Video Article

Isolation of Adult Human Dermal Fibroblasts from Abdominal Skin and Generation of Induced Pluripotent Stem Cells Using a Non-Integrating Method

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Abstract

Induced pluripotent stem cells (iPSCs) could be considered, to date, a promising source of pluripotent cells for the management of currently untreatable diseases, for the reconstitution and regeneration of injured tissues and for the development of new drugs. Despite all the advantages related to the use of iPSCs, such as the low risk of rejection, the lessened ethical issues, and the possibility to obtain them from both young and old patients without any difference in their reprogramming potential, problems to overcome are still numerous. In fact, cell reprogramming conducted with viral and integrating viruses can cause infections and the introduction of required genes can induce a genomic instability of the recipient cell, impairing their use in clinic. In particular, there are many concerns about the use of c-Myc gene, well-known from several studies for its mutation-inducing activity. Fibroblasts have emerged as the suitable cell population for cellular reprogramming as they are easy to isolate and culture and are harvested by a minimally invasive skin punch biopsy. The protocol described here provides a detailed step-by-step description of the whole procedure, from sample processing to obtain cell cultures, choice of reagents and supplies, cleaning and preparation, to cell reprogramming by the means of a commercial non-modified RNAs (NM-RNAs)-based reprogramming kit. The chosen reprogramming kit allows an effective reprogramming of human dermal fibroblast to iPSCs and small colonies can be seen as early as 24 h after the first transfection, even with modifications with the respect to the standard datasheet. The reprogramming procedure used in this protocol offers the advantage of a safe reprogramming, without the risk of infections caused by viral vector-based methods, reduces the cellular defense mechanisms, and allows the generation of xeno-free iPSCs, all critical features that are mandatory for further clinical applications.

Video Link

The video component of this article can be found at https://www.jove.com/video/60629/

Introduction

Cell reprogramming represents a novel technology to transform every somatic cell of the body into a pluripotent stem cell, known as iPSC[1]. The possibility of reprogramming an adult somatic cell back to a pluripotent and undifferentiated state has overcome the limits imposed by the poor availability and ethical issues related to the use of pluripotent cells, previously only derivable from human embryos (embryonic stem cells or ESC)[2,3,4]. In 2006, Kazutoshi Takahashi and Shinya Yamanaka conducted a pioneering study achieving the first conversion of adult somatic cells from skin into pluripotent cells by artificially adding four specific genes (Oct4, Sox2, Klf4, c-Myc)[5]. A year later, work conducted in Thomson's laboratory led to the successful reprogramming of somatic cells into iPSCs by transduction of a different combination of four genes (Oct4, Sox2, Nanog, Lin28)[6].

iPSCs offer a number of opportunities to scientists and researchers of different fields, such as regenerative medicine and pharmacology, being an excellent platform to study and treat different diseases along with a genotypic reflection of the characteristics of the patient they are derived from. The use of iPSCs provides several advantages including: the reduced risk for immune response due to a completely autologous origin of cells; the possibility of creating a cell library, an important tool to predict response to new drugs and their side effects, as they are able to continuously self-renew and generate different cell types; and the chance to develop a customized approach for drug administration[7,8,9].

Diverse techniques are known at present, to induce the expression of the reprogramming factors and they are included in two major categories: non-viral and viral vector-based methods[10,11,12,13]. Non-viral methods include mRNA transfection, miRNA infection/transfection, PiggyBac, minicircle vectors and episomal plasmids and exosomes[10,11,12,13]. Viral-based methods include non-integrating viruses, such as Adenovirus, Sendai virus and proteins, and integrating viruses like Retrovirus and Lentivirus[10,11,12,13].

According to several studies, no significant differences have been noticed among these methods in terms of effectiveness of cell reprogramming, hence, the choice of the suitable method strictly depends on the cell type used and on the subsequent applications of the iPSCs obtained[14,15]. All

the mentioned methods show disadvantages, for example, the Sendai virus is effective on all cell types, but requires a lot of passages to obtain iPSCs; reprogramming by episomes is excellent for blood cells but needs modification of standard culture conditions for fibroblasts; the PiggyBac method could represent an attractive alternative but studies in human cells are still limited and weak[10,11,12,13]. Exosomes are nano-vesicles physiologically secreted into all body fluids by cells. According to recent studies, they are responsible for intercellular communication and can have a role in important biological processes, such as cell proliferation, migration and differentiation. Exosomes can transport and transfer mRNA and miRNA to recipient cells with a completely natural mechanism, as they share the same composition of the cell membrane[16]. Therefore, exosomes are a promising new generation technique for reprogramming, but their potential to reprogram somatic cells by their content is still under investigation. Viral vectors-based methods use viruses modified in order to convey reprogramming genes to recipient cells. This technique, despite the high efficiency of reprogramming, is not considered safe, as the integration of the virus within the cell can be responsible for infection, teratomas and genomic instability[17].

The following protocol to generate iPSCs colonies combines the Yamanaka's and Thompson's reprogramming cocktail and is based upon the use of a method requiring NM-RNAs and immune evasion factors with the possibility to perform it in xeno-free conditions. The rationale behind the use of this method is to spread, within the scientific community, a protocol allowing a rapid, simple and highly effective reprogramming of adult human fibroblasts from abdominal skin into iPSCs[18].

The strengths of the proposed method are, in fact, the ease of performance and the short time needed to obtain iPSCs. Furthermore, the method avoids cellular defense mechanisms and the use of viral vectors, responsible for relevant issues.

With respect to the standard protocol, the following modifications were made: (1) Confluent fibroblasts were synchronized at passage 4 by being placing in 0.1% serum for 48 h before the trypsinization; (2) The cellular density for culture and the volume of reagents were adjusted for the utilization on a 24-well multi-well plate instead of a 6-well plate; (3) The reprogramming experiment was performed using a 5% CO_2 incubator instead of an incubator with atmospheric (21% O_2) or hypoxic (5% O_2) conditions.

Protocol

The specimens from human tissue were collected according to the Declaration of Helsinki while observing University Hospital Federico II guidelines. All patients involved in this study provided written consent.

1. Preparation of Supplies and Culture Media

1. Clean and autoclave one large pair of surgical scissors, two sets of fine forceps, two pairs of microdissecting scissors, 1 L sterile bottle, 500 mL sterile bottle, and a 250 mL sterile bottle.
2. Prepare 100 mm plates, 60 mm plates, 35 mm plates, disposable scalpels, 50 mL sterile tubes, 15 mL sterile tubes, and a 100 mm glass plate. Store instruments under sterile conditions until ready for use.
3. Clean and sterilize 22 mm x 22 mm cover glasses before use. For this, place cover glasses in a glass plate, cover them with 70% ethanol and wait for a few seconds before aspirating the ethanol. Let cover glasses dry in an oven at 37 °C and then autoclave them.
4. Prepare 1 L of Hank's balanced salt solution (HBSS) at pH 7.4 by dissolving commercially available powdered salts in double-distilled sterile water and adding 0.35 g of sodium bicarbonate. Sterilize by filtration under a sterile hood and store at +4 °C until use.
5. Prepare 500 mL of sterile 1x phosphate-buffered-saline (PBS) by dissolving 0.1 g of potassium phosphate monobasic, 0.1 g of potassium chloride, 4.0 g of sodium chloride and 0.575 g of sodium phosphate dibasic in sterile double-distilled water. Check the pH value (7.4) and sterilize under a sterile hood by filtration. Store at +4 °C until use.
6. Prepare 50 mL of trypsin stop solution (TSS) by adding 5 mL of 10% fetal bovine serum (FBS) to 45 mL of HBSS under a sterile hood. Store at +4 °C until use.
7. Prepare the Dulbecco's modified Eagle medium for fibroblast isolation (I-DMEM) using 250 mL of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 0.5% penicillin and streptomycin (pen/strep) under a sterile hood. Store at +4 °C until use.
8. Prepare the DMEM for fibroblasts synchronization (S-DMEM) using 250 mL of DMEM supplemented with 0.1% FBS and 0.5% pen/strep under a sterile hood. Store at +4 °C until use.

2. Isolation of Human Dermal Fibroblasts

NOTE: Steps 2.1 to 2.3 reported below must be performed under a sterile hood. A cylindrical sample measuring about 0.8 cm in diameter yields 2 x 10^6 fibroblasts at passage 1.

1. Wash the freshly obtained sample of human skin from abdomen in a 100 mm dish with HBSS solution. Gently shake to remove blood and other biological fluids. Repeat the step three times, changing the HBSS solution and the plate at each wash.
2. Place the sample in a 100 mm plate, remove hair and fat using fine forceps and scissors, and dissect it with a scalpel to obtain 2 mm x 1 mm fragments (for a total of 16 fragments) avoiding scars, dirty (e.g., as resulting from drawings with dermographic pen) or burned areas of the tissue.
3. Place 4 small fragments in each 35 mm dish, cover with a sterile 22 mm x 22 mm cover glass. Adjust by the means of fine forceps. Add 1.5 mL of I-DMEM.
4. Incubate the plates at 37 °C in 5% CO_2 for about 15 days or until cells reach 85% confluence. Change the culture medium every 3 days and check the outgrowth of cells using an inverted phase-contrast microscope daily.

3. Expansion of Human Skin Fibroblasts

NOTE: The steps reported below must be performed under a sterile hood except the steps performed in the incubator.

1. Lift the cover glasses with fine forceps, place them upside-down in one 100 mm dish and wash with 1x sterile PBS.
2. Remove fragments of the samples and discard them, wash plates with 1x sterile PBS.
3. Remove 1x PBS and add 3 mL and 1 mL of trypsin-EDTA (Ethylenediaminetetraacetic acid) to cover glasses placed in the 100 mm dish and 35 mm dishes, respectively. Incubate for 5 min at 37 °C with 5% CO_2.
4. Block the trypsinization by adding 5 mL of TSS to the 100 mm dish and 2 mL of TSS to each 35 mm dish. Collect the suspension into 15 mL sterile tubes, centrifuge at 400 x *g* at 4 °C for 5 min.
5. Aspirate the supernatant and resuspend the pellet in 12 mL of I-DMEM. Split the cell suspension into 3 mL aliquots for each 60 mm plate.
6. Incubate at 37 °C with 5% CO_2. Change the medium daily. Keep cells in culture until they reach a confluence of 75%.
7. Aspirate the medium from the plates and quickly wash in 1x PBS.
8. Repeat trypsinization (steps 3.3 and 3.4) three times to obtain cells at passage 4. Use 1.5 mL of trypsin-EDTA and 3 mL of TSS in each 60 mm dish. Split the cells 1:3.
9. Synchronize the cells by replacing the I-DMEM with S-DMEM and incubate for 48 h at 37 °C with 5% CO_2.
10. Prepare the following (during the cell synchronization).
    1. Prepare fibroblast expansion medium by adding 5.0 mL of FBS and 0.5 mL of L-glutamine to 44.5 mL of advanced DMEM (A-DMEM), store at +4 °C.
    2. Prepare total NM-RNA-reprogramming cocktail for fibroblast reprogramming by combining the following in each of 9 sterile RNase-free tubes: 32 µL of OSKMNL NM-RNA, 24 µL of EKB NM-RNA, 5.6 µL of NM-microRNAs (61.6 µL final volume for 9 aliquots).
    3. Divide each tube of 61.6 µL of total NM-RNA-reprogramming cocktail for fibroblast reprogramming into four 15.4 µL single-use aliquots in sterile, RNase-free tubes and store at -80 °C (15.4 µL final volume for 36 aliquots).

4. Reprogramming of Dermal Fibroblasts to iPSCs

1. **Day 0: Cell seeding**
   NOTE: Steps 4.1.1 to 4.1.3 and 4.1.8 to 4.1.9 must be performed under a sterile hood. Basement membrane matrix (BMM) gels rapidly at room temperature. Therefore, it is strongly recommended to thaw BMM overnight on ice in a refrigerator, dilute it with ice-cold serum-free medium and use pre-cooled pipets, tips, and tubes.
    1. Prepare a 24-well plate by coating each well with 100 µL of BMM and incubate for at least 1 h at 37 °C before seeding the cells.
    2. Aspirate the medium from plates with cells in culture and quickly wash in 1x PBS.
    3. Repeat trypsinization (steps 3.3 and 3.4) to obtain cells at passage 5. Aspirate the supernatant and resuspend the pellet in an appropriate volume of fibroblasts expansion medium (see step 3.10).
    4. Prepare and clean the hemocytometer with 70% alcohol.
    5. Prepare a solution by gently mixing 10 µl of trypan blue and 10 µL of cell suspension in 1.5 mL tube, and incubate for 1–2 min at room temperature. Be careful not to incubate longer than 5 min.
    6. Pipette 10 µL of the solution into the hemocytometer place it on the stage of an inverted phase contrast microscope to count. Non-viable cells will be blue, while viable cells will be unstained.
    7. Count the cells in at least 2 squares of the hemocytometer chamber. Apply the following calculation to obtain the total number of cells: **total number of cells = (total number of counted cells/square number) x 2 x 10 x total volume of cell suspension**
    8. Perform a dilution to obtain a density of 2.5 x 10^4 cell per 500 µL of fibroblasts expansion medium, based on the total number of counted cells. Resuspend the pellet in an appropriate volume of fibroblasts expansion medium.
    9. Pipette 500 µL of the cell suspension into each well of the 24-well plate. Incubate cells overnight at 37 °C and 5% CO_2.

2. **Day 1: Transfection**
   NOTE: All working surfaces and supplies (e.g., gloves, bottles, sterile hood surfaces, pipettors) MUST be cleaned with cleaning reagent for removing RNase before starting the procedure. Perform steps 4.2.2, 4.2.4–4.2.8 under a sterile hood.
    1. Warm up xeno-free, serum-free,low growth factor human ESC/iPSC culture medium (XF/FF culture medium) in a 37 °C water bath.
    2. Remove the old medium from each well and replace it with 500 µL of pre-warmed XF/FF culture medium.
    3. Incubate at 37 °C under 5% CO_2 for at least 6 h.
    4. Thaw five 15.4 µL aliquots of total NM-RNA reprogramming cocktail at room temperature then place it on ice.
    5. Add 234.6 µL of reduced-serum medium to each aliquot, gently pipette 3–5 times and label each one as **TUBE A** (RNA + reduced-serum medium).
    6. Label 5 sterile, RNase-free 0.5 mL tubes as **TUBE B** and mix 6 µL of synthetic siRNA transfection reagent with 244 µL of reduced-serum medium (RNA-transfection reagent + reduced-serum medium).
    7. Add the contents of each TUBE B to a TUBE A dropwise (to obtain 500 µL final volume of NM-RNA transfection complex solution). Mix by tapping the bottom of the tube. Incubate at room temperature for 15 min.
    8. Add 125 µL of NM-RNA transfection complex solution from each of the five aliquots of 500 µL to 4 wells (to reach a total of 20 wells), tilting the plate and pipetting dropwise into the medium. Mix by rocking gently. Use the remaining 4 wells as a reference.
    9. Incubate for 15 h at 37 °C, 5% CO_2.

3. **Day 2–4: Complete the transfection**
    1. Aspirate the medium. Repeat the transfection procedure as on day 1 under the sterile hood.

4. **Day 5–10: Media changes**
    1. Aspirate the medium and exchange with fresh, pre-warmed XF/FF culture medium under a sterile hood.
    2. Incubate overnight at 37 °C, 5% CO_2.
    3. Keep in culture until observing the formation of iPSC colonies monitoring daily by a phase-contrast microscope.

Representative Results

The aim of the protocol was to reprogram dermal fibroblasts isolated from abdominal skin using non-integrating reprogramming method based on NM-RNAs to induce the expression of specific factors. To achieve this goal, human dermal fibroblasts were isolated from skin specimens of patients undergoing tummy tuck surgery and iPSCs were generated introducing Oct4, Sox2, Klf4, cMyc, Nanog, Lin28 reprogramming factors and E3, K3, B18 immune evasion factors by a commercial ready-to-use reprogramming kit that combines NM-RNA and microRNA technology. The timeline of the protocol is summarized in **Figure 1**.

Human fibroblasts outgrew from samples of abdominal skin within one week of culture (**Figure 2A**) and reached 85% confluence within two weeks (**Figure 2B**). Cells were characterized by adhesive growth on plastic culture dishes and their morphology varied from elongated and spindle-shaped (**Figure 2C**) to flattened and star-shaped (**Figure 2D**). Fibroblast morphology and arrangement in culture dramatically changed after seeding on BMM, when they acquired an elongated morphology and arranged to form thin branched structures (**Figure 3A**). Small colonies were already visible in culture at day 1 from first transfection (**Figure 3B**) and their size grew progressively over time, while their number increased till day 7 and then remained stable between day 7 and day 14 (**Figure 3C,D**), probably as a result of smaller colonies merging to form larger colonies.

Since the reprogramming procedure is performed using antibiotic-free media, microbial contamination is a major issue and it may occur (**Figure 4**) if sterile conditions are not guaranteed Adopt standard procedures to prevent contamination (e.g., wearing gloves, remove dust from all surfaces, clean all surfaces and equipment with 70% ethanol, avoid talking during steps with uncovered cell culture plates).

Figure 1: Protocol timeline. Timeline of isolation and reprogramming of human fibroblasts from abdominal skin. Please click here to view a larger version of this figure.

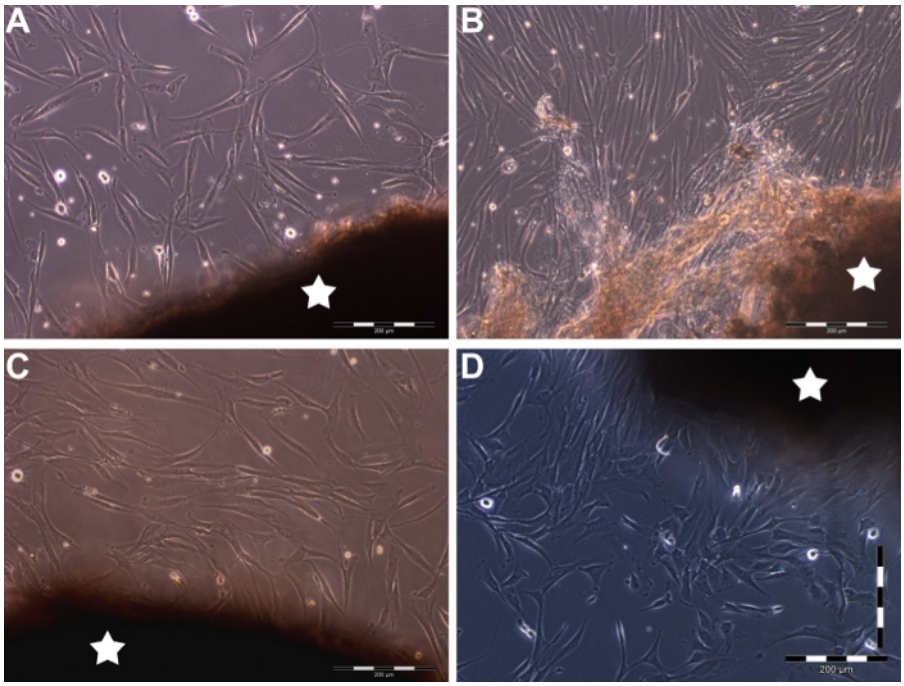

Figure 2: Isolation and culture of dermal fibroblasts. Representative images of fibroblasts outgrowth from the skin fragments (**A**). Fibroblasts reached confluence in about 14 days (**B**) and showed spindle-shaped (**C**) and star shaped (**D**) phenotype. Skin fragments are indicated by a white star. Scale bar = 200 µm. Please click here to view a larger version of this figure.

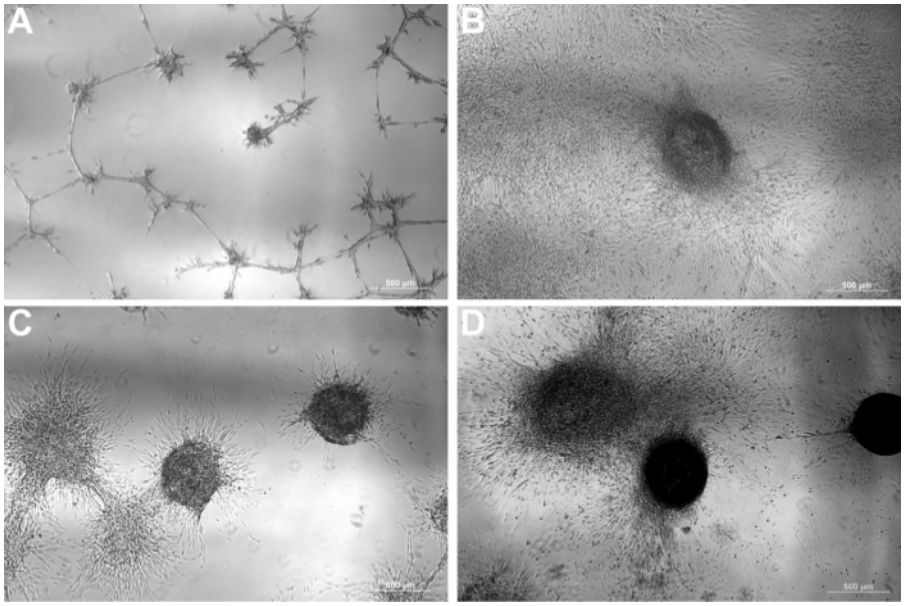

Figure 3: Skin fibroblast reprogramming progression. Cells seeded on BMM showed a marked change in arrangement and morphology (**A**). Small colonies of iPSCs formed as early as 24 h after the first transfection (**B**) and their size increased progressively at day 7 (**C**), and 14 (**D**), while their number increased between day 1 and day 7 but remained stable between day 7 and 14 from first transfection. Scale bar = 500 µm. Please click here to view a larger version of this figure.

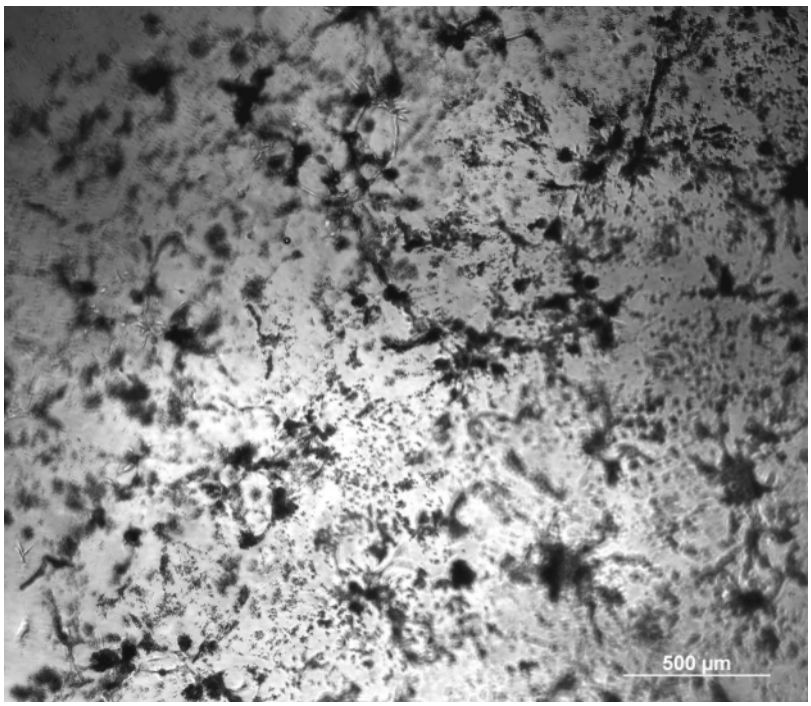

Figure 4: Microbial contamination during reprogramming procedure. Representative image using phase-contrast microscopy showing a microbial contamination of fibroblasts culture during the reprogramming procedure. Scale bar = 500 µm. Please click here to view a larger version of this figure.

Discussion

iPSCs are rapidly emerging as the most promising cell candidate for regenerative medicine applications and as a tremendously useful tool for disease modeling and drug testing[3,8]. The protocol presented here describes the generation of human iPSCs from a sample having the size of a skin punch biopsy with a simple and efficient procedure that does not require any specific equipment or previous experience with reprogramming technology.

It is of primary importance to optimize fibroblast isolation and culture to increase chance of success, as plating density and proliferation rate impact reprogramming efficiency. Further, it has been recently reported that dermal fibroblasts respond differently to reprogramming technology

and, specifically, fibroblasts isolated from the skin of the abdominal region are more easily and readily reprogrammed than dermal fibroblasts isolated from other body regions[18]. Therefore, it is important to accurately select the somatic cells to reprogram and define the plating density on the basis of cell proliferation rate. To this aim, the present protocol also instructs on how to isolate dermal fibroblasts from abdominal skin and how to propagate them in vitro to ease and accelerate the procedure.

Prior to reprogramming, fibroblasts were propagated to reach passage 5 to erase cell memory[18] and comply with reported evidence that passaging could accelerate the induction of pluripotent state[19]. Moreover, according to our experience, fibroblasts in culture exhibit variable proliferative properties, thus we modified the protocol by introducing an additional step to synchronize fibroblasts and reduce variability in the cell population.

Using a commercial NM-RNAs-based kit that introduces a combination of reprogramming factors resulting from Yamanaka's and Thomson's approach[5,6], along with miRNA that have been proven to improve mRNA-based reprogramming[20], human dermal fibroblasts can be successfully reprogrammed to iPSC and small colonies become apparent as early as 24 h after the first transfection. The main advantages of mRNA-based reprogramming are, indeed, the early emergence of colonies even when a low number of cells is reprogrammed[20] along with a very low aneuploidy rate and the complete absence of integration that make iPSCs generated with this method safe for a use in regenerative medicine. Moreover, the commercial kit used for this protocol co-delivers immunomodulating factors that are known to improve the efficiency of reprogramming by preventing the cell death caused by cytotoxic and immunogenic NM-RNAs[21,22].

Although the kit used for reprogramming was designed for 6-well multi-well plate, we optimized cellular density and adjusted the volume of reagents for the utilization on a 24-well multi-well plate to make the protocol effective for the generation of human iPSCs from a skin punch biopsy. Moreover, even though if the need for a tissue culture incubator with O_2 control is reported by several authors[20,23] and recommended by the kit manufacturer, following the protocol described here we reprogrammed human dermal fibroblasts from abdominal skin in a standard 5% CO_2 incubator. Therefore, the procedure can be performed in any cell culture laboratory without the need for atmospheric (21% O_2) or hypoxic (5% O_2) culture conditions, although these might further improve reprogramming efficiency[24,25].

Nonetheless, we used non-human animal-derived reagents to derive from small skin specimens iPSCs that can be used for research purposes. Although the most attractive application is for the regeneration of tissues and organs, iPSCs have been used for modeling different diseases and then both investigate on underlying molecular mechanisms and develop specific drugs and therapies[3,26].

Remarkably, substituting animal-derived reagents for appropriate xeno-free reagents, the same protocol allows the reprogramming of adult human fibroblasts into iPSCs in a complete xeno-free culture environment that warrants their clinical use.

However, the heavy workload needs to be taken into consideration. In our opinion, it is critical to plan the experiment carefully well in advance, including steps that need to be performed during weekends, and to prepare all transfection reagents during the synchronization step. Indeed, transfection is to be performed every day for four days and, afterwards, cells need to be monitored every day and medium is to be replaced on a daily basis.

Furthermore, the accurate identification of iPSC colonies cannot be based solely upon morphological criteria. Hence, newly derived iPSCs need to be characterized searching for the expression of multiple pluripotent markers through cellular and molecular analyses. Widely accepted markers include NANOG, OCT4, SOX2, TRA-1-60, TRA-1-81 and SSEA4, which may be identified by immunocytochemical analysis and by gene expression analysis using semi-quantitative or quantitative RT-PCR. Since alkaline phosphatase activity has been shown to be upregulated in pluripotent stem cells, the detection of such enzymatic activity can be easily carried out to identify iPSCs and verify the occurrence of reprogramming[27,28].

Disclosures

The authors have nothing to disclose.

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