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Non-modified RNA-Based Reprogramming of Human Dermal Fibroblasts into Induced Pluripotent Stem Cells

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Abstract

The generation of pluripotent stem cells from adult somatic cells by cell reprogramming has put a whole new perspective on stem cell biology and stem cell-based regenerative medicine. Cell reprogramming acts through the introduction of key genes that regulate and maintain the pluripotent cell state. In this chapter, we describe the optimized protocol for the efficient isolation of fibroblasts from a skin punch biopsy and the subsequent easy and effective generation of integration-free induced pluripotent stem cell (iPSC) colonies forcing the expression of specific factors by non-modified RNAs.

Key words Cell Reprogramming, Dermal Fibroblasts, Induced Pluripotent Stem Cells (iPSCs), Integration-free, Non-modified RNAs (NM-RNAs), Regenerative Medicine

1 Introduction

Induced Pluripotent Stem Cells (iPSCs) are adult somatic cells forced to dedifferentiate and acquire an embryonic stem (ES) cell-like state by the introduction of specific genes. First obtained in Yamanaka's lab from skin fibroblasts reprogrammed by retroviral transduction of four transcription factors, Oct4, Sox2, Klf-4, and c-Myc [1, 2], iPSCs have rapidly gained recognition for their enormous potential as adult somatic cell-derived pluripotent stem cells whose usage is not restricted by ethical issues [3, 4]. Indeed, iPSCs offer a unique platform for in vitro or in vivo disease modeling and in-depth pathophysiology research and for development and testing of new drugs. Additionally, as pluripotent cells, they can give rise to any cell of the body and then used for personalized regenerative treatment [5–7].

The surge of enthusiasm fired by Yamanaka's groundbreaking work has led to the development of alternate methods to reprogram somatic cells based either on genome-integrating vectors or on non-integrating vectors [8]. Undoubtedly, integration-free techniques ensure better safety of reprogrammed cells and when

procedure is carried out in xeno-free environment the iPSCs generated are suitable for therapeutic applications [9].

Among the non-integrating reprogramming systems, the mRNA-based is probably the most versatile and the most appropriate for clinical production of stem cells, as reprogramming is achieved without genome integration and complete elimination of reprogramming vectors does not require extended culture [10].

The Stemgent StemRNA 3rd Gen Reprogramming Kit (Reprocell) combines non-modified RNAs (NM-RNAs) and microRNA technology to deliver the six reprogramming factors Oct4, SOX2, KLF4, c-Myc, Nanog, and LIN28, in accordance to Yamanaka's and Thompson's approaches [11], along with immune evasion factors to control mRNA immunogenicity and allow repeated transfection [12, 13]. The kit is specifically designed for the reprogramming of human fibroblasts and the procedure can be performed in xeno-free conditions to generate clinically relevant iPSCs.

Dermal fibroblast has been the first human cell reprogrammed to iPSCs [1, 2] and, albeit the reprogramming potential of other somatic cells has been explored [14], the easiness of harvesting and propagation in culture make dermal fibroblasts still the most suitable cell source for cell reprogramming. Skin is the largest organ of the human body and dermal fibroblasts can be derived from several regions. Recent report has demonstrated that positional memory of dermal fibroblasts affects their response to reprogramming technology with fibroblasts isolated from abdominal skin that are more prone to reprogram [15].

We have recently reported a simple, highly effective, and reproducible protocol for the isolation of human dermal fibroblasts from the abdominal skin and their reprogramming into iPSCs by NM-RNA [16]. Here we describe a detailed protocol for the rapid and efficient conversion of human dermal fibroblast isolated from a skin punch biopsy of abdominal skin (Fig. 1) into integration-free iPSCs using the Stemgent StemRNA 3rd Gen Reprogramming Kit from Reprocell. The method, once passage 5 fibroblasts have been obtained, allows the induction of pluripotency as early as 24 h after the first transfection and the generation of iPSC colonies as early as 10 to 14 days after the transfection (Fig. 2). Further, xeno-free iPSCs can be obtained when the procedure is carried out in appropriate environment.

2 Materials

Maintain sterility for all components. Store instruments in sterile conditions until ready for use. Antibiotics (Penicillin and Streptomycin) are not added to all components that come into contact with cells (*see* **Note 1**).

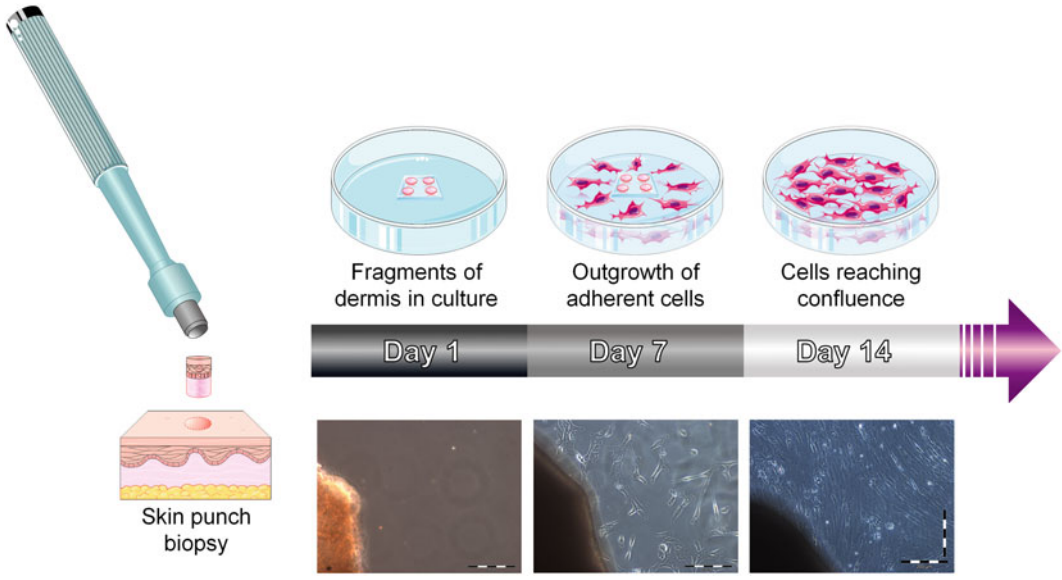

Fig. 1 Skin fibroblasts isolation and expansion. Samples harvested by a skin punch biopsy are dissected in small fragments and placed in culture plates, under coverglasses, with a specific medium to allow fibroblasts outgrowth. The fibroblasts obtained are then expanded in order to reach the passage required to start the reprogramming step. The figure also shows phase-contrast microscope images of fibroblasts during their outgrowth from skin fragments at day 1, day 7, and after 14 days of culture

2.1 Equipment

1. Biosafety cabinet.
2. Phase-contrast microscope.
3. Tissue culture incubator.
4. Autoclave.
5. Ice-maker machine.
6. Pipet-Aid.
7. 1-μL pipetman.
8. 10-μL pipetman.
9. 100-μL pipetman.
10. 1000-μL pipetman.
11. Tube rack.
12. Hemocytometer (Neubauer Chamber).

2.2 Plasticware and Other Disposables

1. 35-mm plates.
2. 60-mm plates.
3. 100-mm plates.
4. 24-well culture plates.
5. 0.22-μM syringe filter.
6. 2-mL cryogenic vials.

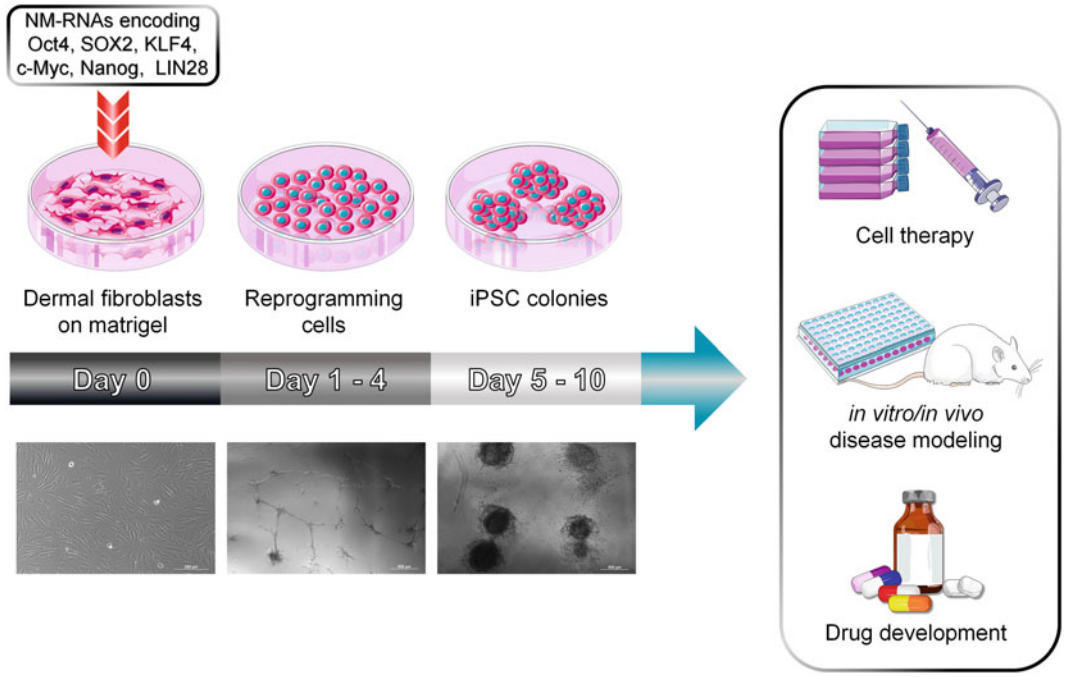

Fig. 2 Reprogramming steps. Previously synchronized fibroblasts are cultured on a Matrigel layer (day 0) and undergo a 4-day-long transfection (day 1–4) with a cocktail of reprogramming factors, then kept in culture and observed until the formation of iPSCs colonies (day 5–10). The first colonies are visible after only one day of transfection. Phase-contrast microscope images show fibroblasts progressively approaching and first arranging in star-shaped and threadlike aggregates, finally in dome-shaped iPSCs colonies. These steps are performed in a 24-well plate, the scheme shows a single well of the plate to simplify. The figure also provides information about the further use of iPSCs obtained for research purposes, disease modeling, and drug testing

7. 22 × 22-mm coverglasses (*see* **Note 2**).
8. 10-μL tips.
9. 100-μL tips.
10. 1000-μL tips.
11. γ-Irradiated individually wrapped polystyrene 5-mL pipettes.
12. γ-Irradiated individually wrapped polystyrene 10-mL pipettes.
13. γ-Irradiated individually wrapped polystyrene 25-mL pipettes.
14. γ-Irradiated 15-mL conical tube.
15. γ-Irradiated 50-mL conical tube.
16. RNase-free 1.5 mL tubes.
17. RNase-free 0.5 mL tubes.
18. Surgical scissors.
19. Fine forceps.
20. Microdissecting scissors.
21. 250-mL sterile bottles

22. 500-mL sterile bottles
23. 1-L sterile bottles
24. Disposable scalpels.
25. Waste container.

2.3 Cell Culture Media

1. Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich; Cat. No. D6429—500 mL) with 10% Fetal Bovine Serum (Sigma-Aldrich; Cat. No. F9665—500 mL) and 0.5% Penicillin and Streptomycin (Sigma-Aldrich; Cat. No. P4333—100 mL): used for dermal fibroblast isolation and culture (I-DMEM).
2. Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich; Cat. No. D6429—500 mL) with 0.1% Fetal Bovine Serum (Sigma-Aldrich; Cat. No. F9665—500 mL) and 0.5% Penicillin and Streptomycin (Sigma-Aldrich; Cat. No. P4333—100 mL): used for fibroblasts synchronization (S-DMEM).
3. Advanced-DMEM (Thermo Fisher; Cat. No. 12491015) with 10% Fetal Bovine Serum (Sigma-Aldrich; Cat. No. F9665—500 mL) and 1% L-glutamine (Lonza; Cat. No.BE17-605E): used for fibroblast expansion during the reprogramming procedure (Fibroblast Expansion Medium).

2.4 Cell Culture Solutions

1. Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich; Cat. No. H1387—1 L) at pH 7.4 with 0.35 g/L of sodium bicarbonate: used for dermal fibroblast isolation.
2. Phosphate-Buffered-Saline (PBS) at pH 7.4: Potassium Phosphate Monobasic 0.2 g/L, Potassium Chloride 0.2 g/L, Sodium Chloride 8 g/L, Sodium Phosphate Dibasic: used for dermal fibroblast culture and reprogramming.
3. Trypsin Stop Solution (TSS): HBSS with 10% Fetal Bovine Serum: used for cell passaging.
4. Trypan Blue (Thermo Fisher Scientific; Cat. No.15250061): used for cell counting.

2.5 Enzymes

1. Trypsin-EDTA (Sigma-Aldrich; Cat. No. T4049—100 mL): used to detach cells during cell passaging.

2.6 Materials for Reprogramming

1. RNaseZap (Invitrogen; Cat. No. AM9780): cleaning agent for removing RNase (*see* **Note 3**).
2. Stemgent StemRNA-NM Reprogramming Kit (Reprocell; Cat. No. 00-0076): used for fibroblast reprogramming, by preparing Total NM-RNA-Reprogramming Cocktail, combining the following in each sterile, RNase-free tube: OSKMNL

NM-RNA 32 μL, EKB NM-RNA 24 μL, NM-microRNAs 5.6 μL (61.6 μL final volume for 9 aliquots).

3. NutriStem XF/FF Culture Medium (Biological Industries; Cat. No. 05-100-1A—500 mL): used for culturing fibroblasts during and after the reprogramming procedure.
4. Opti-MEM Reduced Serum Medium (Thermo Fisher; Cat. No. 31985-062—100 mL): used to prepare the reprogramming cocktail.
5. Lipofectamine RNAiMAX Transfection Reagent (Invitrogen; Cat. No. 13778-030): used to prepare the reprogramming cocktail.

2.7 Extracellular Matrix Components

1. Matrigel (Corning; Cat. No. 354234), basement membrane matrix: used for coating culture dishes during the reprogramming procedure (*see* **Note 4**).

3 Methods

3.1 Isolation, Culture, and Expansion of Fibroblasts from Human Dermis (Fig. 1)

1. Wash the sample of human abdomen dermis in a 100 mm dish with HBSS solution for three times (*see* **Note 5**).
2. Once the sample is washed, place it in a clean 100 mm plate, and remove all the hair and fat by fine forceps.
3. Dissect the sample, using a scalpel, in several 2 × 1 mm fragments (*see* **Note 6**).
4. Place 4 small fragments in each 35 mm dish, cover with a sterile 22 × 22 mm coverglass adjusted by the means of fine forceps and add 1.5 mL of I-DMEM (*see* **Notes 7** and **8**).
5. Incubate the plates at 37 °C in 5% CO_2 for about 15 days, changing the culture medium regularly every 3 days.
6. Check daily the outgrowth of cells at an inverted phase-contrast microscope.
7. Ensure that the cells have reached 85% confluence checking the plates at an inverted phase-contrast microscope.
8. Wash the coverglasses with 1 mL of 1× sterile PBS lifting them from fragments using fine forceps and placing them upside-down in 100 mm dishes.
9. Discard skin fragments and wash plates with 1× sterile PBS.
10. After removing PBS from plates, add 1 mL of trypsin-EDTA and let incubate for 5 min at 37 °C with 5% CO_2.
11. Add 2 mL of TSS to each plate to block the trypsinization and collect all the suspension into 15 mL sterile tubes, centrifugate at 4 °C for 5 min, at 400 × *g*.

12. Discard the supernatant aspirating it and resuspend the pellet collected on the bottom of each tube by adding 12 mL of I-DMEM (*see* **Note 9**).
13. Split the cell suspension into 3 mL for each 60 mm plate (1:3) and incubate at 37 °C with 5% CO_2.
14. Check the cells and change the medium daily, until reaching 75% confluence.
15. Once 75% confluence is reached, aspirate the medium from the plates and make a quick wash in 1× PBS.
16. **Steps 10–13** must be repeated three times to obtain cells at passage 4.
17. Before performing the reprogramming steps, replace the I-DMEM with S-DMEM and incubate the cells for 48 h at 37 °C with 5% CO_2 to allow their synchronization.
18. While synchronizing cells, prepare the Fibroblast Expansion Medium and Total NM-RNA-Reprogramming Cocktail for Fibroblast Reprogramming for the further use.

3.2 Reprogramming Steps (Fig. 2)

3.2.1 Day 0: Cell Seeding

1. Before performing the seeding of the cells, coat 20 wells of a 24-well plate distributing 100 μL of Corning Matrigel Matrix each and incubate for at least 1 h, at 37 °C.
2. Discard the medium from plates containing cell cultures and perform a quick wash in 1× PBS.
3. To detach and collect expanded cells, repeat **steps 10** and **12**, then aspirate the supernatant and resuspend the pellet on the bottom of the tubes in an appropriate volume of Fibroblasts Expansion Medium.
4. To count the cells, clean the Neubauer Chamber with 70% alcohol. Prepare the solution for cell counting by mixing 10 μL of Trypan Blue and 10 μL of cell suspension in 0.5 mL tube, then incubate for 1–2 min at room temperature.
5. Pipette 10 μL of the solution in the Neubauer Chamber, observe and count cells at an inverted phase-contrast microscope (*see* **Note 10**).
6. Calculate the number of cells in the suspension by counting them in at least 2 squares of the chamber (*see* **Note 11**).
7. Once obtained the number of total cells, dissolve the pellet in a volume of Fibroblasts Expansion Medium so as to obtain a density of 2.5×10^4 for each 500 μL.
8. Distribute 500 μL of the cell suspension in each of the 20 wells of the 24-well plate.
9. Incubate cells, now at passage 5, overnight at 37 °C and 5% CO_2.

*3.2.2 Day 1: Transfection (See **Note 12**)*

1. Set the water bath at 37 °C and warm up NutriStem Medium.
2. Discard the medium from each well of the 24-well plate, replacing it with 500 μL of NutriStem Medium and incubate at 37 °C, 5% CO2 for at least 6 h.
3. Prepare Tubes A (RNA + Opti-MEM) as follows: Thaw five 15.4 μL aliquots of Total NM-RNA Reprogramming Cocktail at room temperature and place on ice; add 234.6 μL of Opti-MEM to each aliquot, gently pipetting 3–5 times to mix well and label as Tube A.
4. Prepare 5 sterile, RNase-free 0.5 mL tubes and label as Tubes B, mix 6 μL of Lipofectamine RNAiMAX Transfection Reagent with 244 μL of Opti-MEM (RNAiMAX + Opti-MEM).
5. Prepare 500 μL of NM-RNA Transfection Complex Solution by adding dropwise the content of Tube B to Tube A (250 μL + 250 μL = 500 μL), mix by tapping the bottom of the tube on the shelf of the hood, and incubate at room temperature for 15 min.
6. Pipette 125 μL of NM-RNA Transfection Complex Solution dropwise into the medium of each well, by gently tilting and rocking the plate to mix.
7. Incubate for 15 h at 37 °C, 5% CO_2 in incubator.

3.2.3 Day 2–4: Repeat and Complete the Transfection

1. Replace the old medium and complete the transfection by repeating the procedure as reported in day 1.

3.3 Day 5–10: Media Replacements

1. Pre-warm NutriStem Medium and replace it in each well with fresh one.
2. Incubate at 37 °C, 5% CO_2.

3.3.1 Colony Formation

1. Check the plate daily, monitoring the formation of iPSC colonies by a phase-contrast microscope.

4 Notes

1. The combination of penicillin and streptomycin is very effective against bacterial contamination and it is very helpful when initiating the cultures from the skin samples and during the first passages of the culture. Nevertheless, the reprogramming procedure is carried out in the absence of antibiotics, so sterile conditions are demanded.
2. In order to clean and sterilize them, the coverglasses should be placed in a glass plate, washed with 70% ethanol, dried in oven at 37 °C and autoclaved before use.

3. To ensure the success of the procedure it is extremely important to keep all reagents on ice after thawing and to perform these steps under a sterile hood.
4. As soon as received, Matrigel should be thawed once to 4 °C, aliquoted in order to avoid multiple freeze-thaws, and then stored at −20 °C.
5. Change both the solution and the 100 mm plate at each wash. To successfully remove the excess of blood and other biological fluids, we suggest to gently shake the sample into the solution holding it by surgical forceps.
6. While dissecting the sample to obtain the desired fragments, scar, dirty or burned areas of the tissue must be discarded.
7. To guarantee sterile conditions and avoiding microbial contaminations this step must be performed under a sterile hood, as well as **steps 8–13**, **15**, **17,** and **18** in Subheading 3.1; **steps 1**, **2**, and **9** in, and in Subheading 3.2.1; **steps 3–6** in Subheading 3.2.2; **step 1** in Subheading 3.2.3; and **step 1** in Subheading 3.3.
8. We suggest placing each group of 4 small fragments in 35 mm plate arranging them in a square with a fragment on each angle. This disposition allows the optimal spacing to promote the cell outgrowth from fragments and the adjustment of the coverglass on them.
9. To correctly resuspend the cells, use a pipet-aid and gently move up and down through the tube to obtain a homogeneous cell suspension.
10. Trypan blue is a dye used to quantify live cells by labeling dead cells exclusively. Because live cells have an intact cell membrane, this dye cannot penetrate them, thus non-viable cells will appear blue-colored, while the viable ones will remain unstained.
11. To calculate the number of cells in the suspension, you must apply the following formula:

$$\text{Total number of cells} = (\text{Total number of counted cells/Square number}) \times 2 \times 10.000 \times \text{Total volume of cell suspension}$$

12. As RNA used for transfection steps could be easily degraded, it is highly recommended to clean all working surfaces and supplies (gloves, bottles, sterile hood surfaces, pipetman, pipet-aid, etc.) with RNaseZap before starting the procedure.

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