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Research Article

Co-Electroporation with *Xenopus Laevis* Oocytes Reprograms Normal and Cancerous Human Cells to Resemble Reprogramming Normal and Cancerous Human Cells to Resemble induced Human Pluripotent Stem Cells

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Abstract

Objective

To investigate reprogramming human cells into induced pluripotent stem cells (iPSc) using co-electroporation with *Xenopus laevis* oocytes.

Methods

Human bone marrow stromal cells (BMSC), BJ cells, pre-adipocytes (HPA), CD4+ T-lymphocytes (CD4TLs), buccal mucosa cells, and HeLa and MCF-7 cells were co-electroporated with mature *Xenopus laevis* oocytes, cultured, and assessed for pluripotency marker expression using fluorescent immunohistochemistry.

Results

The co-electroporated human cells formed colonies on irradiated mouse embryonic fibroblast cells (all study cells) and StemAdhere™ substrate (assessed for co-electroporated buccal mucosa cells). Cells derived from BMSC, BJ cells, HPA, CD4TLs, and buccal mucosa cells expressed Oct 3/4, Nanog, SOX-2, Rex-1, TRA-1-60, and SSEA-1. Cells derived from co-electroporated HeLa cells expressed Oct 3/4; cells derived from co-electroporated MCF-7 cells expressed Oct 3/4 and Nanog. Reprogramming efficacy for CD4TLs was 23.4 ± 3.5%. Co-electroporated HPA trans-differentiated into neural progenitor cells in culture conditions that foster neural differentiation. Control experiments suggested that the electroporate conveyed a reprogramming factor(s).

Conclusions

Human cells co-electroporated with *Xenopus laevis* oocytes resembled iPSc in colony formation and pluripotency-associated marker expression.

Keywords: Human iPSc; reprogramming; Pluripotency; Frog oocytes; Co-electroporation; CD4+ T-lymphocytes; Buccal mucosa cells; Pre-adiposites.

Introduction

Induced pluripotent stem cells (iPSc) constitute a potential source of cells for stem cell therapy that avoids the bioethical concerns surrounding the use of embryonic stem cells (ES) [1]. Recent advances in non-viral reprogramming methodology include the use of recombinant proteins, [2] DHP-derivative (novel anti-oxidant) and low oxygen-tension conditions, [3] microRNAs, [4-6,7,8] zinc-finger nucleases, [9] drugs, [10,11,12] hypoxia, [13] silencing the p53 pathway [14] and ES cell-derived protein extracts [15]. Unfortunately, contemporary methods are hampered by the low efficacy of reprogramming human somatic cells into iPSc and the non-autologous nature of the final product [16 17,18].

Many species have evolved mechanisms for cellular reprogramming; pathways for inducing cellular dedifferentiation and redifferentiation exist a wide variety of organisms including some species of bacteria, [19] plants, [20,21] and lower animals [22]. The poor efficacy of reprogramming achieved using current approaches may be due the use of methods that lack vital reprogramming components naturally present in some living organisms.

In the present study, *Xenopus laevis* oocytes were chosen as a source of natural reprogramming factor(s) based on the successful reprogramming events reported for mammalian somatic cells induced by *Xenopus laevis* egg extracts [23]. We show that co-electroporation of living *Xenopus laevis* oocytes with normal human cells and cancerous human cells lines reprogrammed these cells to resemble iPSc with respect to colony morphology and expression of stem-cell associated transcription factors recognized as markers of pluripotency. Furthermore, this co-electroporation technique achieved a relatively high level of reprogramming efficiency.

Materials and Methods

The human subjects who provided buccal mucosa tissue samples (using a non-invasive technique) gave written informed consent. Procedures involving *Xenopus laevis* were conducted in accordance with published laboratory standards [24].

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Preparation of *Xenopus Laevis* Oocytes

Female, egg-bearing *Xenopus laevis* (NASCO) were at kept at ˜18°C using a 12 / 12-hour light/dark cycle in carbon-filtered water supplemented with 13.3 g/gallon NaCl, which was changed daily [24].

Prior to surgical removal of oocytes, frogs were anesthetized in a plastic beaker containing 1 L of 0.2% tricane solution (Sigma) for up to 20 min and, then, placed on a dissecting pan filled with ice. After a 0.5 cm incision through the skin and muscle layers, the bags of ovaries were removed and placed into oocyte washing (OW) solution (82.5 mM NaCl, 5.0 mM 2- [4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid [HEPES], 2.5 mM KCl , 1 mM $MgCl_2$, 1.0 mM Na_2HPO_4, and 0.5% penicillin/streptomycin [pen/strep] at pH 7.4 [penicillin and streptomycin from Gibco; others from Sigma]). Bags containing ovaries were opened with fine forceps, the ovaries were rinsed several times in OW, and treated with a 0.2% collagenase type II solution (Worthington Biochemical Corporation) for ≥ 1 hour at room temperature. The defolliculated oocytes were rinsed in OW solution and incubated overnight in fresh holding buffer (HB) containing 5 mM NaCl, 5.0 mM HEPES, 2.5 mM KCl, 1 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 0.5% pen/strep, 1.0 mM $CaCl_2$ (Sigma), 2.5 mM sodium pyruvate, and 5% heat-inactivated horse serum (Sigma) titrated to pH 7.4. Recovered oocytes in the final stage of maturity were collected in sterile 6-well cell culture clusters (Costar) prefilled with an HB solution and incubated at 17°C for 24 hours before electroporation experiments.

Cell Lines

Human bone marrow stromal Cells (BMSCs) and stably transfected GFP-expressing BMSCs ($BMSC_{GFP}$) were provided by Tulane University Center of Gene Therapy. Prior to release from the source, two trials of frozen, passage-1 cells were analyzed over three passages for colony forming units, cell growth, and differentiation into fat, bone, and chondrocytes. The BMSC and $BMSC_{GFP}$ were cultured in Dulbecco's modified Eagle's Medium (DMEM; Sigma), supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% streptomycin/penicillin (Gibco) and cultured in 25 cm^2 flasks at 37°C with 5% CO_2. At day 4, the cultures were washed with phosphate-buffered saline (PBS; Sigma) to remove the non-adherent cells and further expanded until ˜80% confluence, when they were harvested and expanded in 75 cm^2 flasks.

Human normal foreskin fibroblasts (BJ cells) from American Type Culture Collection (ATCC) were maintained at 37°C and 5% CO_2 in T25 culture flasks in 5 mL of Eagle's Minimum Essential Medium (EMEM; ATCC) supplemented with 10% PBS, 1 mM sodium pyruvate, 0.1m M non-essential amino acids (NEAA), and 1% pen/strep.

Human subcutaneous pre-adipocytes (HPA) from ScienCell Research Laboratories were cultured at 37°C and 5% CO_2 in T25 flasks coated with 0.01% poly-lysine (Sigma) and containing 5 mL of specially formulated pre-adipocyte medium (PAM; ScienCells); PAM was supplemented with 5 % FBS, 1 mM sodium pyruvate, 0.1 mM NEAA, and 1% pen/strep.

Human peripheral blood CD4+ T-lymphocytes (CD4TLs) from Lonza Group, Ltd. (pathogen-free poietics® CD4TLs) were maintained as a cell suspension in T25 culture flasks at 37°C and 5% CO_2 in 5 mL of lymphocyte growth medium-3 (LGM-3®, Lonza Group Ltd.) supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1% pen/strep, and 50 ng/mL recombinant human Interleukin-4 (R&D Systems).

Human buccal mucosa cells were obtained from healthy human subjects approximately 1 hour before the co-electroporation procedure. Subjects abstained from drinking coffee for 1 hour before collection. Subjects' mouths were rinsed twice with Listerine® followed by sterile distilled water before swabbing. Cells were collected by swabbing firmly on the inside of the cheek 20 times on both sides using a MasterAmp™ Buccal Swab Brush (Epicentre Biotechnologies). The brush holding cheek cells was placed into a 50 mL centrifuge tube filled with 20 mL of sterile filtered PBS (Sigma) containing 1% pen/strep. The sample was vigorously twirled for 30 sec and, then centrifuged at 200g for 7 min. Pelleted cells were resuspended in 5 mL of serum-free DMEM (ATCC) supplemented with 1 mM sodium pyruvate, 0.1 mM NEAA, and 1% pen/strep. Buccal mucosa cells were kept in a refrigerator at 4^0C before use.

Human cervical carcinoma (HeLa) cells (routinely maintained at the Bioquark, Inc. facility) were grown at 37°C and 5% CO_2 in T25 flasks filled with 5 mL of Eagle's essential medium (ATCC) supplemented with 10% FBS, 1 mM sodium pyruvate , 0.1 mM NEAA, and 1% pen/strep.

Human breast adenocarcinoma (MCF-7) cells from ATCC were maintained in Eagle's Minimum Essential Medium supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM NEAA, 1% pen/strep, and 0.01 mg/mL recombinant human insulin (Eli Lilly; a gift from North-Suburban Pharmacy, Skokie, IL)

Irradiated mouse embryonic fibroblasts (iMEF; American R&D Systems) were grown at 37°C and 5% CO_2 in non-pyrogenic, sterile 25 cm^2, 0.2 μm ventilated cell culture flasks (T25; Corning) containing 5 mL of high glucose DMEM (Millipore) supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM NEAA, and 1% pen/strep.

Co-Electroporation of Xenopus Laevis Oocytes with Human Cells

Electroporation parameters for *Xenopus Laevis* oocytes were developed from several published studies of electroporation [25-27]. Forty to fifty fresh oocytes from suspensions with ≥ 90% viability (oocytes showing abnormal pigment distribution or signs of damage of equatorial band, patchy gray membranes during the defolliculation process were discarded) were placed in sterile Gene Pulser electroporation cuvettes (Bio-Rad) prefilled with 400 μL of serum-free DMEM containing 1.0 $x10^5$ - $1.5x10^5$ cells/mL of human cells in suspension. Cuvettes were filled to 800 μL with serum-free DMEM and then placed into the shocking chamber. Co-electroporation of frog oocytes with the suspension

of human cells was conducted using the following parameters: 150 v/cm / 25 μF / 7 pulses, with time constant at 0.5 - 0.7 msec. After electroporation, cuvettes containing oocytes and the human cells were incubated at 17°C for three hours to recover. The human cells were transferred to T25 culture flasks containing iMEF feeder cells for culturing.

Culturing of Human Cells Following Co-Electroporation

The co-electroporated human cells were cultured at 37°C on iMEF feeder cells in 0.1% gelatin-coated (gelatin from Sigma) T25 culture flasks containing 5 mL of specially formulated Embryomax® DMEM culture medium (Millipore). Medium was supplemented with 15% FBS, 1 mM sodium pyruvate, 0.1 mM NEAA, 1% pen/strep, 100 μM beta-mercaptoethanol (Gibco), and 1000 U/mL ESGRO® (Millipore). To maintain the cells in an embryonic stem cell-like state, 1000 U ESGRO® per 1.0 mL of tissue culture media was required. After formation of clusters, the human cells were separated from the feeder cells using the differential sedimentation technique previously described by Doetschman, [28] which removed > 99% of contaminating feeder cells from the electroporated human cell suspension. Trypsinized (trypsin from Sigma) human cell cultures containing iMEFs were centrifuged at 200 g, resuspended in 10 mL of complete ES culture medium, and transferred to a new T25 cell culture flask for 30 minutes at 37°C. Following incubation, the culture medium containing mostly human cells was transferred to a new T25 culture flask for 1-hour at 37°C to remove all remaining fibroblast feeders. Following the second incubation, the culture medium containing the human cells was removed, and the cells were counted, centrifuged again at 200 g, and resuspended in the ES culture medium.

Subculturing

After separation from the feeder cells, the human cells were placed on T25 culture flasks containing either iMEF feeder cells or feeder-free StemAdhere™ pluripotency substrate (Primorigen Biosciences). Subcultured human cells were grown in NutriStem™ (StemGent).

Calculation of Reprogramming Efficacy

Fluorescent immunohistochemically detectable expression of the Nanog gene by cells derived from CD4TLs occurred between 12 h – 24 h following co-electroporation with *Xenopus laevis* oocytes. This expression preceded the formation of tight iPSc-like clusters, making it possible to determine the efficiency of reprogramming by calculating the proportion of cells expressing Nanog gene. The mean for the reprogramming efficiency was calculated by counting the total number of Nanog-positive cells per specimen in each T25 flask (3-4 times), subtracting the number of nonspecific binding sites in the control flasks, dividing by the original number of cells having undergone co-electroporation and multiplying by 100%. The standard deviation of the mean was also calculated.

Cryopreservation of Reprogrammed Cells

Cells were cryopreserved using a standard slow-cooling freezing method [29]. One mL of cells was gently resuspended in 1.5 mL cryovials (Nalgene) containing 0.5 mL of 2X hES cell freezing medium (60% FBS, 20% hES cell culture medium, and 20% dimethyl sulfoxide. Cryovials were transferred to 5100 Cryo 1°C Freezing Container (Nalgene), refrigerated at -80°C overnight and then rapidly transferred to liquid nitrogen refrigeration units.

Trans-Differentiation into Neuronal Progenitor Cells

After formation of clusters, reprogrammed cells derived from HPA were separated from the feeder layer using the Doetschman differential sedimentation technique,[28] and were dissociated enzymatically using collagenase IV (Sigma; 200 U/mL) for 30 min at 37°C generating a cell suspension containing small cell aggregates and single cells. Cell culture conditions for growing neural progenitor cells (NPs) from embryonic stem cells were employed [30]. The cells were washed in warm Neurobasal A medium (GibcoBRL/Invitrogen), pelleted and resuspended in pre-warmed (37°C) standard human embryonic stem cell culturing medium (hESC) supplemented with following growth factors and neuronal and other supplements: fibroblast growth factor-2 (10 ng/mL), epidermal growth factor (20 ng/mL), 1% B27, 1% N2, 1% pen/strep, 1% l-glutamine, 1% non-essential amino acids (NEAA), 0.2% beta-mercaptoethanol, and 20% Knockout Serum Replacement (all media components from Gibco- BRL/Invitrogen). The HPA-derived cells in suspension were then seeded at high cell density (150–200×103 cells/cm^2) onto BD BioCoat™ and laminin-coated 150mm petri dishes (Beckson Dickenson), and the medium was supplemented with hESC medium and 4ng/mL fibroblast growth factor-2. Proliferating HPA-derived neural progenitors were observed in 8-10 days. The neural rosettes were dissociated by short (5–10 min) collagenase IV treatment into single cells and re-seeded under the same conditions, thus generating a monolayer population of proliferating neural progenitors.

Qualitative Assessment of Colony Morphology

Assessment of colony morphology (resemblance to iPSc colonies) was performed by Dr. Nikolai Strelchenko, PhD of the hESC Research Lab at Reproductive Genetics Institute, Chicago, IL, USA and Dr. Arshak Alexanian, VMD, PhD, of the Department of Neurosurgery, Neuroscience Research Laboratories, Zablocki Veterans Affairs Medical Center and of Medical College of Wisconsin, Milwaukee, WI, USA.

Alkaline Phosphatase Staining and Fluorescent Immunocytochemistry

Histochemical staining for alkaline phosphatase (AP) was conducted using the Vector® Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Inc.). Expression of several pluripotency factors was assayed using fluorescent immunohistochemistry conducted at room temperature. Samples from all populations of human cells in T25 culture flasks went through the following steps: (a) the growth medium was removed, (b) washed three times with PBS, c) fixed in -10^{0}C methanol, c) washed three times with PBS, d) incubated for 20 min in 10 % normal serum, e) incubated

for 60 min. in primary antibody diluted in 1.5% normal serum, f) washed three times with PBS, g) incubated for 45 min. in the dark with secondary antibody diluted in 1.5% normal serum, h) washed three times with PBS and left in 3rd rinse, I) examined under an inverted-phase contrast fluorescent microscope, j) PBS replaced with the anti-fading reagent 2% DABCO (Sigma), and k) processed T25 flasks with specimens were sealed with parafilm, wrapped in aluminum foil and stored at 4^{0}C.

The primary and secondary antibodies and normal sera (2.5 µg/mL) included polyclonal goat anti-Oct3/4 IgG, polyclonal goat anti-Nanog IgG, polyclonal goat anti-Sox-2 IgG, monoclonal mouse anti-TRA-1-60 IgG, monoclonal mouse anti-SSEA-1 IgM, polyclonal goat anti-Rex-1 IgG, goat-anti mouse lgM-TR, donkey-anti-mouse lgG-FITC, donkey anti-goat IgG-FITC, donkey anti-goat IgG-TR, normal donkey serum, and normal goat serum (all from Santa Cruz Biotechnology, Inc). Anti-sera to the following were used to analyze formation of neural progenitor cells: nestin (1:500 dilution, BD Pharmingen), beta-3 tubulin monoclonal antibody (B3T; 10 µg/mL; Pierce antibodies), neural cell adhesion molecule (NCAM), 1:500 dilution (Abcam), glial fibrillary acidic protein (GFAP, 1:250 dilution (Abcam). DNA staining was performed using 4',6-diamidino-2-phenylindole, 4',6-diamidinophenyl-indole (DAPI; Santa Cruz Biotechnology, Inc.)

Control Experiments

The controls described in (Table 1) were used to test for the effect of the presence of human cells, oocytes, feeder cells, co-electroporation, and the electroporate on reprogramming (expression of Nanog; detected using fluorescent immunohistochemistry).

Results

Controls

Table 1 lists findings from the control experiments conducted on all human cell types used in this study. Nanog was not detected in human cells from controls "a", "b", "c", and "f" A small number of human cells from control "d," in which non-electroporated human cells were exposed for 3 hours to electroporate, expressed the Nanog gene (reprogramming efficiency of ˜0.4%; calculated only for CD4TLs). A similarly low number of human cells from control "e" expressed the Nanog gene (0.9% efficiency, calculated only for CD4TLs); in this control, human cells were electroporated in the absence of oocytes and then were exposed to electroporate for 3 hours.

BMSC and BMSC$_{GFP}$

Within one week of co-electroporation with *Xenopus laevis* oocytes, cells derived from human BMCS strongly co-cultured with iMEF cells expressed the pluripotency-associated transcription factors Oct3/4, SOX-2, Nanog, Rex-1, and SSEA-1 and formed colonies resembling those known to form by iPSc in culture in culture (Figure 1).

	Electroporation Conditions				Post-Electroporation Incubation Conditions		
Control	Human cells*	Oocytes	iMEF cells*	Electroporation	Human cells*	Other	Nanog Expression
(a)	√			√	√		Negative
(b)	√	√			√		Negative
(c)	√						Negative
(d)		√		√	√	Electroporate from oocytes†	0.4%‡
(e)	√	√		√ (human cells electroporated separately)	√	Electroporate from oocytes†	0.9%‡
(f)			√	√		iMEF cells; complete ES growth media	Negative

*Approximately 10^5 of the following: bone marrow stromal cells, BJ cells, human pre-adiposites, CD4TLs, human buccal mucosa cells, HeLa cells, MCF-7 cells (control experiments conducted separately with each human cell type) ; †oocytes removed from the electroporate prior to incubation; ‡calculated using CD4TLs.

CD4TLs, human CD4+ T-lymphocytes; ES, embryonic cell; iMEF, irradiated mouse embryonic fibroblasts.

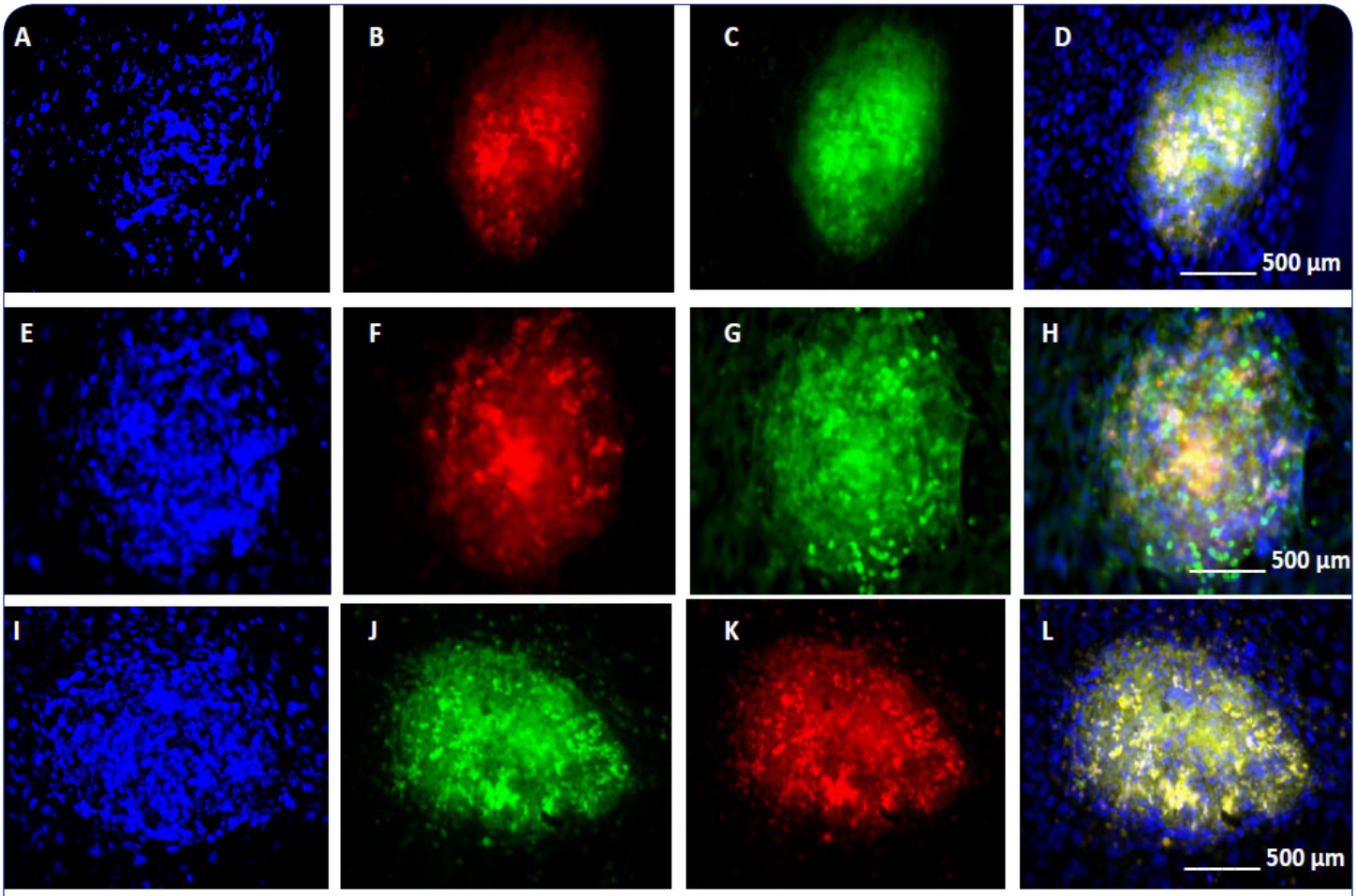

Figure 1. Expression of pluripotency markers by cells derived from human bone marrow stromal cells on d7 following co-electroporation with Xenopus laevis oocytes. (A) – (D) same field; (A) DAPI; (B) Oct 3/4; (C), Sox-2; (D), DAPI, Oct 3/4, and Sox-2 combined; (E) – (H) same field; (E) DAPI; (F) Oct 3/4; (G) Nanog; (H) DAPI, Oct 3/4, and Sox-2 combined; (I) – (L), same field; (I), DAPI; (J) Rex-1; (K) SSEA-1; (L) DAPI, Rex-1, and SSEA-1 combined.

In separate studies, $BMSC_{GFP}$ were co-electroporated with *Xenopus* oocytes and grown on iMEF cells. The resultant cell colonies resembled those of iPSc and contained cells emitting green fluorescence (data not shown).

BJ Cells

Co-electroporation in the presence of *Xenopus* oocytes, followed by co-culture on iMEF feeder cells, resulted in reprogramming of BJ cells, evidenced by a high level of alkaline phosphatase activity and resemblance to iPSc in colony morphology and the expression of Oct3/4, Nanog, SOX-2, TRA-1-60, Rex-1, and SSEA-1 (Figure 2).

HPA Cells–Reprogramming, Cryopreservation, and Trans-differentiation

After co-electroporation of HPA and co-culture on feeder cells, the human cells formed colonies morphologically similar to those of iPSc (Figure 3).The reprogrammed HPA-derived cells displayed strong alkaline phosphatase activity (Figure 3). The cells in these colonies strongly expressed Oct3/4, Nanog, SOX-2, TRA-1-60, Rex-1, and SSEA-1 (Figure 3).

One month after cryopreservation of the reprogrammed HPA-derived cells, the reprogrammed cells were thawed, resulting in 78% viability. By day 4 after subculturing on fresh feeder cells the reprogrammed HPA-derived cells formed secondary clusters resembling those formed by iPSc (data not shown).

Sub culturing cells derived from HPA following co-electroporation in conditions that promote the neural differentiation of embryonic stem cells resulted in formation of cells expressing various immature and mature neural markers including nestin, NCAM, B3T, and GFAP (Figure 4).

CD4TLs – Reprogramming and Efficiency

Within 3 to 5 days after transfer to feeder cell layers following co-electroporation with *Xenopus laevis* oocytes, the human CD4TLs formed colonies similar to those formed by iPSc. Cells in these colonies had high levels of alkaline phosphatase activity (Figure 5) and strongly expressed Oct3/4, Nanog, SOX-2, TRA-1-60, Rex-1, and SSEA- (Figure 6).

Within 12 to 24 hours after co-electroporation with *Xenopus laevis*

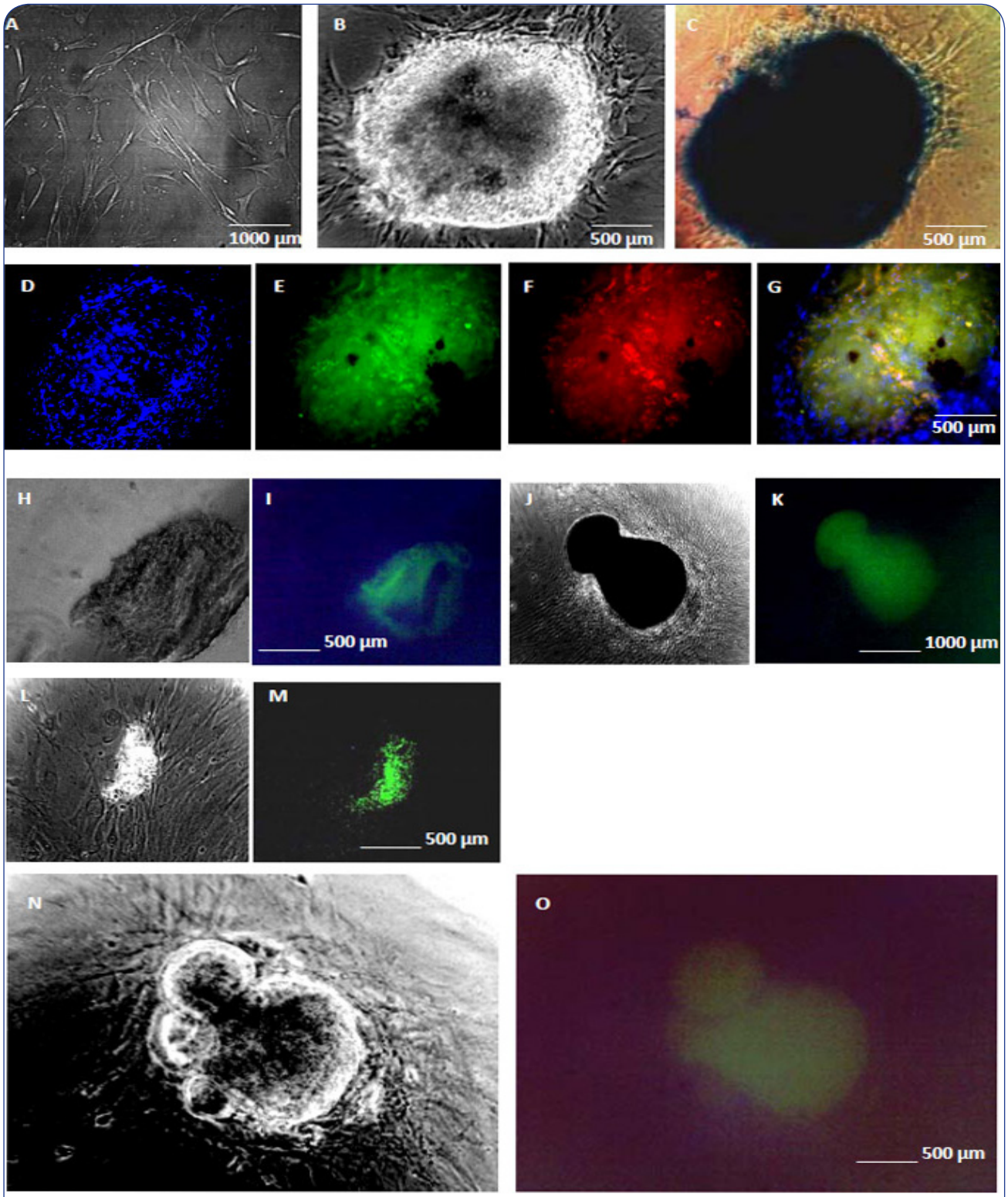

Figure 2. Expression of pluripotency markers by cells derived from BJ cells following co-electroporation with Xenopus laevis oocytes. (A) control cells (no co-electroporation); (B) – (C) same field, d5; (B) phase contrast; (C) alkaline phosphatase; (D) – (G) same field on d5; (D) DAPI; (E) Oct 3/4; (F) Nanog; (G) DAPI, Oct 3/4, and Nanog; (H) – (I) same field, d9; (H) phase contrast, (I) TRA-1-60; (J) – (K) same field, d9; (J) phase contrast; (K) Rex-1; (L) – (M) same, field, d11; (L) phase contrast; (M) SSEA-1; (N) – (O) same field, d5; (M) phase contrast; (N) Sox-2.

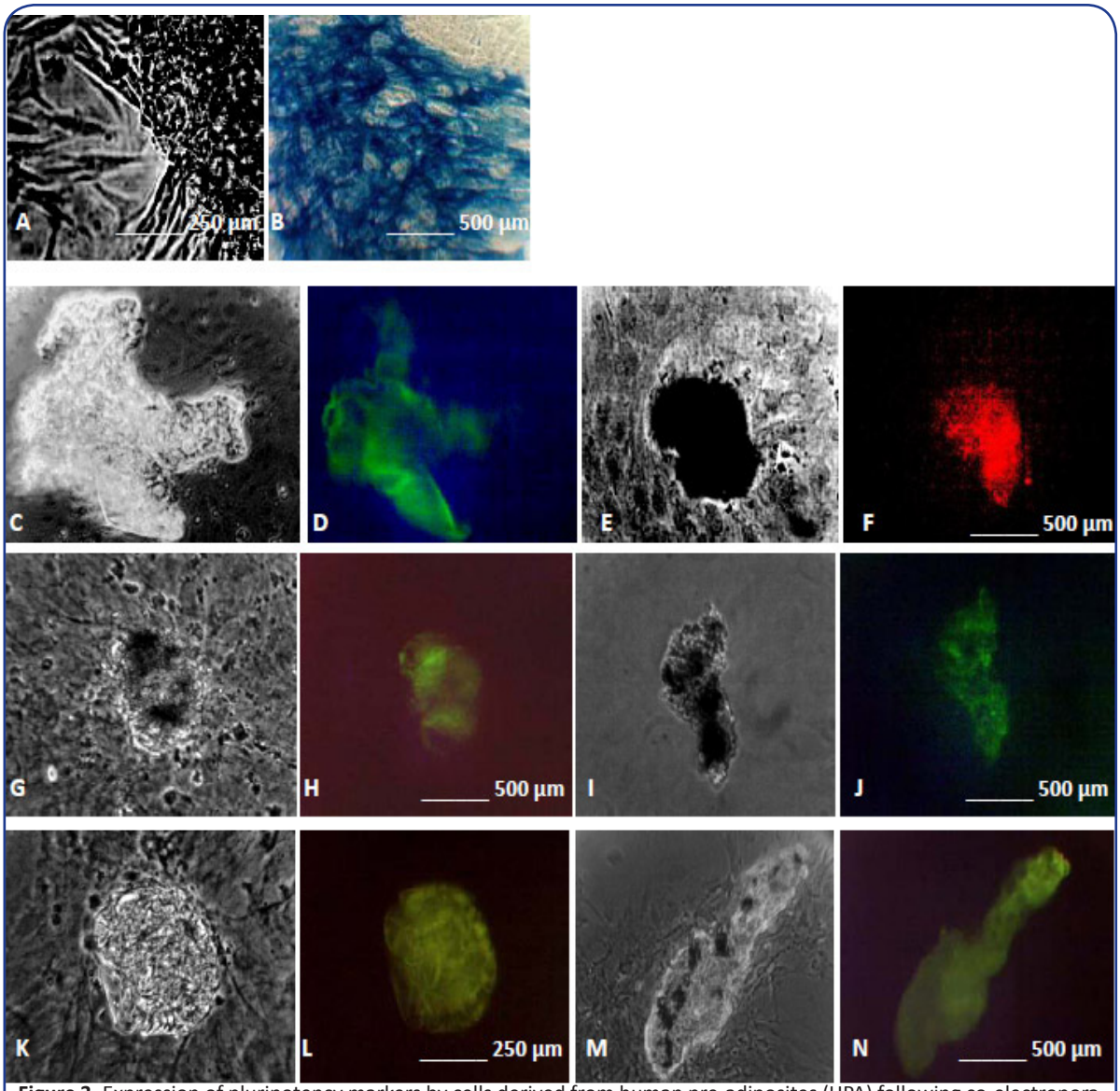

Figure 3. Expression of pluripotency markers by cells derived from human pre-adiposites (HPA) following co-electroporation with Xenopus oocytes. (A) cluster of cells on d5 using phase contrast; (B) alkaline phosphatase; (C) – (D) same field at d5; (C) phase contrast; (D) Oct 3/4; (E) – (F) same field, d5; (E) phase contrast; (F) Nanog; (G) – (H), same field, d10; (G) phase contrast; (H) Sox-2; (I) – (J) same field, d9; (I) phase contrast; (J) TRA-1-60; (K) – (L), same field, d11; (K) phase contrast, (L) Rex-1; (M) – (N) same field, d10; (M) phase contrast, (N) SSEA-1.

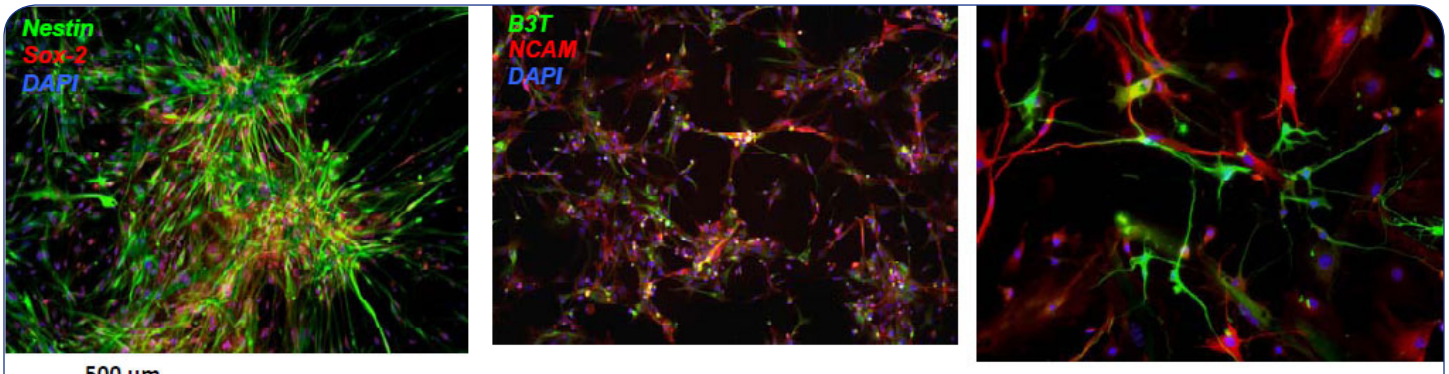

Figure 4. Expression of neural markers by cells derived from human pre-adiposites following culture under conditions that promote neural progenitor differentiation by embryonic stem cells.

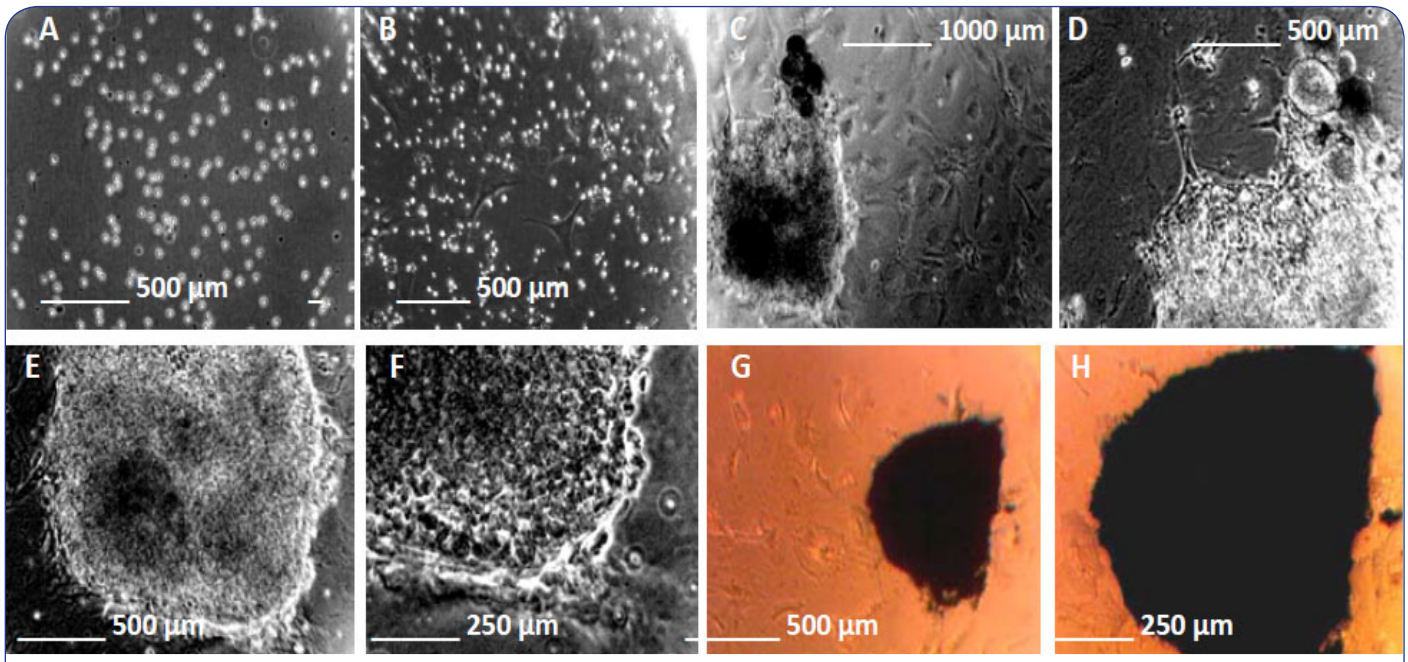

Figure 5. Cells derived from human CD4+ T-lymphocytes following co-electroporation with Xenopus laevis oocytes. (A) control, no co-electroporation; (B) no co-electroporation, culture on irradiated mouse embryonic fibroblasts; (C) – (D) cell culture on d5 following co-electroporation; (E) – (F) lower part of cluster in (D); (G) – (H) alkaline phosphatase on d9.

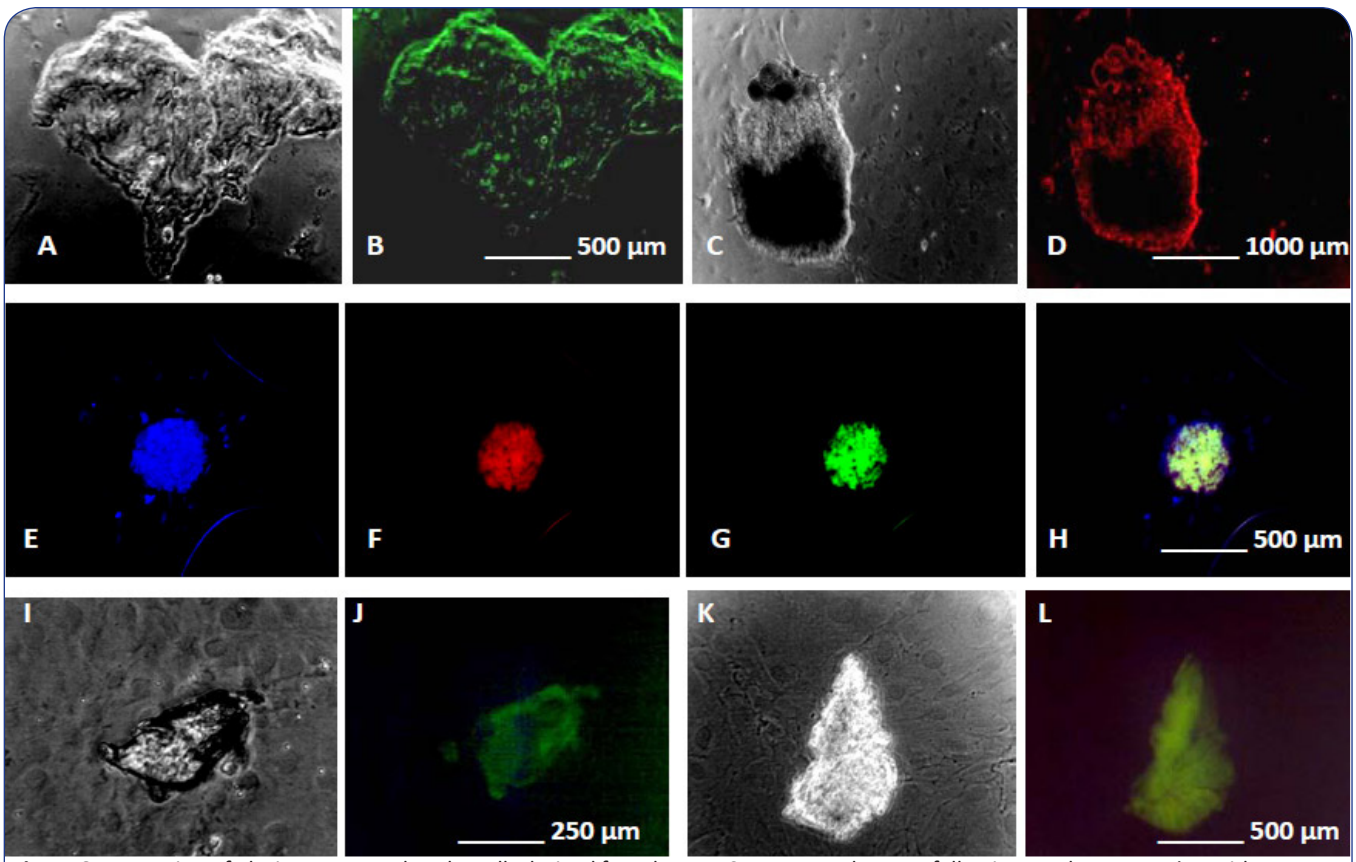

Figure 6. Expression of pluripotency markers by cells derived from human CD4+ T-Lymphocytes following co-electroporation with Xenopus laevis oocytes. (A) – (B), same field, d10; A, phase contrast; (B) Oct 3/4; (C) – (D) same field, d10; (C) phase contrast; (D) Nanog; (E) – (H) same field, d5; (E) DAPI; (F) Rex-1; (G) Sox-2; (H) DAPI, Rex-1, and Sox-2; (I) – (J) same field, d9; (I) phase contrast; (J) TRA-1-60; (K) – (L), same field, d10; (K) phase contrast; (L) SSEA-1.

oocytes, the cells derived from human CD4TLs co-cultured with iMEF started to express the Nanog gene. During this time period, single cells and small iPSc-like clusters in which individual cells could be counted were present (data not shown). The proportion of cells expressing Nanog and the total number of cells were counted for calculation of reprogramming efficacy, which was 23.4 ±3.5%.

Human Buccal Mucosa Cells

Freshly obtained human buccal mucosa cells, co-electroporated in the presence of *Xenopus* oocytes and cultured on iMEF and on feeder cell-free StemAdhere™ substrate, gave rise to cells that formed colonies similar to those of iPSc (Figure 7). Cells in these colonies expressed Oct3/4, Nanog, SOX-2, TRA-1-60, Rex-1, and SSEA-1 (Figure 8).

HeLa and MCF-7 Cells

Two human cancer cell lines, HeLa and MCF-7, were subjected to co-electroporation with *Xenopus laevis* oocytes followed by co-culture on iMEF. The cells derived from co-electroporation of these tumor cells showed partial dedifferentiation, with formation of clusters and expression of Oct 3/4 (HeLa-derived cells and MCF-7-derived cells. Figure 9) and Nanog (MCF-7-derived cells; Figure 9). The cell clusters tended to be smaller than those derived from co-electroporation of non-tumor cells (data not shown).

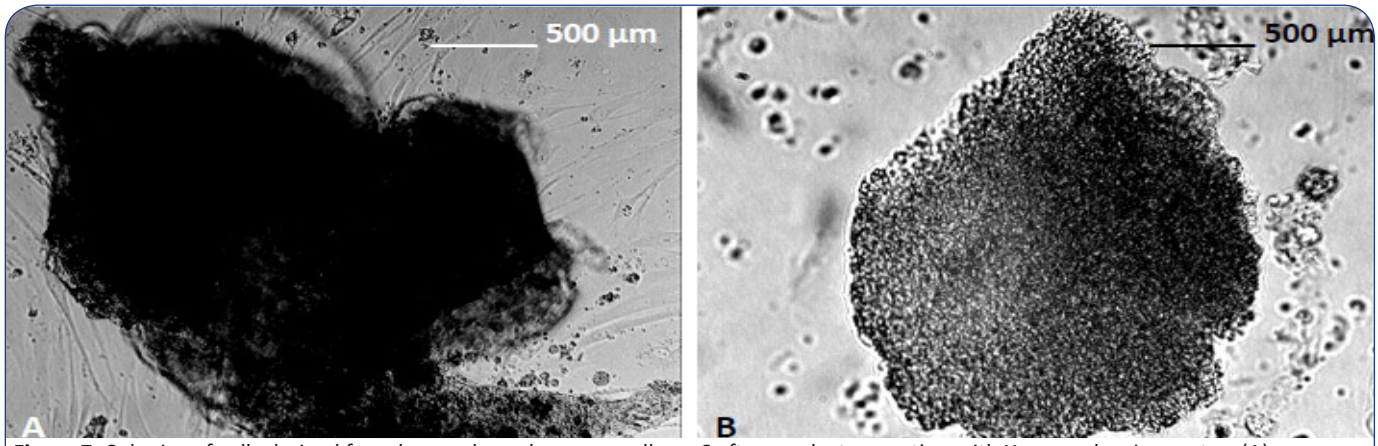

Figure 7. Colonies of cells derived from human buccal mucosa cells on 6 after co-electroporation with Xenopus laevis oocytes. (A) grown on irradiated mouse embryonic fibroblast substrate; (B) grown on StemAdhere™ substrate.

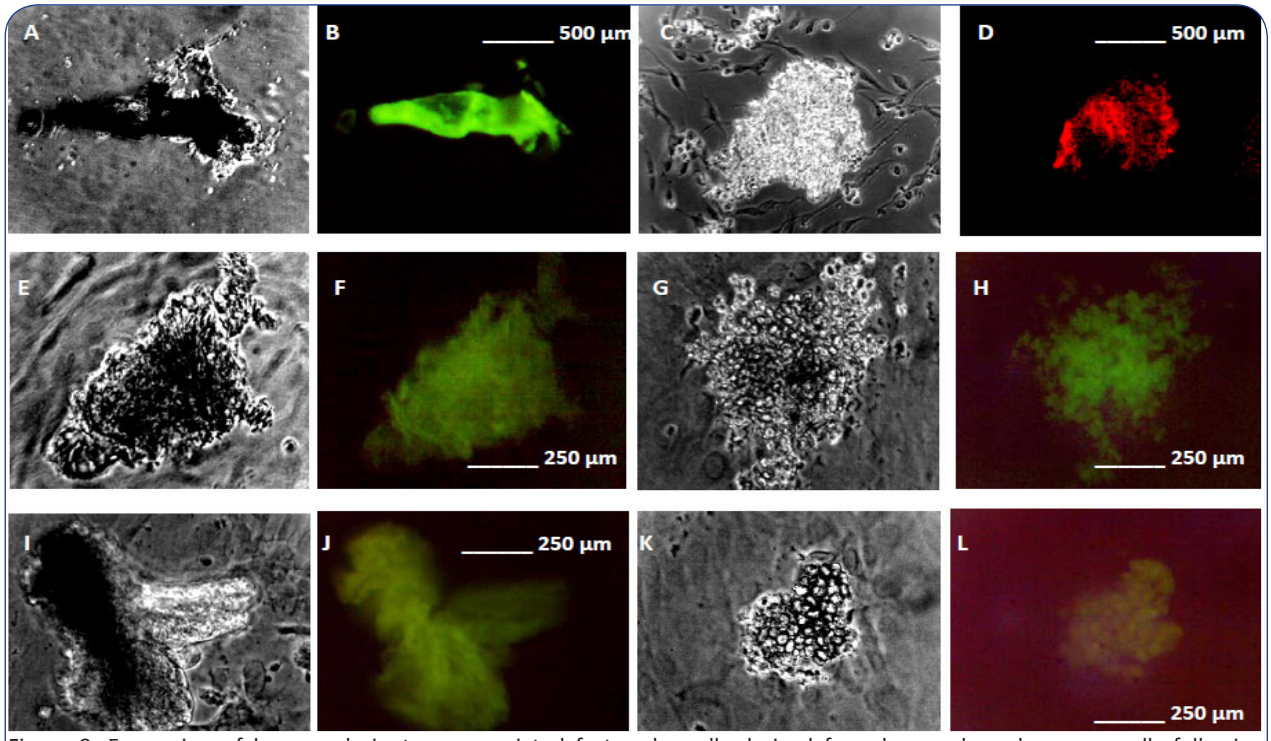

Figure 8. Expression of human pluripotency-associated factors by cells derived from human buccal mucosa cells following co-electroporation with Xenopus laevis oocytes. (A) – (B) same field, 96 h; (A) phase contrast; (B) Oct 3/4; (C) – (D) same field, d10; (C) phase contrast; (D) Nanog; (E) – (F) same field, d10; (E) phase contrast; (F) Sox-2; (G) – (H) same field, d9, (G) phase contrast; (H) TRA-1-60; (I) – (J), same field, d11; (I) phase contrast; (J) Rex-1; (K) – (L) same field, d11; (K) phase contrast; (L) SSEA-1.

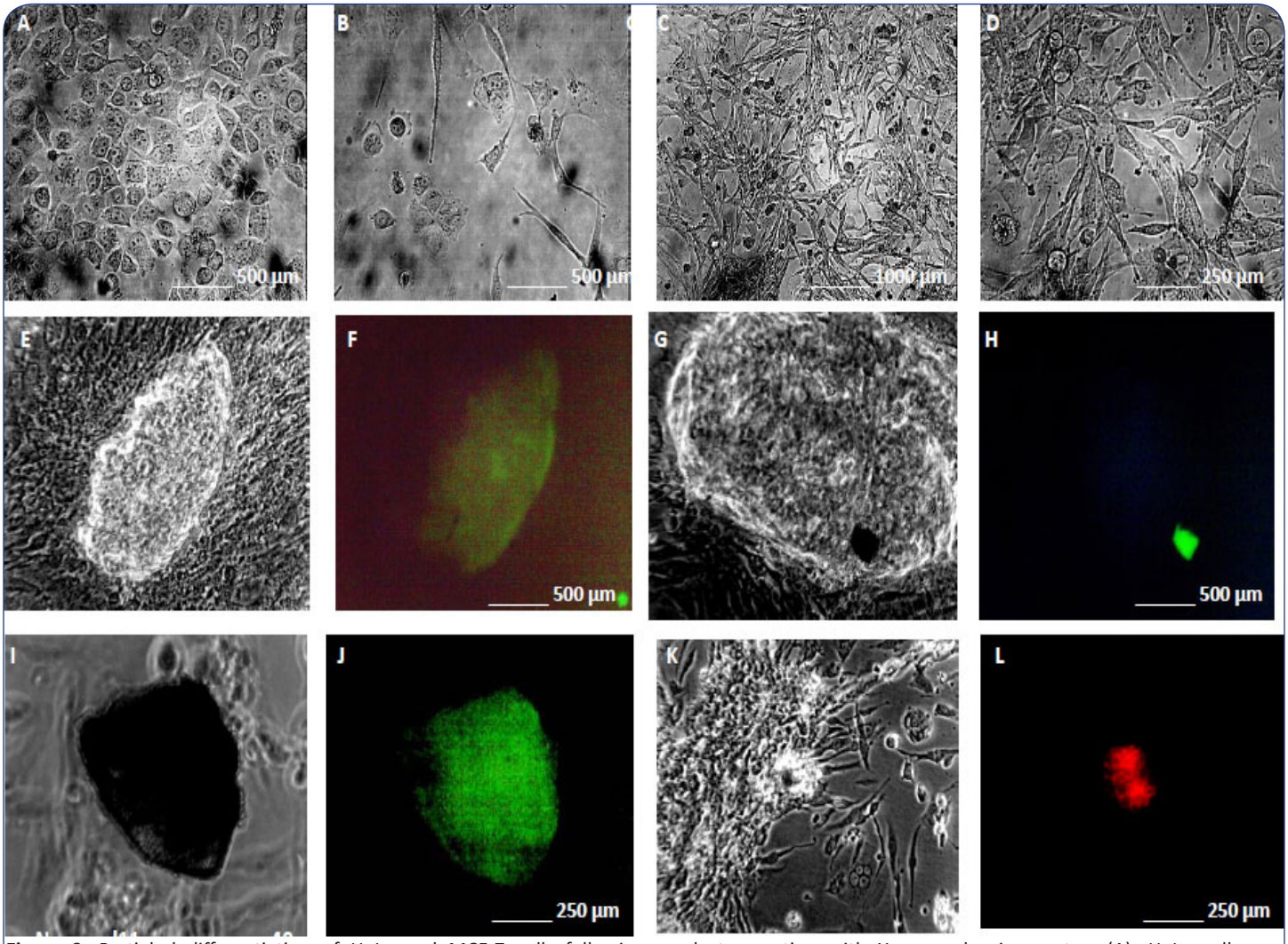

Figure 9. Partial dedifferentiation of HeLa and MCF-7 cells following co-electroporation with Xenopus laevis oocytes. (A), HeLa cells, no co-electroporation; (B) HeLa cells grown on irradiated mouse embryonic fibroblast cells, no co-electroporation; (C) MCF-7 cells, no co-electroporation; (D) MCF-7 cells grown on irradiated mouse embryonic fibroblast cells, no co-electroporation; (E) – (H) cells derived from HeLa cells following co-electroporation with Xenopus laevis oocytes; (E) – (F), same field, d11; (E) phase contrast; (F) Oct 3/4; (G) phase contrast; (H) Oct 3/4; (I) – (L) MCF-7 cells following co-electroporation with Xenopus laevis oocytes; (G) – (H) same field, d11; (G) phase contrast; (H) Oct 3/4; (I) – (J) same field, d11; (I) phase contrast; (J) Nanog.

Discussion

The limited ability of many human tissues to regenerate has spurred interest in methods to produce iPSc for therapeutic applications. We evaluated a new methodology for the non-viral reprogramming of cells into iPSc. Using co-electroporation of living *Xenopus laevis* oocytes with various human normal and cancer cell lines, we obtained cells resembling iPSc as evidenced by colony morphology and expression of human iPSc markers.

Human bone marrow stromal cells showed signs of reprogramming into cells resembling iPSc, with colony formation and strong expression the pluripotency-associated transcription factors Oct3/4, Nanog, SOX-2, TRA-1-60, Rex-1, and SSEA-1. The isolation of human BMSCs represents a routine procedure at many hospitals, and this new method for the generation of human BMSC-derived iPSc may present opportunities for their therapeutic applications in humans.

Because of the pioneering studies on successful retroviral vector-mediated reprogramming of fibroblast cell lines, [31] we tested reprogramming of BJ cells using *Xenopus laevis* oocyte co-electroporation. Cultured BJ cells were reprogrammed into cells that resembled iPSc. These cells formed clusters with high AP activity and strong expression of major stem-cell markers, namely Oct3/4, Nanog, SOX-2, TRA-1-60, Rex-1, SSEA-1. The successful reprogramming of BJ cells provided further evidence of the effectiveness of this reprogramming method.

We also tested this reprogramming method using HPA because

these cells can be safely isolated. The HPA were purchased from a commercial source that obtained HPA from less than 50 g of human adipose tissue obtained from a clinic that performs liposuction. Co-electroporation with *Xenopus laevis* oocytes resulted in reprogramming the HPA into cells resembling iPSc. We also showed that these HPA-derived deprogrammed cells could be cryopreserved, thawed, subcultured, and trans-differentiated into cells expressing neural and neural progenitor markers. These data suggest that this reprogramming technology may have the potential for large-scale production of inexpensive human iPSc from adipose tissue.

Reprogramming of T cells to produce stem cells for adoptive transfer constitutes an important area of interest in immune-based oncology therapy [32]. While human CD4TL can be easily isolated from as little as 5 mL of peripheral blood, this specific cell type has not been a common research target for producing iPSc [33]. We demonstrated that our protocol reprogrammed CD4TLs into cells resembling iPSc with formation of clusters on iMEF feeder cells, high alkaline phosphatase activity, and expression of pluripotency-associated transcription factors.

The oral mucosa contains cells that can be obtained without invasive techniques, but the literature on reprogramming of these cells is limited. Miyoshi et al reported production of iPSc from oral mucosa cells (obtained from oral biopsy tissue) using retroviral transfer of Oct-4, Sox-2, c-Myc, and KLF4 [34]. Using co-electroporation with *Xenopus laevis* oocytes, we were able to reprogram cells from the buccal mucosa (obtained non-invasively) to resemble iPSc. Furthermore, these reprogrammed cells could be subcultured and were able to grow on the feeder-free StemAdhere™ substrate. The success with reprogramming of buccal mucosa cells suggests the possibility of generation of large amounts of human autologous stem cells from this easily obtained tissue.

The prospect for converting cancer cells into normal or benign quiescent cells using a reprogramming approach, which can alter cellular transcription programs, is widely discussed in the scientific literature. Experimental approaches includes reverting adult neoplasms, [35] epigenetic reprogramming of breast cancer cells by valproic acid, [36] miRNA reprogramming of human skin cancer, [37] reprogramming of human cancer cells in the mouse mammary gland by exposure to mammary epithelial cells, [38] and viral-mediated transfer of stem cell transcription factors to reprogram colorectal cells [39]. Using co-electroporation with *Xenopus laevis* oocytes, we observed that cells from these human cervical carcinoma and breast adenocarcinoma cell lines partially de-differentiated. The cells formed iPSc-like clusters, with some cells expressing Oct 3/4. This partially reprogramming may provide a transitional point for potential redifferentiation into normalized cells. Partially reprogrammed cells may also be amenable to trans-differentiational reprogramming [40].

The relatively high rate of reprogramming achieved using his co-electroporation method, 23.4%, is of note. The efficiency of reprogramming reported in the literature includes 0.5% with standard, four-factor retroviral methodology, [41,42] 0.98 %-2.34% when adding two more reprogramming factors ,[10] 2-4% with the use of dox-inducible lentiviruses , [12] and 18% with cell-to-cell extracts. [43] Because our main objective was to test reprogramming using co-electroporation with *Xenopus laevis* oocytes in multiple types of human cell and tissues lines, we limited the evaluation of reprogramming efficacy to human CD4TLs, which could be evaluated for Nanog expression at early stages before formation of tightly clustered colonies. Modifications of the method may improve the reprogramming efficiency. For example, preliminary studies suggest that the reprogramming efficiency can be modulated by fluctuations in barometric pressure and environmental temperature (Bioquark Inc., data on file).

The absence of natural reprogramming signals may result in failure or inconsistency of cellular reprogramming. Compromise of signaling factors may occur during the preparation of crude extracts from *Xenopus laevis* oocytes, in which potentially vital nuclear and cytoplasmic components present in living eggs could be disrupted. Ganier et al observed similarly low efficacy of nuclear transfer and reprogramming of mouse embryonic fibroblasts using pretreatment with *Xenopus laevis* oocyte extracts and with viral-mediated expression of Oct-4, Sox-2, Klf4, and cMyc (OSKM). However, reprogramming efficiency was improved approximately 10-fold when extract pretreatment and viral transfer of the transcription factors were both performed [44]. Using a process that promotes the natural order of reprogramming signals also appears important. Grad et al reported that reprogramming that deviates from what is known of the normal sequence of events (induction of Nanog before OSKM) produced abnormal cells [45].

The results of control experiments strongly suggest that during co-electroporation of living *Xenopus laevis* oocytes, there is transfer of a vital, soluble reprogramming factor or factors to the human cells. This interpretation is supported by low but detectable level of reprogrammimg in controls “d” and “e”, in which human cells that were not co-electroporated with *Xenopus laevis* oocytes were exposed to electroporate from *Xenopus laevis* oocytes. The possibility of multiple factors being necessary is logical considering such a biologically significant cellular event as reprogramming would be regulated through multiple factors and pathways. In addition, successful retroviral and other molecular reprogramming techniques require transfer of multiple transcription factors [31]. Identification, purification, and amplification of active reprogramming components transferred during co-electroporation may provide opportunities for investigation of therapeutic potential.

The results presented above need to be confirmed by independent studies, and further research is needed for full proof of concept of reprogramming cells to pluripotency. Ongoing activities include assessment of redifferentiation and trans-differentiation, molecular karyotyping, DNA fingerprinting, and teratoma formation. Biochemical and molecular analysis of the intrinsic

molecular mechanisms underlying the *Xenopus oocyte*-mediated reprogramming phenomena are in progress.

Conclusion

The data obtained from analysis of human cells subjected to co-electroporation with *Xenopus laevis* oocytes show that this reprogramming system induced human somatic cell reprogramming in a fast, efficient, highly reproducible, standardized fashion in different cell types. The system also provides for easy separation of reprogrammed cells from oocytes and subculture of these cells in the absence of feeder cells.

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Disclosure of Potential Conflicts of Interest

The author is an employee of Bioquark Inc.

References

1. Liras A (2010) Future research and therapeutic applications of human stem cells: general, regulatory, and bioethical aspects. J Transl Med 8: 131.
2. Zhou H, Wu S, Joo JY, Zhu S, Han DW, et al. (2009) Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4(5): 381-384.
3. Jee MK, Kim JH, Han YM, Jung SJ, Kang KS, et al. (2010) DHP-derivative and low oxygen tension effectively induces human adipose stromal cell reprogramming. PLoS One 5(2): e9026.
4. Judson RL, Babiarz JE, Venere M,, et al. (2009) Embryonic stem cell-specific microRNAs promote induced pluripotency. Nat Biotechnol 27(5): 459-461.
5. Lin SL, Chang DC, Ying SY, Leu D, Wu DT (2010) MicroRNA miR-302 inhibits the tumorigenecity of human pluripotent stem cells by coordinate suppression of the CDK2 and CDK4/6 cell cycle pathways. Cancer Res 70(22): 9473-9482.
6. Lin SL, Chang DC, Lin CH, Ying SY, Leu D, et al. (2011) Regulation of somatic cell reprogramming through inducible mir-302 expression. Nucleic Acids Res 39(3): 1054-1065.
7. Anokye-Danso F, Trivedi CM, Juhr D, Gupta M, Cui Z, et al. (2011) Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell 8(4): 376-388.
8. Miyoshi N, Ishii H, Nagano H, Haraguchi N, Dewi DL, et al. (2011) Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell 8(6): 633-638.
9. Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, et al. (2009) Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat Biotechnol 27(9): 851-857.
10. Markoulaki S, Hanna J, Beard C, Carey BW, Cheng AW, et al. (2009) Transgenic mice with defined combinations of drug-inducible reprogramming factors. Nat Biotechnol 27(2): 169-171.
11. Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, et al. (2008) Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol 26(11): 1269-1275.
12. Wernig M, Lengner CJ, Hanna J, Lodato MA, Steine E, et al. (2008) A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. Nat Biotechnol 26(8): 916-924.
13. Yoshida Y, Takahashi K, Okita K, Ichisaka T, Yamanaka S (2009) Hypoxia enhances the generation of induced pluripotent stem cells. Cell Stem Cell 5(3): 237-241.
14. Hong H, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, et al. (2009) Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature 460(7259): 1132-1135.
15. Cho HJ, Lee CS, Kwon YW, Paek JS, Lee SH, et al. (2010) Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. Blood 116(3): 386-395.
16. Oh SI, Lee CK, Cho KJ, Lee KO, Cho SG,, et al. (2012) Technological progress in generation of induced pluripotent stem cells for clinical applications. Scientific World Journal 2012: 417809.
17. Kastenberg ZJ, Odorico JS (2008) Alternative sources of pluripotency: science, ethics, and stem cells. Transplant Rev (Orlando) 22(3): 215-222.
18. Barrilleaux B, Knoepfler PS (2011) Inducing iPSCs to escape the dish. Cell Stem Cell 9(2): 103-111.
19. Katoh M, Chen G, Roberge E, Shaulsky G, Kuspa A (2007) Developmental commitment in Dictyostelium discoideum. Eukaryot Cell 6(11): 2038-2045.
20. Zhao J, Morozova N, Williams L, Libs L, Avivi Y,, et al. (2001) Two phases of chromatin decondensation during dedifferentiation of plant cells: distinction between competence for cell fate switch and a commitment for S phase. J Biol Chem 276(25): 22772-22778.
21. Kim YD, Kang SM, Min JY, Choi WK, Jeong MJ, et al. (2010) Production of tropane alkaloids during de-differentiation of Scopolia parviflora calli. J Nat Prod 73(2): 147-150.
22. Straube WL, Tanaka EM (2006) Reversibility of the differentiated state: regeneration in amphibians. Artif Organs 30(10): 743 –755.
23. Miyamoto K, Furusawa T, Ohnuki M, Goel S, Tokunaga T, et al. (2007) Reprogramming events of mammalian somatic cells induced by Xenopus laevis egg extracts. Mol Reprod Dev 74(10): 1268-1277.
24. Rand M, Kalishman J (2001) Xenopus care, health & disease: A brief overview. New York, NY: Columbia University.
25. Buono RJ, Linser PJ (1992) Transgenic zebrafish by electroporation. Bio-Rad. EU/EG Bulletin 1354.
26. Hostetler HA, Peck SL, Muir WM (2003) High efficiency production of germ-line transgenic Japanese medaka (Oryzias latipes) by electroporation with direct current-shifted radio frequency pulses. Transgenic Res 12(4): 413-424.

27. Granneman JG, Li P, Lu Y, Tilak J (2004) Seeing the trees in the forest: selective electroporation of adipocytes within adipose tissue. Am J Physiol Endocrinol Metab 287(3): E574-E582.

28. Doetschman T (2002) Gene Targeting in Embryonic Stem Cells. A Lab Handbook. San Diego, CA: Academic Press.

29. Peterson S, Loring JF, Wesselschmidt RL, Schwartz PH (2007) Human Stem Cell Manual, A Laboratory Guide: Academic Press.

30. Axell MZ, Zlateva S, Curtis M (2009) A method for rapid derivation and propagation of neural progenitors from human embryonic stem cells. J Neurosci Methods 184(2): 275-284.

31. Okita K, Yamanaka S (2011) Induced pluripotent stem cells: opportunities and challenges. Philos Trans R Soc Lond B Biol Sci 366(1575): 2198-2207.

32. Stroncek DF, Berger C, Cheever MA, Childs RW, Dudley ME, et al. (2012) New directions in cellular therapy of cancer: a summary of the summit on cellular therapy for cancer. J Transl Med 10: 48.

33. Chun YS, Byun K, Lee B (2011) Induced pluripotent stem cells and personalized medicine: current progress and future perspectives. Anat Cell Biol 44(4): 245-255.

34. Miyoshi K, Tsuji D, Kudoh K, Satomura K, Muto T, et al. (2010) Generation of human induced pluripotent stem cells from oral mucosa. J Biosci Bioeng 110(3): 345-350.

35. Frenster JH, Hovsepian JA (2007) Models of embryonic gene-induced initiation and reversion of adult neoplasms. Presented at the AACR-NCI-EORTC International Conference: "Molecular Targets and Cancer Therapeutics: Discovery, Biology, and Clinical Applications".

36. Travaglini L, Vian L, Billi M, Grignani F, Nervi C (2009) Epigenetic reprogramming of breast cancer cells by valproic acid occurs regardless of estrogen receptor status. Int J Biochem Cell Biol 41(1): 225-234.

37. Lin SL, Chang DC, Chang-Lin S, Lin CH, Wu DT, et al. (2008) Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. RNA 14(10): 2115-24.

38. Bussard KM, Boulanger CA, Booth BW, Bruno RD, Smith GH (2010) Reprogramming human cancer cells in the mouse mammary gland. Cancer Res 70(15): 6336-6343.

39. Dewi DL, Ishii H, Haraguchi N, Nishikawa S, Kano Y, et al. (2012) Dicer 1, ribonuclease type III modulates a reprogramming effect in colorectal cancer cells. Int J Mol Med 29(6): 1060-1064.

40. Sareen D, Svendsen CN (2010) Stem cell biologists sure play a mean pinball. Nat Biotechnol 28: 333-335.

41. Meissner A, Wernig M, Jaenisch R (2007) Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat Biotechnol 25(10): 1177-1181.

42. Condic ML, Rao M (2008) Regulatory issues for personalized pluripotent cells. Stem Cells 26(11): 2753-2758.

43. Hakelien AM, Landsverk HB, Robl JM, Skalhegg BS, Collas P (2002) Reprogramming fibroblasts to express T-cell functions using cell extracts. Nat Biotechnol 20(5): 460-466.

44. Ganier O, Bocquet S, Peiffer I, Brochard V, Arnaud P, et al. (2011) Synergic reprogramming of mammalian cells by combined exposure to mitotic Xenopus egg extracts and transcription factors. Proc Natl Acad Sci USA 108(42):17331-17336.

45. Grad I, Hibaoui Y, Jaconi M, Chicha L, Bergstrom-Tengzelius R, et al. (2011) NANOG priming before full reprogramming may generate germ cell tumours. Eur Cell Mater 22: 258-274.