Title: A two-step protocol to erase human skin fibroblasts and convert them into trophoblast-like cells

Running Title: Direct conversion of fibroblasts in Trophoblast-like cells

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Abstract

The first differentiation event in mammalian embryos is the formation of the trophectoderm, which is the progenitor of the outer epithelial component of the placenta and supports the fetus during intrauterine life. Our understanding of these events is limited, particularly in human, because of ethical and legal restrictions and availability of adequate in vitro models would be very advantageous. Here we describe a method that converts human fibroblasts into trophoblast-like cells, combining the use of 5-azacytidine-CR (5-aza-CR) to erase the original cell phenotype and a cocktail containing bone morphogenetic protein 4 (BMP4) with inhibitors of the Activin/Nodal/ERK signaling pathways, to drive erased fibroblasts into the trophoblastic differentiation. This innovative method uses very easily accessible cells to derive trophoblast-like cells and it can be useful to study embryo implantation disorders related to aging.

Key words: 5-aza-CR, Activin/Nodal/ERK inhibitors, BMP4, epigenetics, fibroblast, trophoblast.

1. Introduction

In mammals, trophectoderm is the progenitor tissue of the entire outer epithelial component of the placenta that supports the fetus during intrauterine life. Its primary function is to allow implant of the embryo to the uterine wall, to protect the fetus from immunological response, to secrete hormones for pregnancy maintenance and to allow gasses and nutrient exchange ***(1)***. Trophectoderm is composed by two subpopulations: villous cytotrophoblast that layers the basement membrane surrounding placental villi and syncytio-trophoblast, a cell population that makes direct contact with maternal blood and is characterized by production of chorionic gonadotropin (CG) and other placental hormones ***(2)***. Although placental dysfunction seems to be the main disorder of pregnancy, with immediate consequences for the mother and child, aging is also one of the main causes of infertility. In this perspective, it comes as no surprise that the percentage of infertility has increased dramatically in recent years, given the fact that women's empowerment causes the postponement of motherhood, with an increase in placental and embryonic implantation defect. Recent evidence has shown that aged uterine environment is decisively involved, causing a reduced uterus decidualization and increased severe placentation defects resulting in abnormal embryonic development ***(3)***.

At the moment, only few in vitro models are available to study the main mechanisms involved in reproductive disorders and, all of them, are obtained from embryonic stem cells (ESCs). In particular, Roberts et.al has demonstrated that human pluripotent stem cells (hPSCs), bone morphogenetic protein 4 (BMP4) and hypoxic environment allow to derive trophoblast cells ***(2)***; Wang et al. has described that hESC seems to be a robust model to acquire trophoblast ***(4)*** and Turco et al. has highlighted how hESC together with BMP4, 3D matrix and hypoxic environment permits to obtain trophoblast organoids ***(5)***. Here we describe a model that uses a two-step protocol to derive trophoblast-like cells. Step one involves the use of epigenetic erasing to drive human adult somatic cells into, into a high plasticity state ***(6***, ***7)***. Step two takes

advantage of this induced high plasticity window and uses a cocktail of trophoblast inducers to encourage the acquisition of the trophoblastic phenotype ***(8)***. More in details, step 2 is based on the use of mouse embryonic fibroblasts **(**MEF) conditioned medium combined with a cocktail of BMP4 and inhibitors of the Activin/Nodal/ERK signaling pathways as previously described by Roberts et al, Schulz et al and Wang et al to drive cells into the new phenotype ***(2***, ***4)***. This method allows the generation of trophoblast cells from easily accessible cells, such as dermal fibroblasts that can be simply propagated in vitro. It is free of any genetic modifications that make cells prone to instability and transformation. Because of their stable phenotype, the cells generated with this procedure are more easily applied in regenerative medicine.

The in vitro model generated is efficient and reproducible. It can be used for the acquisition of useful information on the pathogenesis of developmental disorders based on trophoblast defects and aging, as well as drug discovery and regenerative medicine

2. Materials

2.1. Mouse embryonic fibroblast (MEF) thawing and inactivation

1. MEF cells.
2. Water bath.
3. 15 mL sterile tubes.
4. Centrifuge.
5. 35mm Petri dishes.
6. T25 flasks.
7. 0.20 µm filter.
8. Inverted Microscope.
9. CO_2 incubator.

10. Trypsin-EDTA solution.

11. MEF culture medium: 88% (v/v) Dulbecco's modified Eagle medium, 10% (v/v) fetal bovine serum (FBS), 2mM (v/v) L-glutamine solution, 1% antibiotics.

12. MEF inactivating medium: 6 mL of fibroblast culture medium with 60 µL of Mytomicin C.

13. ESC culture medium without bFGF: 40% Ham's F-10 Nutrient mix, 40% DMEM Low glucose, 10% Knock-out Serum Replacement, 5% FCS, 1% antibiotics, 2mM L-glutamine, 1% Nucleoside Mix, 1% MEM Non essential Amino Acids Solution, 0,1 mM 2-Mercaptoethanol, 1 unit/mL LIF.

2.2. Isolation of human skin fibroblasts

1. Skin biopsy collected from adult women.
2. 15 mL sterile tubes.
3. Sterile surgical instruments.
4. 35mm Petri dishes.
5. T25 flasks.
6. CO_2 incubator.
7. Inverted Microscope.
8. Dulbecco's phosphate-buffered saline (PBS) containing 2% antibiotics.
9. 0,1% porcine gelatin: dissolve 0,1 g of porcine gelatin in 100 mL of sterile water. Autoclave and stock at +4°C.
10. Fibroblast culture medium: 77% (v/v) Dulbecco's modified Eagle medium, 20% (v/v) fetal bovine serum (FBS), 2mM (v/v) L-glutamine solution, 1% antibiotics.

2.3. Seeding of human skin fibroblasts

1. 4-well multidishes.
2. 15 mL sterile tubes.
3. Cell counter.
4. Centrifuge.
5. Inverted Microscope.
6. CO_2 incubator.
7. Water bath.
8. 0,1% porcine gelatin: dissolve 0,1g of porcine gelatin in 100 mL of sterile water. Autoclave and stock at +4°C.
9. Fibroblast culture medium: 88% (v/v) Dulbecco's modified Eagle medium, 10% (v/v) fetal bovine serum (FBS), 2mM (v/v) L-glutamine solution, 1% antibiotics.

2.4. 5-azacytidine-CR (5-aza-CR) treatment

1. 15 mL sterile tubes.
2. Water bath.
3. CO_2 incubator.
4. 1 mM 5-aza-CR: dissolve 0,0024 g of 5-aza-CR in 10 mL of warm DMEM; vortex the solution and sterilize using 0,22 µm filter.
5. Fibroblast culture medium: fresh culture medium: 88% (v/v) Dulbecco's modified Eagle medium, 10% (v/v) fetal bovine serum (FBS), 2mM (v/v) L-glutamine solution, 1% antibiotics.
6. ESC culture medium: 40% Ham's F-10 Nutrient mix, 40% DMEM Low glucose, 10% Knock-out Serum Replacement, 5% FCS, 1% antibiotics, 2mM L-glutamine, 1% Nucleoside Mix, 1% MEM Non-essential Amino Acids Solution, 0,1 mM 2-Mercaptoethanol, 1 unit/mL LIF, 5 ng/ml human basic fibroblast growth factor (bFGF).

2.5. Trophoblastic induction

1. Water bath.
2. Inverted microscope.
3. Tri-gas incubator.
4. Trophoblast induction medium: 10 mL of conditioned MEF inactivated medium, 10µl of BMP4 (50 ng/ml), 10µl A83-01 (1µM), 10µl PD173074 (0.1 µM).

3. Methods

3.1. MEF thawing and inactivation

1. Thaw a cryo-vial containing MEFs using a 37°C water bath.
2. Add 2 mL of fibroblast culture medium in a 15 mL sterile tube.
3. Transfer MEFs in the 15 mL sterile tube containing the 2 mL of fibroblast culture medium.
4. Centrifuge at 300 g for 5 minutes.
5. Resuspend the pellet in 2 mL of fibroblast culture medium.
6. Seed in a 35 mm petri dish and incubate at 37°C in CO_2 incubator.
7. Change fibroblast culture medium every day.
8. When they reach confluency, remove culture medium from the dish.
9. Wash 3 times in sterile PBS supplemented with 1% antibiotic antimycotic solution.
10. Add 500 µL of trypsin-EDTA solution and incubate at 37 °C until cell monolayer begins to detach from the bottom of the tissue culture dish and cells dissociate.
11. Neutralize Tripsin-EDTA using 1.5 mL of MEF culture medium.
12. Dislodge cells by repeatedly and gently pipetting.
13. Plate cells in a new culture dish using a 1:2 passage ratio and culture at 37 °C in 5% CO2 incubator.
14. Change fibroblast culture medium every day and passage every 2 days.

15. Seed MEFs at a density of 2×10^6 in T25 flask and incubate for 24 hours in CO_2 incubator.

16. Rinse cells 3 times in sterile PBS and expose sub-confluent monolayers to the MEF inactivating medium for 3 hours.

17. Eliminate MEF inactivating medium and incubate cells with ESC medium without b-FGF for 24 hours.

18. Collect conditioned medium, filter using 0.20 µm filter and storage at -20°C until use.

3.2. Isolation of human skin fibroblasts

1. Isolate skin biopsy and transfer it in a 50 mL sterile tube containing sterile PBS with 2% antibiotics.

2. Prepare 2 mL of 0.1% of porcine gelatin in a 35mm petri dish. Wait 45 minutes to coat the surface at room temperature.

3. Wash the biopsy in sterile PBS and cut it in small fragments of approximately 2 mm^3.

4. Transfer 5 skin fragments in a petri dish.

5. Add 20 µL of fibroblast culture medium onto each fragment and incubate at 37°C in CO_2 incubator.

6. Add around 20-50 µL of fibroblast culture medium daily for the first 5 days.

7. Add 1 mL in each petri dish and culture until cells reach confluency.

8. When cells reach confluency, trypsinize (see Sect 3.1., steps 9-12) and transfer cells in a new T25 flask.

9. Incubate at 37°C

10. Passage every 3 days with a ratio of 1:2

3.3. Seeding of human skin fibroblasts

1. Add 0.5 mL of 0.1% of porcine gelatin in each well of 4-well multidish and wait 20 minutes to coat the surface at room temperature.

2. Eliminate gelatin and let it dry for 45 minutes.

3. Remove the medium from T25 flask, and ten trypsinized using 0.6 mL of Trypsin-EDTA (see Sect 3.1, step 9-12).

4. Collect cell suspension in a 15 mL sterile tube.

5. Count cells using a counting chamber under an optical microscope at room temperature. Calculate the volume of medium needed to re-suspend cells in order to obtain 7.8 X 10^4 cells/cm^2.

6. Centrifuge cells at 300 g for 5 minutes.

11. Resuspend the pellet in a calculated volume by pipetting carefully.

12. Plate cells in 4-well multidish.

13. Incubate at 37°C for 24 hours.

3.4. 5-aza-CR treatment

1. Rinse cells in a 4-well multidish using 0.5 mL of sterile PBS/well.

7. Dilute 1 µL of 5-aza-CR stock solution in 1 mL of fibroblast culture medium.

8. Remove the medium from the dish.

9. Add 0.5 mL of fibroblast culture medium containing 5-aza-CR in three wells. Use one as a negative control.

2. Incubate at 37°C for 18 hours in CO_2 incubator.

3. Remove 5-aza-CR.

4. Rinse cells 3 times in sterile PBS and add 0.5 mL of ESC medium for 3 hours at 37°C in CO_2 incubator.

3.5. Trophoblastic induction

1. Rinse cells 3 times in sterile PBS.
2. Expose cells to the trophoblast induction medium and incubate in a hypoxic environment (5% O_2) in the tri-gas incubator for 11 days.
3. Refresh culture medium every other day.

NOTES

1. It is crucial to cut very small pieces of skin tissue in order to facilitate fibroblasts to grow out of the tissue fragments.
2. Before seeding cells for 5-aza-CR treatment, it is important that they are in sub-confluency.
3. Prepare 5-aza-CR solution just before use.
4. Aliquot differentiation factors in small volumes.
5. It is very important to add differentiation factors just before use.

Acknowledgement

This work was supported by Carraresi Foundation T.A.L.B., F.G., S.A are members of the COST Actions CA16119.

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Figure legends

Figure 1. An overview of the steps involved in the protocol.

Figure 2. Morphological changes of fibroblasts (A) after 5-azacytidine treatment (B). Trophoblast-like cells obtained after 11 days of chemical induction in hypoxic environment: cytotrophoblast cells (C) and syncytio-trophoblast cells (D).