***ex vivo* culture for preimplantation mouse embryo to analyse pluripotency**

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Running Head

Preimplantation embryo culture

Summary/Abstract

A couple of days after fertilization of a mouse oocyte by a sperm, two sequential cell differentiation events segregate pluripotent cells that can be identified by the presence of specific markers. Early mammalian embryos are relatively easy to recover as they are not yet implanted in the uterus matrix. Several decades of experimentation have enabled to find appropriate media to culture them, and therefore provide an excellent way to test different experimental set-up such as the use of signalling inhibitors. We provide here a commonly used protocol to culture preimplantation embryos as well as a method to detect pluripotent cells in blastocysts.

Key Words

blastocyst, immunofluorescence, epiblast, primitive endoderm, trophectoderm

1. Introduction

Mammalian preimplantation development has received a lot of attention in the last decades. As the onset of a new organism, it has been shown to pass through critical and/or unique steps of development such as embryonic genome activation, epigenetic remodelling and the first cell lineages differentiation. Early mammalian embryos have been the source of technological developments with the production of transgenic mouse lines, the isolation of embryonic stem (ES) cells that enabled the production of hundreds of KO mice and are now studied for stem cell therapies.

Before their implantation in the uterus, mammalian embryos can be easily collected and cultured. The first embryo cultures were reported in the 1950s [1], and rapidly validated by a successful development after embryo transfer into surrogate females [2]. Since then, several modifications were brought in media composition, taking into account energy source and amino acids requirements as well as pH and osmolarity changes in order to improve the quality of cultures [3]. As such, pyruvate is first needed after fertilization, then lactate from the two-cell stage onward, and finally glucose can support embryo development after compaction. Further optimization led to the KSOM medium [4] enabling to overcome the two-cell block observed with several mouse strains, which was improved by adding non-essential and essential amino acids (AA) [3, 5]. The KSOM+AA is widely used for developing mouse preimplantation embryos, and indeed comparing their transcriptome shows that they are close to freshly collected embryos [6]. Other media have been developed, essentially for human ART (Assisted Reproductive Technologies), with various composition (sometimes not completely disclosed) [7–9].

For practical reasons, mouse embryos are generally cultured with atmospheric oxygen tension that is around 20%. However it is thought that the female reproductive tract is hypoxic (around 3-5 %) [10], and a high oxygen tension can create reactive oxygen species (ROS) that can alter embryo development. When culturing in a 20% oxygen atmosphere, inclusion of the chelator EDTA improves blastocyst development, however with a lower cell number compared to a 5% atmosphere [11]. Correlated to embryonic growth, there is an impact of oxygen concentration on the transcriptome of cultured embryos [12].

In mice, three cell lineages are segregated before implantation, in two sequential steps: first the Inner Cell Mass (ICM) and the trophectoderm (TE) segregate, followed by differentiation of the ICM into Epiblast (Epi) and Primitive Endoderm (PE) cells during the blastocyst formation [13]. These two cell types originate from common ICM precursors that differentiate asynchronously between E3.25 and E3.75. Cell specification occurs in an apparent random manner, characterized by a "salt and pepper" pattern [14, 15]. From E3.75-4.0, Epi and PE cells sort to constitute two separated tissues. While TE and PE are essentially extraembryonic tissues, Epi cells are producing all the cells of the future individual and are therefore pluripotent. They are the source of ES cells, with whom they share many characteristics at E4.5 [16].

Variation of the culture medium composition is known to affect the balance in cell lineage distribution. Similarly culture conditions have an impact on ES cell state and differentiation

abilities[18] and thus may have an effect on Epiblast differentiation. Moreover, culturing *Tead4*^{-/-} embryos in 5% oxygen tension rescues trophectoderm formation compared to a 20% atmosphere [17], demonstrating that ROS can have an effect on cell lineage differentiation.

Thus, in the blastocyst four cell types can be found at the same time: outside TE cells, ICM precursor cells, PE and Epi cells. As Epi and PrE cells are intermingled and the differentiation is asynchronous, it is difficult to distinguish between ICM (precursors), PE and Epi states before cell sorting. *In situ*, the most reliable method is to examine by immunofluorescence the relative levels of the transcription factors NANOG and GATA6. The NANOG/GATA6 level ratio is high in Epi cells and low in PE cells, whereas in ICM state cells have relatively equal levels of NANOG and GATA6 [19, 20]. Other markers such as SOX2 (Epi), SOX17, GATA4 or PDGFRα (PE) can be used only after E3.75 as their expression is initiated or is specifically restricted at later time points [15, 21, 22]. Around E4.5, NANOG levels decrease [23], it is thus advised to use another marker such as SOX2 to identify the pluripotent Epi cells. Alternatively, individual cells can be characterized by single-cell RNA analyses [24–26]. During blastocyst formation, the pluripotent Epi cells can be identified by the high *Nanog/Gata6* RNA levels ratio, but also by expression of *Fgf4* and higher RNA levels of *Tdgf1*, *Klf2*, *Klf4*, *Prdm14* compared to PrE cells [27, 28].

We provide here a method to recover and culture preimplantation embryos, as well as a protocol to detect the pluripotent Epi cells by immunofluorescence when they appear during blastocyst formation (E3.25 - E4.0).

2. Materials

1. Stereomicroscope
2. Incubator: regular cell/tissue culture incubator (37°C, 5% CO₂)
3. Three dimensional rotating, rocking mixer
4. Fine stainless steel forceps (tip width 0.1 x 0.06mm)
5. Fine stainless steel scissors sharp blunt
6. Burner
7. 1x Phosphate buffer saline (PBS)
8. Triton 100X
9. 70% ethanol
10. M2 culture medium (embryo tested) (64.66 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂·2H₂O, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄·7H₂O, 4.15 mM NaHCO₃, 20.85 mM HEPES, 23.28 mM Sodium lactate, 0.33 mM Sodium pyruvate, 5.56 mM glucose, 1 g/l BSA, 4 g/l)
11. KSOM+AA culture medium (embryo tested) supplemented with amino acids (95 mM NaCl, 2.5 mM KCl, 0.35 mM KH₂PO₄, 0.20 mM MgSO₄·7H₂O, 10.0 mM Sodium Lactate, 0.3 mM Sodium Pyruvate, 2.8 mM Glucose, 25 mM NaHCO₃, 1.71 mM CaCl₂, 1.0 mM Glutamine, 0.01 mM EDTA,

0.5 mM Non essential amino acids, 0.5 mM Essential amino acids, 100 IU/ml Penicillin G, 0.5 g/ml Streptomycin sulphate, 0.5 g/ml Streptomycin Sulphate, 5.0 mg/ml BSA)

12. Aspirator tube assembly (see Fig1C)

13. 30G needles

14. 26G needles

15. Bacterial Petri dishes

16. Foetal Bovine Serum

17. Mineral oil, embryo tested

18. 4-well plates

19. 35 mm tissue culture dishes

20. Pasteur pipettes

21. Paraformaldehyde 32%

22. 35 mm Petri dishes (non adherent are preferable)

23. Terasaki microwell plate (60 conical wells of 10 μ l)

24. μ -Slide 18 Well - Flat slides (slides with glass or confocal-compatible plastic bottom)

25. Primary antibodies (see Table 1)

26. Secondary antibodies directed against the different hosts of the primary antibodies, coupled with different fluorophores compatible with the confocal microscope setup.

27. DAPI (4',6-diamidino-2-phenylindole) DNA dye

28. Confocal microscope

[insert Table 1 near here]

3. Methods

From the 2- to the 16-cell stage, embryos are recovered by flushing the oviducts. From the 32-cell to blastocyst stage, embryos are collected from the uterine horns. When embryos are collected for culture, flush with M2 medium. If embryos are directly processed for analysis, they can be flushed with PBS. All manipulations are carried out under a stereomicroscope.

[insert Figure 1 here]

3.1 Dissection and recovery of embryos from the oviduct:

1-After euthanasia, soak the abdomen with 70% ethanol. Using forceps and scissors make a large incision through skin, muscles and peritoneum of the abdomen, enabling to see the whole intestine. Push the gut away to uncover the two uterus horns, oviducts and ovaries underneath.

2- to collect the oviduct, hold the uterus horn close to the oviduct with forceps. With fine scissors cut the uterus horn and then between the ovary and the oviduct (see Fig 1A). Collect the oviduct in a bacterial Petri dish. The oviduct can remain without buffer for around 15 min.

3- place the oviduct in a drop of M2 medium (*see Note 1*).

4- Fill a 1 ml syringe with M2 and adjust a 30G needle, making sure there are no air bubbles left. The needle can be blunted with robust scissors to avoid piercing the oviduct, however it is more difficult to introduce into the infundibulum.

5- Locate the infundibulum (opened extremity of the oviduct) within the loops of the oviduct. With the help of forceps, introduce the needle into the infundibulum (Fig 1B). Maintain the needle inside with the forceps and flush around 0.1 ml of M2. The oviduct should swell, and the flush should exit from the uterine horn side.

6- Remove the oviduct and collect the embryos using a mouth pipette (*see Note2*) and a pulled Pasteur pipette (*see Note 3*) and transfer them into a clean M2 drop (around 50 μ l). Embryos are washed twice in drops of clean M2.

3.2- Uterine horn flush

1- Using forceps and fine scissors, cut each uterine horn at the cervix and oviduct extremities (Fig 1A). It is better to trim away the mesometrium and the fat beforehand with the scissors. Place the horns in a bacterial Petri dish. The horns can remain without buffer for around 15 min.

2- place individual horns in a drop of M2 medium

3- Fill a 1 ml syringe with M2 and adjust a 26G needle, making sure there are no air bubbles left.

4-With the help of forceps, insert the needle into one extremity of the horn. Maintain the needle inside with the forceps and flush 0.2-0.3 ml.

5- Remove the horn and collect the embryos using a pulled Pasteur pipette. Transfer them into a clean M2 drop (around 50 μ l). Embryos are washed twice in drops of clean M2.

3.3- Embryo culture

Culturing mouse embryos in groups is beneficial compared to individual cultures probably due to unknown paracrine factors [3, 29]. Embryos can be cultured in drops covered by mineral oil or in open culture systems such as 4-well plates [3]. Murine embryos are susceptible to temperature and pH variations, thus a minimum of time in manipulating embryos is beneficial. Embryos can be cultured in a classic incubator at 37°C, 5% CO₂.

1- rinse rapidly the embryos in 2 drops of culture medium and place them in 400 to 500 µl of medium in a 4-well plate. Alternatively, embryos can be cultured in 10 µl medium drops (about 10 embryos per drop) covered by 2 ml of mineral oil in a 35 mm tissue culture dish.

2- incubate for the desired time. Staging can be appreciated with the morphology (compaction, cavity size, hatching...). In a successful culture there are very small or no delays compared to freshly collected embryos.

3.4- Characterization of cell types to identify pluripotent cells *in situ* by immunofluorescence.

To identify the pluripotent epiblast cells, we use antibodies directed against NANOG and GATA6, as well as CDX2. Indeed, there can be TE cells that still express NANOG, leading to misinterpretation. Thus it is strongly advised to examine a TE marker such as CDX2 to clearly identify TE versus ICM cells. A nuclear dye can be used for visualisation of all the cells and for fluorescence normalisation.

Unless otherwise indicated, experiments are carried out at room temperature (RT) in four-well plates with 0.5 ml of solution and under gentle agitation. At each step, embryos are either transferred into the next solution with a pulled Pasteur pipette, or the solution can be replaced in the same well, paying attention to the embryos.

1-After flushing or embryo culture, transfer embryos with pulled Pasteur pipette into a four-well plate containing the fixation solution (4% PFA, *see Note 5*). Fix embryos for 10 min at RT to overnight at 4°C (this can depend on your epitope).

2- Wash the embryos in PBT (*see Note 6*) twice for 5 min. For long-term storage, embryos can be dehydrated (*see Note 7*)

3-Permeabilize with PBT-0,5% (*see Note 6*) for 5 min.

4- Block antibody- aspecific sites by incubating with blocking solution (10% FBS in PBT) for 15 min.

5-Incubate with primary antibodies diluted in blocking solution, overnight at 4°C (*see Note 8*)

6- Wash embryos once for 5 min and twice for 25 min in PBT.

7- Incubate with secondary antibodies and with the DNA dye diluted in blocking solution (Dapi 0.1 µg/ml) for 1 hour. From this step on, to avoid fluorescence decay, embryos are protected from the light (in a dark chamber or cover with aluminium foil)

8- Wash twice for 5 min in PBT.

9- Transfer embryos into a confocal-compatible slide and scan embryos at the confocal microscope (*see Note 9*).

10- Analyse markers labelling. An example of interoretation is given in Fig. 2

[insert Figure 2 near here]

Notes

1- M2 is a working medium buffered with HEPES. It can be aliquoted and kept at -20°C for several months. Once thawed, keep at +4°C and use within 3 days.

2 - A mouth pipette (Fig 1C) is composed of an aspirator tube in latex (3 mm x 5 mm in diameter, ~50 cm long), a mouth piece, a pulled Pasteur pipette and a pipette holder (1000 µl tip). The mouth piece can be home-made by cutting the extremity of the barrel of a 1 ml syringe. Pieces are assembled as in Fig 1C.

3 - Disposable glass Pasteur pipettes are used to transfer embryos. Their narrow part is pulled on a flame and a blunt tip is broken up by applying a twist on the flexible and tapered end of the Pasteur pipette. The break must be blunt to prevent damaging the embryos. To avoid embryo sticking to the walls of the pipette, we coat the Pasteur pipette with FBS. Pneumatic pressure is adjusted by picking a few air bubbles alternated with the medium used.

4 - KSOM+AA medium is aliquoted and kept frozen at -20°C for several month. Once thawed keep at +4°C and use within 2 days. The KSOM culture medium needs to be equilibrated to allow temperature and gas equilibration for a minimum of 2 hours before starting embryo culture. Make holes in the tube cap for gas exchange and place aliquots directly into the incubator.

5 - 4% PFA in PBS 1X. Aliquots are kept at -20°C for several months. Once thawed, 4% PFA can be kept at +4°C for one week. When transferring embryos into PFA, they tend to move to the surface, due to the difference in solution composition, where they could be damaged. This can be prevented by making swirls with the pipette to maintain embryos down.

6 -PBT (0.1% (vol/vol) Triton X-100 in PBS 1X) and PBT-0,5% (0.5% (vol/vol) Triton X-100 in PBS 1X) can be stored at RT for several months. Washes can also be performed with Tween-20 at the same dilution instead of Triton X-100.

7- For long-term storage, embryos can be dehydrated in increasing concentrations of ethanol in PBT (25%, 50% and 75% ethanol (vol/vol) and twice in ethanol 100%) 5 min each and stored at -20°C in four-well plate with parafilm. Caution to prevent full evaporation when stored for several weeks. When needed, embryos are rehydrated in decreasing concentrations of ethanol in PBT (75%, 50% and 25% ethanol (vol/vol)) and twice in PBT, 5 min each.

This freezing step can damage some epitopes. The antibodies we report here are working after a dehydration step. Many epitopes are not perturbed by this treatment however this should be tested and validated for each primary antibody.

8- In order to use smaller amounts of antibodies, we carry out incubations in 10 µl in terasaki microwell plates (humidified with a wet pad).

9 - We generally scan embryos in PBT in confocal-compatible microwell plates (maximum 100 µm thick bottom). This allows to easily recover embryos for genotyping by PCR after imaging.

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Table1: Primary antibodies used.

Epitope	Host	Supplier	Reference	Dilution
CDX2	Mouse	Abcam	Ab89949	1/1
GATA6	Goat	R&D	AF1700	1/300
NANOG	Rabbit	Abcam	Ab80892	1/100

Figure 1:

A- After opening the abdomen, uncover the uterine horn-oviduct-ovary on one side. Cut (dashed lines) to recover either the oviduct or the uterine horn. Proceed to the other side. B- To flush the oviduct, locate the infundibulum (naturally loose opening). Use the forceps to introduce the needle. Maintain the needle with the forceps while flushing. C- Mouth pipette set-up. A latex aspirator tube is connected with a mouth piece (or a cut syringe barrel) on one side, and a 1 ml micropipette tip on the other side, which is the holder of the pulled Pasteur pipette.

Figure 2

Labelling of NANOG, GATA6 and CDX2 by immunofluorescence coupled with nuclear staining by Dapi. The image shows a single Z plane through a E3.75 embryo. The bottom right panel depicts the interpretation of the cell identities: Epi (pluripotent) cells are labelled by NANOG (green), PrE cells are labelled by GATA6 (red), ICM precursor (pluripotent) cells are labelled by both NANOG and GATA6 (yellow), TE cells are labelled by CDX2 (blue). On this section there are cells in mitosis (purple) that cannot be attributed to a lineage with these markers. In grey, there is a weak labelling with Dapi, indicating that it will be necessary to change Z plane to correctly identify this cell.