure 5C). The results in Figure 5 show the presence of ALP on the ESCs cultured on the feeder cells, but absence of detectable levels of ALP on the ESCs cultured without feeder cells or the feeder cells alone (blank control). Since the ALP staining assay requires several washing and fixing steps as described in the method, we did observe the partially removal of some cellular aggregates (ESCs) in Figure 5A. Nonetheless, the red-orange stains of ALP on ESCs in Figure 5A are unambiguous, which illustrates that ESCs cultured on the growth-arrested feeder cells prepared using the electric pulses indeed remain undifferentiated over time. Taken together, the results demonstrate that the pulsed electric fields are well suitable to prepare viable growth-arrested feeder cells for culturing and sustaining of undifferentiated ESCs over time.

4. Summary

In summary, we have developed a new, simple, green, and effective technique (electric pulses) to produce viable and growth-arrested cells (RTS34st), and used them as high-quality feeder cells to culture and sustain undifferentiated zebrafish ESCs over time. The pulsed electric fields (PEFs) can be generated using either the 10nsEP generator or widely available inexpensive conventional electroporators, demonstrating the possibility of using freshly prepared feeder cells to culture and sustain undifferentiated ESCs in a wide variety of research laboratories for expansion and advance of ESC research. We found that the effects of PEFs on the cellular functions highly depended upon the duration, number and E of the pulses, suggesting that PEFs penetrated into the cells and inactivated mitotic processes. This study offers a powerful new tool for one to produce a wide variety of high quality growth-arrested feeder cells for culture of various ESCs. Notably, it is simple and easy to scale up the preparation of growth-arrested feeder cells using PEFs. The work is in progress for probing the molecular mechanisms of the effects of PEFs on the cells.

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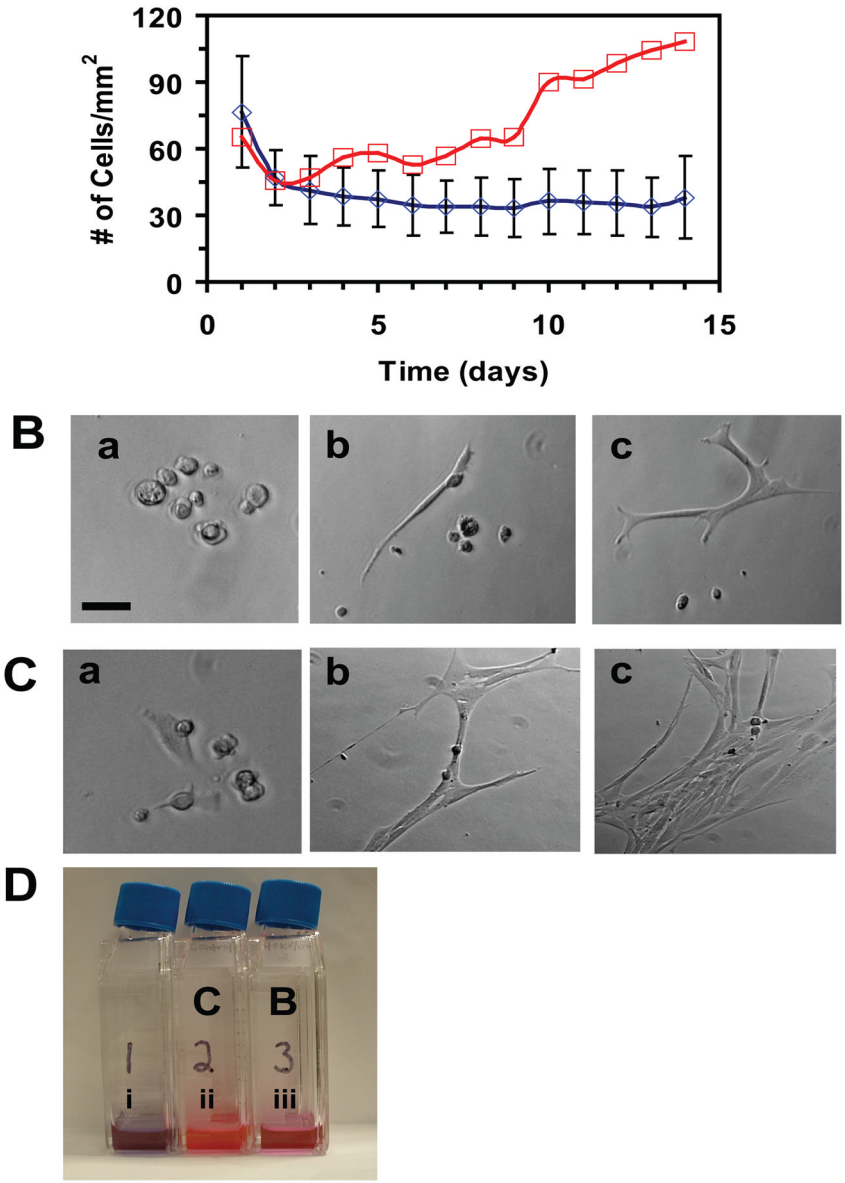

Figure 1. Preparation and characterization of viable growth-arrested feeder cells (RTS34st)

(A) The growth curves of (a) the cells that had been exposed to 25 sequential 10-nsEPs of 40 kV/cm with 1s pulse interval, show that the number of cells decreases and then remains constant (growth-arrested) after the third day of culture; and (b) the unexposed cells (control) show that the number of the cells increases with time.

(B) Optical images of the cells in (A–a) cultured on (a) day 1, (b) day 7 and (c) day 14, show that the number of the cells remains nearly unchanged.

(C) Optical images of the unexposed cells (control experiment) in (A–b) cultured on (a) day 1, (b) day 7 and (c) day 14, show that the cells grow with time. Scale bars in (B – C) = 500 μm.

(D) Photos of the flasks containing: (i) alamar-blue with medium alone (blank control), (ii) the cells cultured on day 14 in (C), and (iii) the cells on day 14 in (B), show blue color in (i), and pink colors in (ii) and (iii), indicating that the cells in (ii) and (iii) are viable.

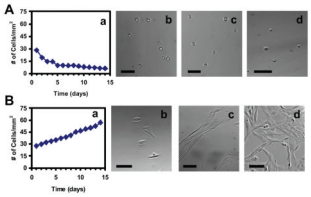

Figure 2.

The effects of electric field strength (E) of 10nsEPs on the cell growth. (a) Growth curves of the cells that had been exposed to 25 sequential 10-nsEPs with 1s pulse interval, at E of (A) 150 and (B) 25 kV/cm and (b–d) their optical images on (b) day 1, (c) day 7, and (d) day 14. Scale bars = 100 μm.

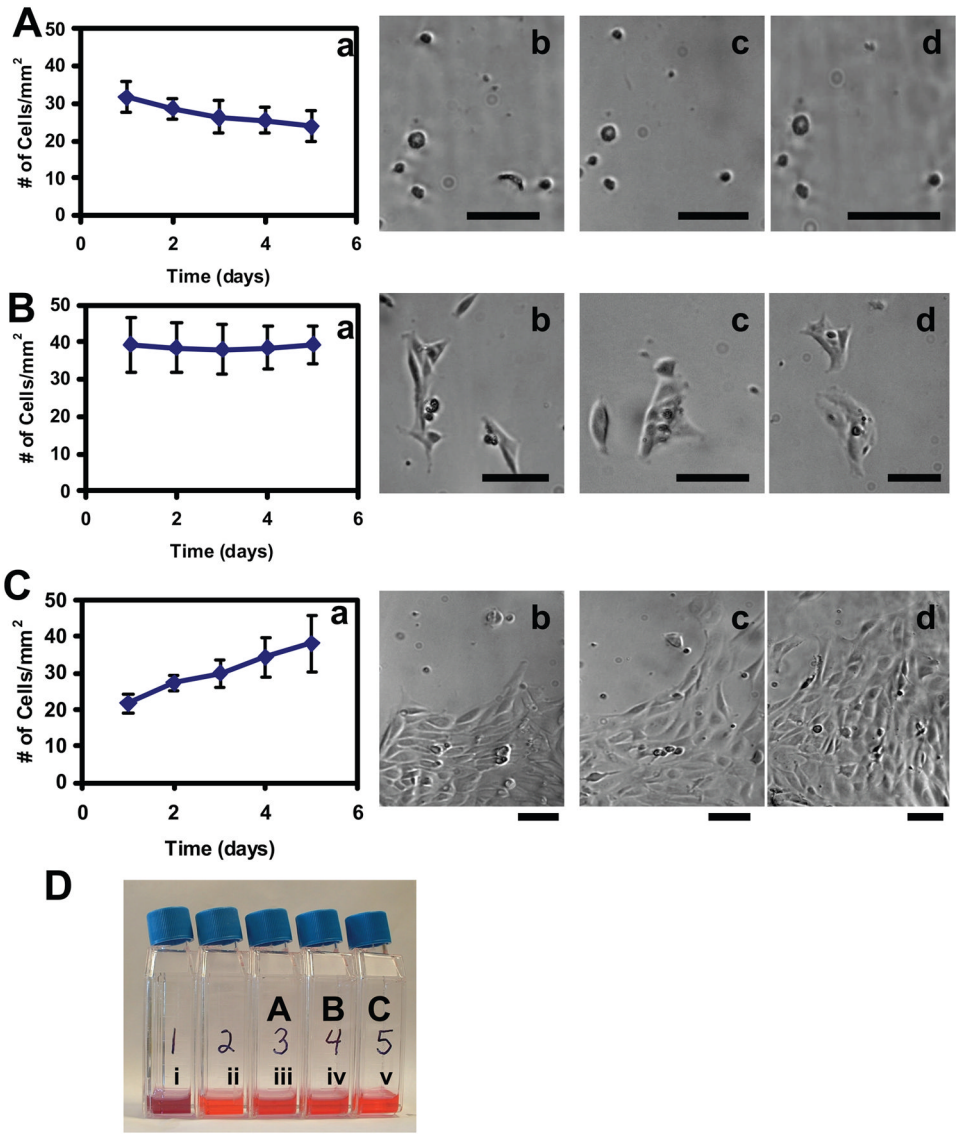

Figure 3. Effects of electric field strength (E) of 50μsEPs on growth and viability of the cells (RTS34st)

(a) Growth curves of the cells that had been exposed to 2 sequential 50μsEP with 5s pulse interval at E of (A) 2.82, (B) 1.78 and (C) 0.75 kV/cm, and (b–d) their optical images on (b) day 1, (c) day 3, and (d) day 5. Scale bars in (A–C) in 100 μm

(D) Photos of the flasks containing: (i) alamar-blue with medium alone (blank control), (ii) the unexposed cells on day 6 (positive control), (iii–v) the cells on day 6 in (A–C), respectively, show blue color in (i) and pink colors in (ii–v), indicating that the cells in (ii–v) are viable.

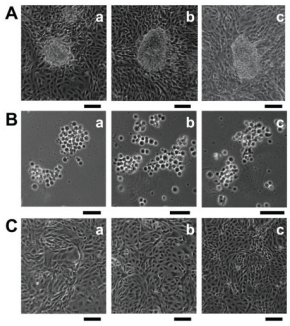

Figure 4.

Characterization of the morphologies of undifferentiated ESCs cultured on the growth-arrested feeder cells prepared using PEFs. Optical images of ESCs cultured on (A) the monolayer of feeder cells, and (B) on a bare surface of wells without the feeder cells on (a) day 1, (b) day 3 and (c) day 6 of ESC culture (days 6, 8 and 11 of feeder cell culture), show that the feeder cells sustain ESC growth and proliferation. Optical images of (C) the feeder cells (RTS34st) cultured on the wells on (a) day 6, (b) day 8, and (c) day 11, show the formation of monolayer and that number of the cells remains unchanged over time. Scale bars = 100 μm.

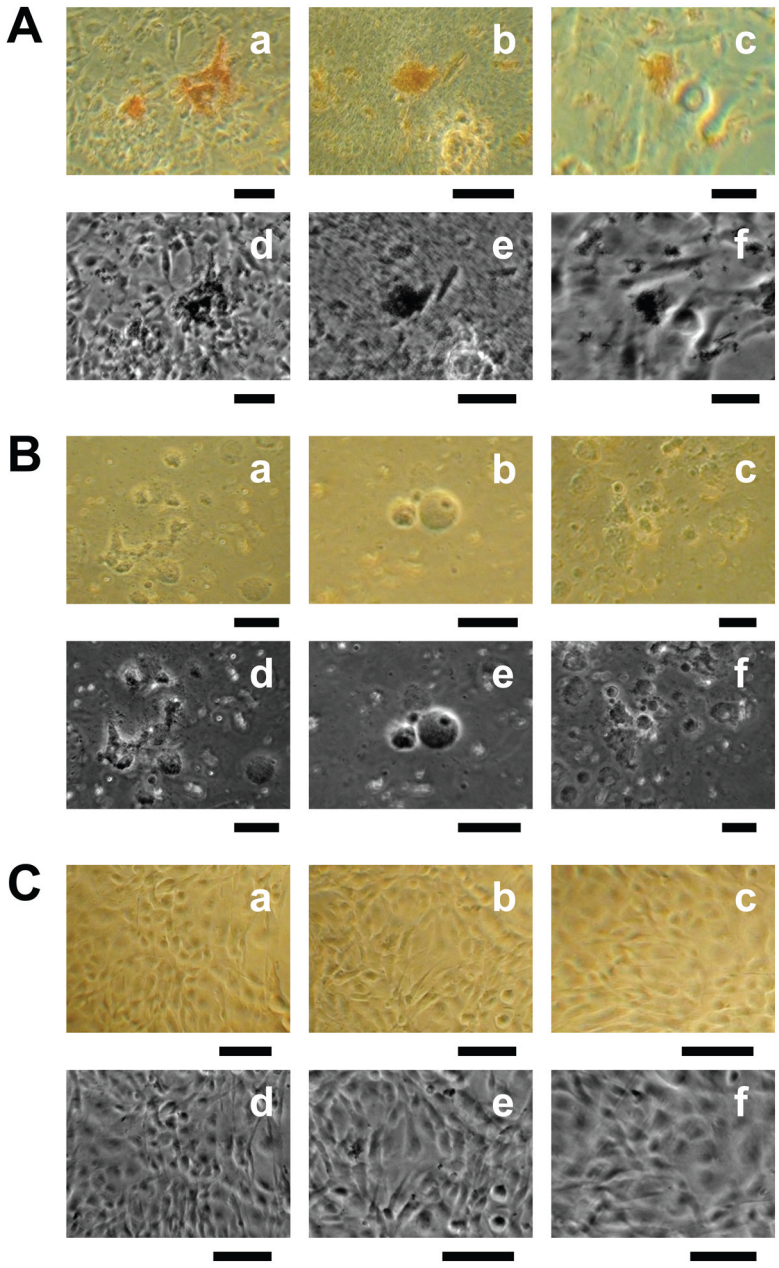

Figure 5.

Characterization of the undifferentiated ESCs using alkaline phosphatase (ALP) staining assay. ALP staining assay of (A) ECSs cultured on the growth-arrested feeder cells, (B) ESCs cultured on the bare surface without the feeder cells, and (C) growth-arrested feeder alone cells, show the red-orange colonies in (A) and colorless in (B–C). Images in (ac) and (d–f) were acquired using digital color camera and CCD camera, respectively. Scale bars in (A), (B) and (C) are 25, 50 and 100 μm, respectively.