Evaluation of a laser technique to isolate the inner cell mass of murine blastocysts

José Luis Cortés^{*†}, Fernando Cobo^{*}, Purificación Catalina^{*}, Ana Nieto^{*}, Carmen Cabrera^{*}, Rosa Montes^{*}, Ángel Concha[†] and Pablo Menendez^{*†}

^{*}Andalusian Stem Cell Bank (Spanish Central Node), Centro de Investigaciones Biomédicas, Parque Tecnológico de las Ciencias de la Salud, Avenida del Conocimiento s/n, Granada, Spain, and [†]Servicio de Anatomía Patológica, Hospital Universitario Virgen de la Nieves, Avda Fuerzas Armadas 2, Granada, Spain

hESCs (human embryonic stem cells) are pluripotent cells derived from the ICM (inner cell mass) of blastocysts that can be used to derive several kinds of cells of the human body for the treatment of some previously untreated diseases. In considering the future use of hESCs in regenerative medicine and cell-therapy programmes, several research centres have begun projects involving the derivation of hESC lines using spare human embryos from IVF (*in vitro* fertilization) cycles. In some stem-cell banks, such as ours, the law also permits us to obtain these cell lines. The low availability of spare IVF human embryos, and the low rate of success in the derivation of hESC lines, give these embryos a great research value that limits experiments with new techniques. The use of murine embryos would be a good model with which to do research to discover the best methodologies to use in order to derive new hESC lines. The aim of the present study was to evaluate a new method of isolation of the ICM and derivation of ESC lines in a murine blastocyst model using laser drilling to eliminate the trophectoderm cells and compare it with the usual control method consisting of culturing the whole murine blastocyst. We also tested the adhesion and growth of primary colonies of mESCs (murine ESCs) over two different growth surfaces, namely an MEF (inactive murine fibroblastic feeder layer) or gelatin-coated dishes, in order to achieve the best culture conditions for future derivation of human stem-cell lines for application in human transplantation.

Introduction

Transplantation of cells of human origin is a sector of medicine which shows increasing opportunities for the treatment of diseases, some of which, until now, have been incurable. Thus, with the introduction of regenerative medicine and cell therapy programmes using human stem cells, a great number of research centres have attempted to derive cell lines using hESCs (human embryonic stem cells) [1]. Moreover, in some

stem-cell banks, such as ours (the Andalusian Stem Cell Bank – Spanish Central Node), the law also permits us to obtain these cell lines [2]. As these cells may be able to provide an unlimited cell source for transplantation therapies, it is necessary to establish reliable methods for their handling and manipulation. One of the most critical stages in this process is the isolation of the ICM (inner cell mass) of the blastocysts, where these pluripotent cells can be found.

The low availability of spare IVF (*in vitro* fertilization) human embryos, and the low rate of success in the derivation of hESC lines, give these embryos a great research value and limit the experiments with new techniques. Thus the use of murine models [3,4] in our centre permits us to do research about the best methodologies to use, although there is a need to establish the differences between both the human and the murine blastocysts.

The ICM could usually be separated and isolated from the trophectoderm using immunosurgery [5], with mechanical processes [6], with whole-embryo culture of the blastocysts and partial-embryo culture methods [7] or single blastomeres [8].

Laser technology is commonly used for assisted hatching with some applications. One of them consists of making the embryonic membrane weaker to make the exit of the future blastocyst easier and so favour the derivation [9]. Another consists of blastomere biopsy for preimplantational diagnosis [10].

Until now, this new laser-drilling method has been suggested to derive stem-cell lines by one group, although without presenting any conclusive results [11]. A recent paper [12] reported preliminary results for a murine model using this method; subsequent culture of ESCs in a serum/cell-free culture system was achieved.

Key words: embryonic stem cell (ESC), inner cell mass (ICM), *in vitro* fertilization (IVF), laser drill, murine blastocyst, whole-embryo culture.

Abbreviations used: hESC, human embryonic stem cell; ICM, inner cell mass; IVF, *in vitro* fertilization; MEF, inactive murine fibroblastic feeder layer; mESC, murine ESC.

[†] To whom correspondence should be addressed (email josel.cortes.sspa@juntadeandalucia.es or pablo.menendez@juntadeandalucia.es).

Figure 1 Examples of murine embryos obtained in the present study

The Figure shows murine embryos at the cell (A) and compacting (B) stages.

In our opinion, some notion of which of these methods are the most efficient for the isolation of ICM is very important for future research in this field. Accordingly we have compared the ICM isolation method using laser drilling in a murine blastocyst model with one of the most commonly used methods (until now, the whole-embryo culture [7]), and we have carried out the culture on two different growth surfaces, namely MEF (inactive murine fibroblastic feeder layer) [13] and gelatin-coated dishes [14].

Materials and methods

Female 3–6-month-old mice (C57BL/CBA) were killed by cervical dislocation on day 4.5 of pregnancy (midday of the day that the copulation plug is found was deemed to be day 0.5). The oviducts were dissected and placed in warm (37 °C) PBS (Gibco–Invitrogen, Carlsbad, CA, U.S.A.). Then the oviducts were manipulated in a plastic Petri dish with warm Mops-buffered medium (G-MOPS™; Vitrolife Sweden AB, Kungsbacka, Sweden) the oviduct being removed by first cutting through the top of the uterus, leaving a small portion of it attached to the oviduct, and then dissecting the oviduct away from the ovary. This must be done very carefully to ensure that the opening of the oviduct remains intact, since it is extremely difficult to insert a needle once it has been damaged. This is done with the use of a binocular dissecting microscope (SZ-61; Olympus Spain, Barcelona, Spain) and an inverted microscope with Hoffman optics (XI-71; Olympus

Spain) to determine the embryo stage and the quality. The embryos found were placed in warm G-MOPS™ until the culture in sequential medium G-2 V.III (Vitrolife) to achieve the blastocyst stage. These embryos were cultured in an incubator at 37 °C and under a CO₂/air (6:94) atmosphere.

Quality of murine blastocysts

We included 31 murine embryos, both in the cell stage (days 2–3 after fertilization) (Figure 1A), as well as in the compacting stage (day 4 after fertilization) (Figure 1B). In all the cases these embryos were cultured until the development of expanded blastocysts (day 5–6 after fertilization) (Figure 2). In IVF programmes, blastocyst quality is one of the most important factors in the determination of both implantation and pregnancy. However, experiments that assess the developmental potential of embryos that are days behind normal development and which have very few ICM cells, are not feasible, and so these embryos are discarded. Mitalipova et al. [15] reported the establishment of four hESC lines from 19 such embryos. However, we have considered the blastocyst quality for this study in order to select the most effective derivation method. Both human and murine blastocysts are morphologically very similar, except in size, so, we adopted the human blastocysts grading system developed by the Cornell University (Ithaca, NY, U.S.A.) programme [16]. The expanded murine blastocysts have been classified into three categories according to the morphological aspect of the ICM, such as expanded human blastocysts (Figures 2A, 2B and 2C):

- Good quality blastocysts with large and distinct ICM
- Blastocysts with distinct but smaller ICM
- Bad-quality blastocysts with indistinguishable ICM

After the assessment of the blastocyst quality, all the murine blastocysts were treated with acid Tyrode's solution (Irvine Scientific, Santa Ana, CA, U.S.A.) for no more than 1 min to assure the complete dissolution of zona pellucida (Figure 3A).

Figure 2 Classification of expanded murine blastocysts

(A) Good quality murine blastocyst with large and distinct ICM; (B) murine blastocyst with distinct, but smaller, ICM. The arrow indicates the ICM region; (C) bad-quality murine blastocyst with indistinguishable ICM.

Figure 3 Examples of murine blastocysts included in the study

Methods of ICM isolation were evaluated. (A) Expanded murine blastocyst treated with Tyrode Acid to assure the complete dissolution of zona pellucida. The black arrow indicates the ICM region; (B) primary colony of a murine blastocyst adhered to gelatin-coated dishes using the whole-embryo culture method. The black arrow indicates the ICM region; (C) good-quality murine blastocyst with large and distinct ICM before laser drilling. The black arrows indicate the ICM regions; (D) murine embryos treated with the laser drilling. The white arrows indicate the ICM regions. The white asterisk indicates the lysed trophoctoderm; (E) murine blastocyst treated with the laser at day +1 formed a pseudo-trophoctoderm with a few cells not destroyed by the laser because they were near the ICM; cells were cultured over a fibroblastic feeder layer; the white arrow indicates the ICM region; the white asterisk indicates the lysed trophoctoderm and the white closed circle indicates the pseudo-trophoctoderm; (F) primary colony of a murine blastocyst adhered to gelatin-coated dishes using the laser drilling technique. The black arrow indicates the ICM region.

ESC medium

The ESC medium used consisted of Dulbecco's modified Eagle's medium (Gibco–Invitrogen), 20% (v/v) fetal-bovine serum (PAA, Colbe, Germany), 1% L-glutamine (Gibco–Invitrogen), 0.1 mM non-essential amino acids (Gibco–Invitrogen), supplemented with mouse leukaemia inhibitory factor; 2000 i.u./ml (Sigma–Aldrich), 0.1 mM 2-mercaptoethanol (Sigma–Aldrich), 50 units/ml penicillin (PAA) and 50 µg/mg streptomycin (PAA).

ICM isolation methods

We used the whole-embryo culture method to isolate the ICM and to establish mESC (murine ESC) lines in cases where the blastocysts had a distinct, but smaller or indistinguishable, ICM, in such a way that the trophoctoderm cells and the ICM tend to adhere to the growth surface used.

Using this method and after 2 days had passed, the attachment of the whole embryo to the growth surface was confirmed and the old medium was replaced with fresh medium. The trophoctoderm layer began to expand on the third day of culturing. On day 6, the ICM-like cells formed a dome structure, surrounded by trophoctoderm (Figure 3B). At this point, the ICM was removed, after which it was transferred to a freshly prepared growth surface. The whole-embryo culture tends to manifest an abundance of both trophoctoderm and differentiated cells, and the isolation of mESC-like colonies from differentiated cells requires a great deal of care and caution. However, the poor quality of the blastocysts often results in failures in the attachment of the ICM to the dish, so this procedure runs a much greater risk of trophoctoderm overgrowth than other methods because the entire trophoctoderm is cultured along with the ICM.

However, with good quality blastocysts with a large and distinct ICM, we used the laser drill (Eyeware™; Octax, Herborn, Germany), which was connected to an inverted microscope (XI-71; Olympus) and processed by a computer program which permits data analysis. We did not use poor-quality blastocysts in this method for this study because we cannot shoot the ICM if we cannot see it. In our experiment, the murine blastocyst to be treated with laser drill was positioned at the centre of the field of view under low magnification; this was done either with an object guide which moves the Petri dish over the microscope table in an x–y direction or using a mechanical or motorized stage which brings the Petri dish and thereby the blastocyst into the desired position by moving the table. The blastocyst position was moved when necessary. Blastocysts can be secured by holding two pipettes with the ICM positioned at '9 o'clock' if desired [12]. The special gold-coloured and octagonally shaped Laser Shot™ laser objective was turned inwards. It was necessary to lower the objective revolver to prevent the objective from touching anything prior to turning it. After focusing on the trophoctoderm cells, the object had to be moved so that the part of the trophoctoderm to be treated was located at the cross-hair position displayed on the monitor as the impact location of the laser focus. Thus this new mechanical method destroyed the trophoctoderm cells by shooting the laser over them carefully without damaging the ICM (Figures 3C and 3D). The length of radiation time of the laser and the number of laser shots were controlled by the embryologist. So the ICM cells were the ones that achieved adhesion (Figure 3F).

Growth surfaces

Both types of blastocyst groups were cultured individually in plates using two different growth surfaces in equal proportions {MEF [13] and an extracellular growth surface using gelatin-coated dishes (Sigma–Aldrich) [14]} to compare the

Table 1 Number of blastocysts used in each derivation method over each growth surface

ICM isolation method	Number of blastocysts used	Murine feeder layer	Gelatin-coated dishes
Whole-embryo culture; bad-quality blastocysts	10/31 (32.2%)	6/10 (60.0%)	4/10 (40.0%)
Laser-drilling method; good quality blastocysts	21/31 (67.7%)	10/21 (47.6%)	11/21 (52.4%)

efficiency. Confluent active MEF were treated with 10 µg/ml of mitomycin C (Sigma–Aldrich) for 3 h. The monolayer was washed with PBS and incubated with 0.05 % trypsin/0.53 mM EDTA solution for 2 min at 37 °C. When the cells detached from the flask surface, complete medium was added to inactivate the trypsin/EDTA solution. After centrifugation, the single-cell suspension of inactive MEF was resuspended in completed medium and plated in cell culture dishes of 3.5 cm pre-coated with 0.2 % of gelatin (Sigma–Aldrich) to maintain the feeder attachment on the concentration necessary to obtain a confluent layer. Only 3.5-cm-diameter dishes precoated with 0.2 % gelatin were used for the other surface test.

Results

Ten blastocysts out of the 31 obtained had a smaller, but distinct or indistinguishable, ICM (32.2%), so we tried ICM isolation by the whole-embryo culture method. Six out of ten were placed over an inactive MEF (60%) and four over gelatin-coated dishes (40%). By contrast, 21 blastocysts with good quality were derived using the laser drill (67.7%). Ten blastocysts were placed over an inactive MEF (47.6%) and 11 over gelatin (52.4%) (Table 1).

Using the whole-embryo culture we obtained adhesion and isolation of the ICM in four blastocysts over an inactive MEF (66.7%) and three over gelatin (75%). So the total adhesion/isolation rate was 70%. Using the laser-drilling method we obtained adhesion and isolation of the ICM in eight blastocysts over an inactive MEF (80%) and three

over gelatin (27.3%). So the total adhesion/isolation rate was 52.4% (Table 2).

We derived two lines which were morphologically similar to mESC lines using the whole-embryo culture method over MEF (33.3%) and none over the gelatin-coated dishes. One line which was morphologically similar to mESC line using the laser drill over MEF (10%) and one over gelatin (9.1%) (Table 2).

Discussion

According to the adhesion and isolation of the ICM, we observed that the whole-embryo culture method of the blastocysts is more effective than the laser method (70 versus 52.4%). Taking into account that we had used the laser drill for the good-quality blastocysts and that the concealment of the ICM by the trophectoderm cells is the only disadvantage of the whole-embryo culture method [6], we must continue to improve the laser-drill technique so that the trophectoderm cells are destroyed and do not interfere with ICM adhesion [12]. Curiously, the blastocysts treated with the laser at day +1 formed a pseudo-trophectoderm, with a few cells not destroyed by the laser because they were near the ICM (Figure 3E). These trophectoderm cells disappeared after repeated passages.

In addition, we think that murine blastocyst quality is important for selecting the most adequate derivation method for future application in the derivation of hESC lines [7].

On the other hand, regardless of the isolation method used, some groups have come to the general conclusion that cells grown on mouse feeder cells would not be appropriate for clinical applications, and this has lead to significant efforts to grow ESC lines on human feeders [17], immortalized cells [18], feeder cell lines that have been derived from the original ESC themselves [19] and the culture on Matrigel™ [20]. We obtained better adhesion rates when we used the MEF instead of the gelatin growth surface using the laser drill (80 versus 27.3%). Using whole-embryo culture we obtained a better adhesion rate using gelatin, but with only a small

Table 2 Comparison of the methods to isolate ICM from murine blastocysts

ICM isolation method	Growth surface ...		Murine feeder layer		Gelatin-coated dishes	
	Adhesion/isolation ICM	Establishment mESC	Adhesion/isolation ICM	Establishment mESC	Total adhesion isolation rate	
Whole-embryo culture; bad-quality blastocysts	4/6 (66.7% ^a)	2/6 (33.3%)	3/4 (75.0%)	0/4 (0%)	7/10 (70.0%)	
Laser-drilling method; good-quality blastocysts	8/10 (80.0%)	1/10 (10.0%)	3/11 (27.3%)	1/11 (9.1%)	11/21 (52.4%)	

^aOwing to a limitation in the number of the murine embryos used in the present study, these percentages have to be considered as only very approximate.

difference with respect to the use of an inactive MEF (66.7 versus 75%). If our purpose is not to be dependent on the culture and the inactivation of murine fibroblasts to maintain our stem-cell cultures, we need to do more research on the use of extracellular growth surfaces using gelatin-coated dishes. Furthermore, the mouse feeder layers could present special risks of microbiological contamination in the culture of hESC lines (e.g. mycoplasma, bacteria, yeast and fungi) [21]. However, viral contamination of feeder cells is a common risk in all biotechnological products derived from cell lines, irrespective of the species of origin [22]. For future experiments, we want to carry out the culture of ESCs in a serum-free system in order to eliminate dependence on the culture and the inactivation of murine fibroblasts as well as the risk of contamination with animal pathogens [23].

Our study must be completed with the assessment of the following data concerning the establishment of the mESC line carrying out the ESC characterization, ESC gene expression, embryoid-body formation and determination of cellular differentiation [12]. Moreover, our study should adopt a randomized point-of-view.

The future direction of our group is research on the use of the laser drill for the whole-embryo culture method, using the laser drill in the destruction of the trophectoderm cells that adhere to the ICM before the first passage (results not shown). Finally, research and experiments with animal models [3,4] must form part of the tasks of research centres and stem-cell banks, because, in the future, this will help us find new strategies in human regenerative medicine and cell therapy.

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