Whole-blastocyst culture followed by laser drilling technology enhances the efficiency of ICM isolation and ESC derivation from good and poor-quality mouse embryos: new insights for derivation of hESC lines

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Running title: Whole-embryo culture followed by laser drilling improves ESC derivation

Key words: mESC, inner cell mass, expanded blastocyst, laser drill technology, whole-blastocyst culture.

Funding Support: This work was funded by the Fundación Progreso y Salud (grants reference 0028/2006, 0029/2006 and 0030/2006 to C.B and P.M), Consejería de Salud, Junta de Andalucía, Spain and The International Jose Carreras Foundation against the Leukemia Award to P.M/C.B (EDThomas-05) and the Spanish Ministry of Health (FIS-PI070026 to PM).

ABSTRACT

The optimization of hESC line derivation methods is challenging because many laboratories have no access to spare human embryos and even if the embryos were available for laboratories closely linked to IVF clinics, studies performed directly on human embryos imply a waste of precious human biological material. Here, we developed a new strategy based on the combination of whole-blastocyst culture followed by laser drilling destruction of the trophoectoderm for improving the efficiency of ICM isolation and ESC derivation, using murine embryos. Embryos were divided into good- and poor-quality embryos. We demonstrate that the efficiency of both ICM isolation and ESC derivation using this novel strategy is significantly superior to whole-blastocyst culture or laser drilling technology itself. Regardless of the ICM isolation method, the ESC derivation process depends on a feeder cell growth surface. Importantly, this combined whole-blastocyst culture followed by laser drilling technology can be successfully applied to poor-quality blastocyts which, otherwise would not have even been used for laser drilling itself nor immunosurgery in an attempt to derive ESC lines due to the inability to distinguish the ICM. The ESC lines derived by this combined method were characterized and shown to maintain a typical morphology, undifferentiated phenotype and three germ layer differentiation potential. Finally, all ESC lines established using either technology acquired an aneuploid karyotype after extended culture periods, suggesting that the method used for ESC derivation does not seem to influence the karyotype of the ESCs after long-term culture. This methodology may open up new avenues for further improvements for the derivation of hESCs, the majority of which are derived from frozen, poor-quality human embryos.

INTRODUCTION

ESC lines were first generated in 1981 from mouse blastocysts (1,2). They are endowed with two unique properties that distinguish them from all other organ-specific stem cells identified thus far. First, they self-renew continuously and can be maintained and expanded for extended periods of time while maintaining their undifferentiated status. Second, they are pluripotent, capable of differentiating into every cell type in the body (3,4). The first human embryonic stem cell (hESC) lines were derived in 1998 from the inner cell mass (ICM) of donated, pre-implantation embryos (5,6) and are also endowed with these biological properties of self-renewal and pluripotency (7-20). Several laboratories have since reported the derivation of additional hESC lines from pre-implantation embryos, using either mouse or human feeders (reviewed in 21). Human ESCs are expected to open up new avenues in regenerative medicine by permitting the in vitro generation of transplantable cells to be used in future cell replacement therapies but are also anticipated to become a promising system to study stages of early human development that are inaccessible in vivo (3,22-29). Human ESC research represents a nascent area of investigation and therefore, many fundamental questions regarding the cellular and molecular mechanisms controlling pluripotency must be addressed and more efficient ESC derivation methods still need to be developed.

The blastocyst quality and the ICM isolation method used are the two main factors dictating either ESC derivation failure or success. Regarding blastocyst quality, human blastocyts available for ESC derivation are of poorer quality than their mouse counterparts. This is owing to i) in many countries human embryos must be used frozen at the time of hESC line derivation because of ethical constraints (30) whereas mouse blastocysts are always used fresh, and ii) in countries allowing the use of fresh embryos for hESC derivation the best quality embryos are transferred into the woman's uterus leaving the poor-quality leftover embryos for hESC derivation.

This explains not only the extremely low hESC derivation success rates (31) but also implies that in many countries (i.e. U.S and many European Countries) surplus poor quality fresh human embryos have to be either frozen or directly discarded without even being used for ESC derivation (30).

The current methods to isolate the ICM are still controversial. The ICM is usually isolated from the expanded blastocysts using a variety of techniques including: immunosurgery (32), mechanical processes (33) and whole-blastocyst culture methods (34). Alternatively, ESCs were established from single blastomeres obtained from cell stage embryos (35,36). These ICM isolation methods are associated with three main downsides: i) the use of xeno-components which may prevent the use of hESC derivatives in potential future therapeutic applications (32), ii) karyotypic changes after prolonged culture (37) and iii) low ESC establishment efficiency.

Culture of ESCs has been associated with long term karyotypic changes, although it remains to be elucidated whether the ESC derivation process itself or the culture conditions used might be responsible for this loss of genetic stability. Clearly, further studies on blastocyst quality, ICM isolation methods and subsequent ESC derivation and genomic stability are urgently required for hESC research to meet its expectations.

Ideally, the optimization of new ESC derivation methods should be done using human embryos as starting material. However, this represents a scientific/logistic challenge because: i) many laboratories have no access to enough spare human embryos from in vitro fertilization (IVF) cycles and ii) even if the embryos were available for laboratories closely linked to IVF clinics, the experimental optimization on human embryos would imply a great waste of precious human biological material.

The laser drilling technology is commonly used for assisted reproduction (38-40). This laser technology also facilitates the somatic cell nuclear transfer, by speeding up the process of enucleation to a matter of seconds, without eliciting damage to the egg (41). Very recently, we and

others have harnessed this laser drilling technology to destroy the trophoectoderm of good quality expanded murine blastocysts allowing for ICM isolation and mouse ESC derivation (42,43). Importantly, however, it needs to be stressed that the use of laser drill technology to shoot and breakdown the trophoectoderm requires the use of good quality expanded blastocysts with large and clearly distinguishable ICM, otherwise there is no way the embryologist can precisely identify and distinguish the ICM from the trophoectoderm cells, making the laser-shooting process inaccurate and random (42,43).

Here, we have developed a two-step ESC derivation method based on the combination of whole-blastocyst culture followed by laser drilling destruction of the trophoectoderm cells. We demonstrate that the efficiency of ICM isolation and ESC derivation using this strategy is significantly superior to both whole-blastocyst culture and laser drilling technology itself. Regardless of the ICM isolation method employed, the ESC derivation process seems to be dependent on the use of a feeder cell layer as growth surface. Most importantly, this combined whole-blastocyst culture followed by laser drilling technology can be successfully applied to poor-quality blastocyts which, otherwise would not have even been used by alternative methods for ESC line derivation due to the inability to distinguish the ICM. The ESC lines derived by this combined method were fully characterized and shown to maintain an undifferentiated state and three germ layer differentiation potential. Finally, ESC lines established using either methodology acquired an abnormal aneuploid karyotype after extended culture, suggesting that the method used for ESC derivation does not seem to make the ESC lines more vulnerable to genetic instability (44,45,46). We envision this two-step ESC derivation strategy will open up new avenues to further improve the derivation of hESCs, which are commonly derived from frozen, poor-quality human embryos.

MATERIALS AND METHODS

Blastocyst recovery

Blastocysts were flushed from oviducts of 3-6 month old female mice (C57BL/CBA) which were sacrificed by cervical dislocation on day 4.5 of pregnancy. The oviducts were dissected and placed in pre-warmed PBS (Gibco-Invitrogen, CA, USA) and then were carefully manipulated into a plastic Petri dish with warm G-MOPS (Vitrolife, Sweden) as previously described (43). Briefly, the oviducts were removed by first cutting through the top of the uterus, leaving a small portion of the uterus attached to the oviduct and the oviduct eventually dissected away from the ovary as previously described (43).

Blastocyst quality assessment

One hundred and thirty murine blastocysts were included in the present study. They were harvested either as a compacting blastocyst (**Fig. 1A**) or as an expanded blastocyst (**Fig. 1B**). Compacting blastocysts were cultured until they achieved the expanded blastocysts stage (**Fig. 1B**). In order to identify the most effective ICM isolation and ESC derivation method, the blastocyst quality was taken into account. Owing to the similar morphology between human and murine blastocysts, the human blastocyst grading system developed by the Cornell University (Ithaca, NY) programme was adopted (47). In our mESC derivation process, blastocysts were classified as good or poor quality blastocysts: good blastocyst had large and clearly distinguishable ICM (**Fig. 1C**). Poor blastocysts were classified as having distinguishable but smaller ICM (**Fig. 1D**) and those with indistinguishable ICM (**Fig. 1E**).

Inner Cell Mass Isolation Methods

The following three different experimental approaches were used for ICM isolation and further ESC line derivation. **Fig. 2** depicts our experimental design to enhance the efficiency of ICM isolation and ESC derivation.

Whole-blastocyst culture

The whole-blastocyst culture method was used regardless of the quality of the blastocysts as previously described (34, 43) (**Fig. 2**). Briefly, the blastocyst was cultured in such a way that the trophoectoderm cells and the ICM adhered to either MEFs (**Fig. 3A**) or gelatin (**Fig. 3B**). Two days later, the attachment of the blastocyst was confirmed and the medium replaced by fresh pre-warmed medium. By day 3 of culture, the trophoectoderm outgrowth was clearly visible (**Fig. 3 A,B**) providing a support for the ICM to grow and form a dome-like structure. At this time, the ICM was carefully plucked and transferred to a freshly prepared growth surface (43).

The whole-blastocyst culture method has the risk of trophoectoderm overgrowth because the entire trophoectoderm is cultured along with the ICM, preventing the embryologist from accessing the ICM in poor quality blastocysts.

Laser drill technology

The laser drill technology was first reported by Palermo´s Lab (42) and very recently reproduced in our lab (43). The laser (OCTAX Eyeware™, Germany) is connected to an inverted microscope (XI-71, Olympus) and processed by computer software which permits data analysis. The rationale of using laser drill technology is to laser-shoot the unwanted trophoectoderm cells in order to get rid of them. The laser drilling technology may be used by itself only when good quality expanded blastocysts are available. Unfortunately, however, the laser drilling technology itself is

not feasible for poor quality blastocysts because the ICM is not distinguishable and therefore can not be identified by the embryologist .

As previously described (42), expanded blastocysts are secured by holding two pipettes with the ICM positioned at 9 o'clock (42). The length of laser exposure, and the number of laser shots are controlled by the embryologist and may slightly vary among embryos. This laser drilling method, allows the ICM to adhere to the growth surface while getting rid of the trophoectoderm cells (**Fig. 3C-E**).

Whole-blastocyst culture followed by Laser drill technology

In an attempt to improve the methodology to isolate the ICM and to establish ESC lines from poor quality expanded blastocysts, we have developed a two-step method based on the whole-blastocyst culture followed by laser drilling. With this method, all murine expanded blastocysts were treated with Tyrode´s Acid (Irvine Scientific, CA, USA) for no more than one minute, to assure the complete dissolution of zona pellucida, in such a way that the trophoectoderm cells and the ICM adhere to the growth surface (**Fig. 3F**). Two days later, the attachment of the whole embryo was confirmed and the medium replaced by fresh pre-warmed medium. By day 3 of culture the trophoectoderm cells began to expand leaving the ICM accessible, forming a dome-like structure (**Fig. 3F**). At this point, we used the laser to shoot the trophoectoderm cells which surround the ICM, leaving the area near the ICM free of trophoectoderm cells (**Fig. 3G**) and reducing the risk of dragging the trophoectoderm cells when the ICM was plucked and transferred to a freshly prepared growth surface **(Fig. 3F-H)**.

Embryonic outgrowth culture

Embryonic outgrowths were cultured either on MEFs or in gelatin-coated plates in medium consisting of DMEM (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS) (PAA, Colbe, Germany), 1% L-glutamine (Gibco), 0.1 mM non-essential amino acids

(Gibco-Invitrogen), mouse leukemia inhibitory factor (LIF; 2000 IU/ml) (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), and 50 U/ml penicillin and 50 μg/ml streptomycin (PAA).

Growth Surfaces

In order to determine the extent to which the growth surface facilitates the ICM isolation and mESC establishment, the embryonic outgrowths were cultured individually either on MEFs (48) or in 0.2% gelatin-coated dishes (Sigma Aldrich) (49), as previously described (43).

Immunocytochemistry characterization of established mESCs

Established mESCs were characterized by indirect immunocytochemistry using antibodies against SSEA-1 (Developmental Studies Hybridoma Bank, University of Iowa, USA) and Oct3/4 (Santa Cruz Biotechnology, CA, USA). Briefly, mESC colonies were cultured in chamber slides coated with gelatin. Cells were fixed in 4% of paraformaldehyde for 20 minutes followed by 30 minutes incubation in 10% normal goat serum in PBS. For Oct3/4 immunostaining, cells are then permeabilized with Triton X100 (Sigma). Colonies were incubated with primary antibodies for 1 hour at RT. Conjugated secondary antibodies were used for 30 minutes at RT as follows: FITC-conjugated anti-mouse IgM for detecting SSEA-1 and anti-mouse IgG for Oct3/4 (Jackson Laboratories Inc). The slides were mounted in Vectashield containing DAPI. For negative control staining the primary antibodies were replaced by PBS.

Embryoid body (EB) formation and differentiation analysis

Near confluent mESCs were treated with 0.05% trypsin for 5 min at 37ºC, transferred ($2x10^2$ cells/cm^2) to non-adherent plates and allowed to differentiate spontaneously by EB formation in DMEM supplemented with 20% FBS, 1% L-glutamine, 0.1 mM non-essential amino acids and 0.1 mM β-mercaptoethanol but without LIF with media changes every 2-3 days. After 21 days in differentiation media, the EBs were transferred to tissue-treated plates where they grew and gave

rise to a confluent monolayer. Three germ layer differentiation was determined by immunocytochemistry. EB cells were fixed with 4% paraformaldehyde for 10 minutes. Then, cells were incubated (1 hour at RT) with primary antibodies for alpha-fetoprotein (Santa Cruz Biotechnology), anti-nestin (Chemicon) and anti-actin (Chemicon). Slides were then incubated with a byotinylated secondary antibody (30 minutes at RT) and a streptavidin peroxidase complex (30 minutes at RT) (Vector Laboratories Inc). The immunostaining was visualized using diaminobenzidine and counterstained with hematoxylin.

Cytogenetic analysis

Conventional karyotyping analysis was performed between passages (p12-p35) after establishment of the new ESC lines (44,45,50). Thirty metaphase spreads per mESC line were analyzed (44,45). The metasystem software (Izasa, Barcelona, Spain) was used for karyotyping analysis.

Statistical analysis

To explore the significance in the differences found between groups The Pearson chi-square test and Yates correlation were used. All analyses were done with the Statgraphic program. Differences were considered significant when p-values were less than 0.05.

RESULTS

Experimental strategy for the development of an approach combining whole-blastocyst culture followed by laser drilling technology considering the blastocyst quality

Our Spanish Stem Cell Bank is developing new methods to enhance the efficiency of hESC line derivation for future applications in basic biology and cell replacement therapies. The optimization of new ESC derivation methods using human embryos would be ideal but it is a scientific and logistic challenge because: i) many laboratories have no access to enough spare human embryos

from IVF cycles and ii) even if the embryos were available to laboratories closely linked to IVF clinics, the experimental optimization on human embryos would imply a great loss of precious human biological material.

To the best of our knowledge, the blastocyst quality is rarely (34) taken into account in a prospective manner before making a decision about the ESC derivation method to be used. Accordingly, we have used 130 murine embryos as starting material to develop a new technique based on whole-blastocyst culture followed by laser shooting of the trophoectoderm cells. One hundred and six out of the 130 embryos were good-quality expanded blastocysts and 24 out of 130 blastocysts were poor-quality expanded blastocysts (**Fig. 1**). Based on this blastocyst quality distribution, we designed the experimental strategy illustrated in **Fig. 2** to determine whether our proposed two-step method combining whole-blastocyst culture and laser drilling i) enhances the efficiency of ICM isolation/ESC establishment as compared to the whole-blastocyst culture (34, 43) and the laser drilling itself (42,43) and ii) may be successfully applied to poor-quality blastocyst which otherwise would not have even been employed by laser drilling technology itself since the ICM is not distinguishable for the embryologist (**Fig. 1,2**). ICM outgrowths were allowed to expand either over MEFs or gelatin in order to i) ascertain which growth surface best facilitates the establishment of ESC lines and ii) determine the potential interference between the ICM isolation method, growth surface and blastocyst quality for ESC derivation. Finally, established ESC lines were fully characterized by morphology, immunocytochemistry, cytogenetic analysis and in vitro differentiation potential.

Whole-blastocyst culture followed by laser drilling enhances the efficiency of ICM isolation regardless of the blastocyst quality and growth surface

Using the whole-blastocyst method, the ICM isolation efficiency was not affected neither by the growth surface used (average in MEFs 59% vs gelatin 70%; $p>0.05$) nor by the blastocyst quality (average: good-quality 67% vs poor-quality embryos: 63%, $p>0.05$) (**Table 1**). This ICM isolation methodology has a few disadvantages. Firstly, trophoectoderm cells proliferate very fast suppressing the outgrowth of the ICM. Secondly, there is a risk of trophoectoderm overgrowth and is very likely to drag trophoectoderm cells when plucking the ICM (**Fig. 3A,B**).

To get around these disadvantages, we used the recently developed laser drilling technique (42,43) based on laser-shooting of the expanded blastocysts in order to get rid of the unwanted trophoectoderm cells (**Fig. 3C-E**). The ICM isolation efficiency on MEFs was identical between the whole-blastocyst culture and the laser drilling technique (53% vs 47%, $p>0.05$) (**Table 1**). On gelatin, however, rather than the laser drilling technique the whole-embryo culture facilitated the ICM isolation (80% vs 16%; $p<0.05$%). Unfortunately, however, although this novel laser-based approach allows for a cleaner trophoectoderm removal, it can only be applied to good-quality embryos (**Fig. 3C and Tables 1**) and is rarely applicable to poor-quality embryos (**Fig. 1D,E**) where the ICM is not distinguishable, making its use not feasible when attempting to derive hESC lines from human embryos which are normally frozen and are of poorer quality.

In an attempt to enhance the efficiency of ICM isolation and ESC derivation in comparison with the whole-blastocyst culture or laser drill technique and, more importantly, in order to harness the poor-quality embryos which are normally discarded or not even considered for ESC derivation, we developed a novel two-step approach based on embryo attachment to either MEFs or gelatin by whole-blastocyst culture (**Fig. 3F**) followed by laser-shooting of the trophoectoderm (**Fig. 3G,H**). **Fig. 3G** illustrates the isolated ICM free of trophoectoderm cells whilst **Fig. 3H** shows the rest of

laser-shot destroyed trophoectoderm. Intriguingly, using good-quality embryos, this novel two-step approach significantly increased the ICM isolation efficiency on MEFs (100%) as compared to whole-blastocyst culture (53%; p=0.005) and laser drilling alone (47%; p=0.001), respectively (**Table 1**). Moreover, on gelatin this newly developed method enhanced the ICM isolation efficiency by 7-fold as compared to laser drilling (100% vs 16%; p=0.0001) and slightly as compared to whole-blastocyst culture (100% vs 80%) (**Table 1**).

More importantly, in an attempt to rescue the poor-quality embryos and make them suitable and useful for ESC derivation, we observed that the attachment of the poor-quality embryo to a monolayer of MEFs followed by laser destruction of the trophoectoderm resulted in a significantly superior (almost 2-fold) ICM isolation success as compared with whole-blastocyst culture itself (100% vs 65%; p=0.05) (**Table 1**). On gelatin as a growth surface, this combined method equally resulted in a slight increase of ICM isolation (75% vs 60%) (**Table 1**). This data indicates that regardless of the blastocyst quality and growth surface this newly developed whole-blastocyst culture helped by laser drilling technique clearly facilitates the ICM isolation.

Whole-blastocyst culture followed by Laser drilling enhances the efficiency of ESC derivation regardless of the blastocyst quality in a growth surface-dependent manner

Among the good-quality embryos, 33% of the initially isolated ICMs on MEFs expanded and gave rise to ESC lines when the whole-blastocyst culture followed by laser drilling strategy was employed. In contrast, only 16% (2-fold decrease; p=0.04) and 6% (6-fold decrease; p=0.0001) of the isolated ICMs expanded and gave rise to ESC lines using the whole-blastocyst culture itself or the laser drilling technology alone, respectively (**Table 2**).Importantly, an almost 2-fold increase in the efficiency of ESC derivation was observed among the poor-quality embryos when the whole-

blastocyst culture followed by laser drilling was used as compared with whole-blastocyst culture (50% vs 30%; p=0.09) (**Table 2**).

Despite the ICMs being successfully isolated using gelatin as a growth surface (**Table 1**), it is remarkable that no ESC line could be derived in such a feeder-free system, regardless the blastocyst quality and/or the ICM isolation method used (**Table 2**). On gelatin, an extensive trophoectoderm overgrowth was always observed (**Fig. 3A**) preventing us from plucking a clean ICM without dragging trophoectodem cells. Taken together, this data suggests that whole-blastocyst culture combined with laser drilling technology significantly enhances the efficiency of ESC derivation regardless the blastocyst quality but in a feeder-dependent manner. The implementation of this newly developed method provides an alternative to immunosurgery and whole-blastocyst culture to standardize ESC line derivation in a way that permits the usage of poor-quality human blastocysts (fresh and specially frozen) which are the main source of starting material for hESC line derivation.

Characterization of ESC lines established using whole-blastocyst culture followed by laser drilling technology

In order to ensure that the new technology does not impair the intrinsic biological properties of the derived ESC lines, we characterized the ESC lines derived with this novel approach. The ESC lines showed a typical ESC morphology (**Fig. 4 A,B**) and expressed the markers associated with an undifferentiated stage SSEA-1 (**Fig. 4C**) and Oct3/4 (**Fig. 4D**).

To assess their in vitro differentiation potential, EBs (**Fig. 4E**) were formed and allowed to differentiate under conditions promoting the specification into tissues representing the three germ layers. Accordingly, ESC lines derived using whole-blastocyst culture helped by laser drilling showed homogeneous expression of the endoderm-associated pan-marker alpha-fetoprotein (**Fig.**

4F), the mesoderm marker actin (**Fig. 4G**) and the ectoderm marker nestin (**Fig. 4H**). Taken together, this data demonstrates that the newly developed methodology does not compromise the capacity of the derived ESC lines to maintain an undifferentiated stage in culture and three germ-layer differentiation potential.

The method used for ESC derivation does not seem to determine the karyotype of the ESCs after extended culture

Long-term culture of ESCs is associated with karyotypic changes, although it remains to be elucidated whether or not the ESC derivation process itself is responsible for the loss of genetic stability. Accordingly, here we analyzed whether the methodology used for ESC derivation makes the ESCs more vulnerable to genetic instability after long-term culture. Using conventional karyotyping, we assessed the frequency of aneuploidy of ESC lines derived either using a whole-blastocyst culture or laser drilling technology.

ESC lines established using either method were diploid (40 chromosomes) after short culture (passage 12) (**Fig. 5A,B**). However, regardless of the derivation method used, all ESC lines established acquired an abnormal aneuploid karyotype (> 40 chromosomes) after extended culture (passage 35) (**Fig. 5C-E**). This data suggests that the method used for ESC derivation does not seem to influence the likelihood of karyotypic changes, although further studies are still needed to determine whether the frequently observed karyotypic changes are due to the routine ESC maintenance and culture adaptation.

DISCUSSION

The successful derivation of hESC lines by Thomson and colleagues (6) opened a new area of investigation in regenerative medicine and early human development. Human ESCs are anticipated to serve as an invaluable experimental model to investigate stages of human development that are inaccessible *in vivo* (27-29,44). However, hESC research represents a nascent area of research and therefore, many fundamental questions regarding the cellular and molecular mechanisms controlling proliferation, self-renewal, differentiation and cellular transformation must be addressed.

In addition, the current methods to isolate the ICM are still controversial. The ICM is usually isolated from the expanded blastocysts using a variety of techniques including immunosurgery (2), mechanical processes (33) and whole-blastocyst culture methods (34) which are associated with three main drawbacks: i) the use of xeno-components which may prevent the use of hESC derivatives in potential future therapeutic applications (32), ii) karyotypic changes after prolonged culture (37) and iii) low ESC establishment efficiency. Our Spanish Stem Cell Bank is optimizing new methods to enhance the efficiency of hESC line derivation. Ideally, the optimization of ESC derivation methods should be carried out using human embryos. However, this represents a scientific and logistic challenge because: i) many laboratories have no access to enough spare human embryos from IVF clinics and ii) even if the embryos were available for laboratories closely linked to IVF clinics, the experimental optimization on human embryos would imply a great loss of precious human biological material. Although there are differences between the mouse and the human embryogenesis, as with any scientific research using animal models, it is envisioned that experience gained from mESC work will supply answers to challenges still vexing the hESC field. Here, we have developed a new strategy based on the combination of whole-blastocyst culture followed by laser drilling destruction of the trophoectoderm for improving

the efficiency of ICM isolation and ESC derivation. We demonstrate that the efficiency of both ICM isolation and ESC derivation using this new technology is significantly superior to both whole-blastocyst culture and laser drilling technology itself. The ESC lines derived by this two-step method were characterized and shown to maintain a typical ESC morphology, an undifferentiated phenotype and being capable of three germ layer differentiation potential.

Apart from the ICM isolation method, the blastocyst quality used represents a key factor influencing ESC derivation success. Human blastocyts available for ESC derivation are of poorer quality than their mouse counterparts owing to, at least in part, i) in many countries human embryos must be used frozen at the time of hESC line derivation because of ethical constraints (30) and, ii) in countries allowing the use of fresh embryos for hESC derivation the best quality embryos are transferred into the woman's uterus leaving the poor-quality leftover embryos for hESC derivation. This explains not only the extremely low hESC derivation success rates (31) but also implies that in many countries surplus poor-quality fresh human embryos have to be either frozen or directly discarded without being even used for ESC derivation (30). Accordingly, to the best of our knowledge, the blastocyst quality is rarely (34) taken into account in a prospective manner before making a decision about the ESC derivation method to be used.

Here, regardless of the blastocyst quality and the ICM isolation method, the ESC derivation process seems to be fully dependent on the use of a feeder cell layer as growth surface. Whilst we and others may routinely maintain established mESCs and hESCs under feeder-free conditions (gelatin and matrigel), the present work, however, suggests that a cell feeder layer is still required to derive ESC lines since we were unable to establish a single mESC colony using gelatin as a growth surface. In particular, when using gelatin as growth surface, we observed an enormous trophoectoderm overgrowth impeding the plucking of the ICM without dragging trophoectoderm cells and the subsequent derivation of ESC lines (**Fig. 3B**). Thus, this study offers a newly

developed technique as an alternative to immunosurgery in an attempt to reduce the use of xeno-components and whole-embryo culture in order to clean up the ICM from trophoectoderm cells but indicates that feeder cells are still required for ESC derivation.

From our point of view, the major contribution of this study is the fact that this combined whole-blastocyst culture followed by laser drilling technology can be equally applied with success to both good and poor-quality blastocyts which, otherwise would not have even been used for laser drilling itself in an attempt to derive ESC lines due to the inability to distinguish the ICM. This technological advance should encourage further initiatives aimed at deriving hESC lines from frozen or poor-quality human surplus embryos which are normally discarded or not even considered for ESC derivation.

Finally, long-term culture of mESCs (46) and hESCs (37,44) is associated with karyotypic changes, although it remains to be elucidated whether or not the ESC derivation process itself influences such a loss of genetic stability. Here, we have analyzed whether whole-blastocyst culture or laser drilling technology used for ESC derivation makes the ESCs more vulnerable to karyotypic changes after long-term culture. All our ESC lines established, acquired an abnormal aneuploid karyotype after extended culture periods, regardless of the derivation method used, suggesting that the ESC derivation method does not influence the karyotype of the ESC lines. As it has been previously described by Jaenisch`s group (44) trisomy 8 was a common chromosomal abnormality in all our ESC lines established by whole-embryo culture. However, ESC lines derived using a laser shooting method, showed gains of chromosomes 1 and 2 but never showed trisomy of chromosome 8. Future studies may reveal the molecular and cellular mechanisms underlying the different karyotypic changes observed in ESCs obtained by different methodologies. In our humble opinion, we speculate that rather than the ESC derivation method, the routine culture methods used for maintenance entails an ESC culture adaptation and subsequent clonal evolution

which is likely to contribute to these karyotypic changes. However, we must be cautious and recognize that further genetic studies are needed to clarify whether the ESC derivation process itself or the culture methods used for ESC maintenance and passage are responsible for the loss of genetic stability.

On the basis of these studies, prospective experiments are ongoing in our laboratory to refine and apply this new technique to the derivation of hESC lines from frozen poor-quality embryos that represent very valuable human material for research in cell therapy and basic biology. It should be recognized that although differences between mESCs and hESCs exist (51), caution is required when extrapolating findings between species. However, this data on mESC derivation, underlines the relevance of taking into account the blastocyst quality before one or another derivation method is applied (34).

ACKNOWLEDGMENTS

We thank Dr.Jose Luis García (Michigan University, USA) and Dr. Javier García-Castro (BACM, Granada, Spain) for critically reading the manuscript. We are indebted to Dr. Antonia Collado for the outstanding technical support and advice on animal experimentation.

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LEGEND TO FIGURES

Figure 1. Representative pictures depicting the quality classification of murine blastocysts obtained in this study. A) Embryo in compacting blastocyst stage. **B)** Embryo in expanded blastocyst stage. **C)** Good quality expanded blastocyst with large and clearly distinguishable ICM. **D)** Poor expanded blastocysts with distinguishable but small ICM and **E)** Poor blastocysts with indistinguishable ICM. The black arrows indicate the ICM region.

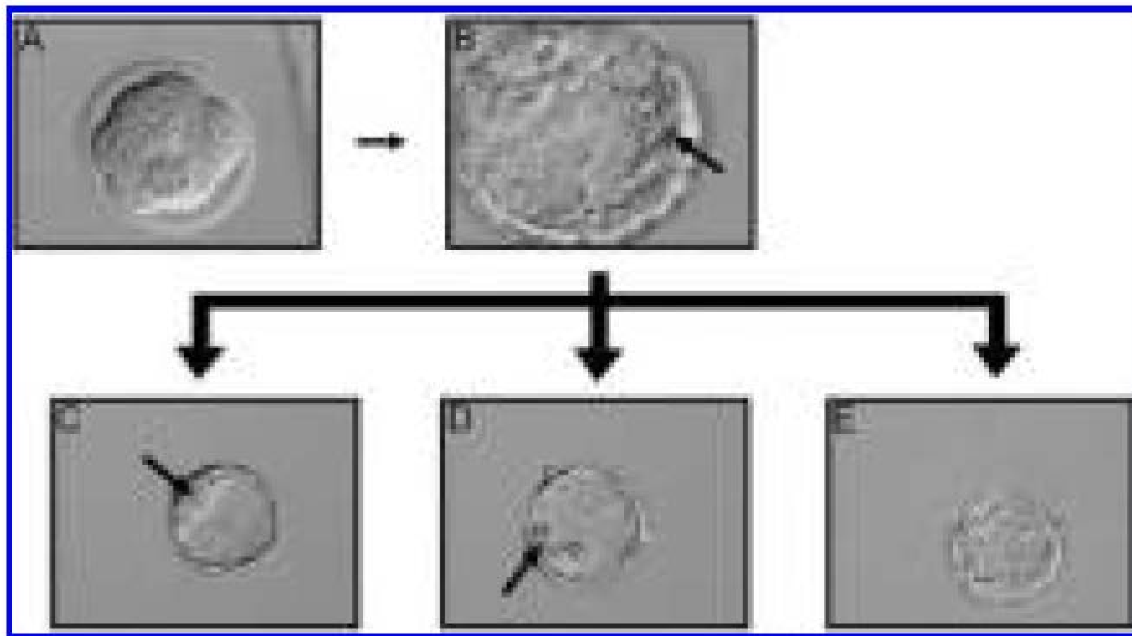


Figure 2. Experimental design for the optimization of the efficiency of ESC derivation based on the impact of blastocyst quality, ICM isolation method and growth surface. One hundred and thirty murine expanded blastocysts were used in the present study. Blastocysts were classified as good quality blastocyst (n=106) or poor quality blastocyst (n=24). Good quality blastocyst had large and clearly distinguishable ICM whereas poor quality blastocyst had distinguishable but small ICM or indistinguishable ICM. Good quality blastocysts were subdivided into three groups to isolate the ICM and derive mESC lines based on three different ICM isolation methods: i) whole-blastocyst

culture (n=48) (42); ii) Laser drilling (n=36) (41) and iii) a new strategy combining whole-blastocyst culture and laser drilling technology (n=22). Poor quality blastocysts, however, were subdivided into two groups: i) whole-blastocyst culture (n=11) (42)and ii) whole-blastocyst culture followed by laser drilling (n=13). It should be noted that the laser drill technology alone could never be applied to poor quality blastocysts because the ICM is not distinguishable and therefore it can not be identified by the embryologyst. Isolated ICMs were cultured either on MEFs or gelatin-coated plates to ascertain which of these growth surfaces best support the expansion of the ICMs and subsequent establishment of mESC lines. The efficiency of ICM isolation and mESC establishment were analyzed under each experimental condition. In addition, established mESC lines were characterized by immunocytochemistry, cytogenetic analysis, and their in vitro differentiation potential into the three germ layers (ectoderm, mesoderm, and endoderm).

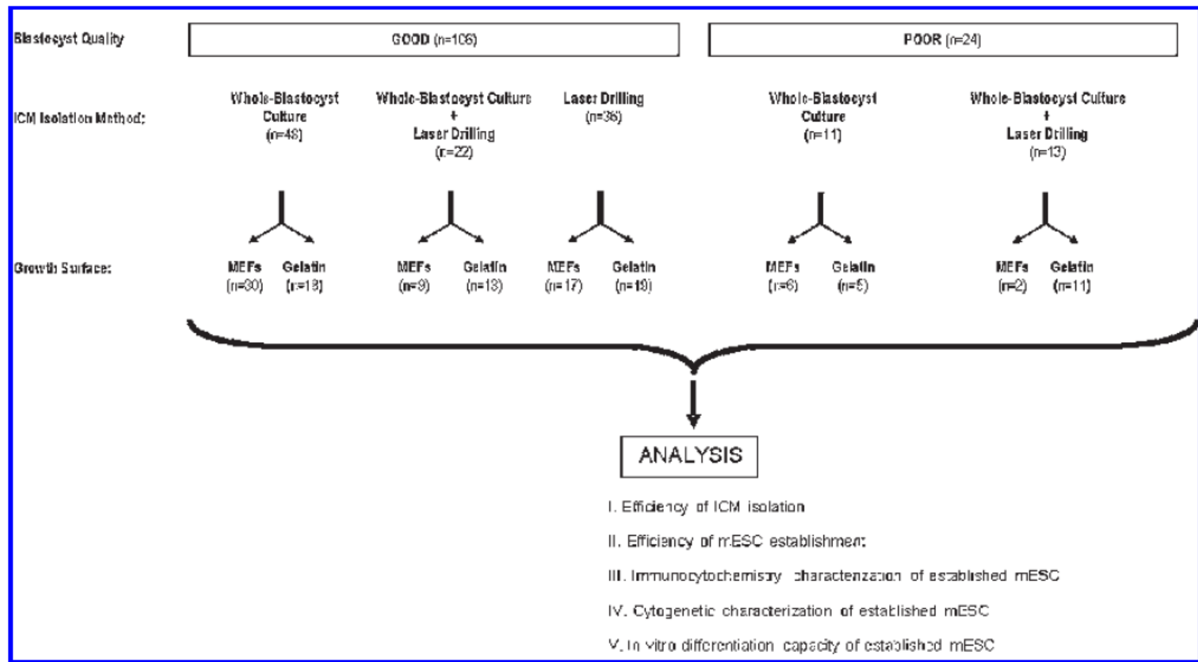


Figure 3. Representative pictures showing the assessment of ICM isolation methods compared in the present study. (A-B) Isolation of the ICM by whole-blastocyst culture method alone. **A)** Outgrowth of a blastocyst adhered to MEFs 5 days after whole-blastocyst culture. **B)** Outgrowth of a blastocyst adhered to gelatin 5 days after whole-blastocyst culture. Please note the outgrowth of the trophoectoderm in gelatin. **(C-E)** Isolation of the ICM by laser drilling technology itself. **C)** Blastocyst secured by two holding pipettes with the ICM being released by laser destruction of the trophoectoderm and zona pellucida. The black arrowheads represent the laser shots. **D)** Blastocyst shown in C right after trophoectoderm breakdown by laser shot. The black arrow indicates the laser-lysed trophoectoderm. **E)** Outgrowth of the same blastocyst 2 days after direct laser drilling. **(F-G)** Isolation of the ICM by whole-blastocyst culture and subsequent laser drilling of the trophoectoderm. **F)** Outgrowth of a blastocyst 5 days after whole-blastocyst culture. **G)** Clean ICM free of trophoectoderm cells after laser shots. **H)** Residual trophoectoderm after laser drilling. The asterisks represent the ICMs. The wide white arrows denote the embryo zona pellucida. The fine white arrows show examples of the exact laser shots.

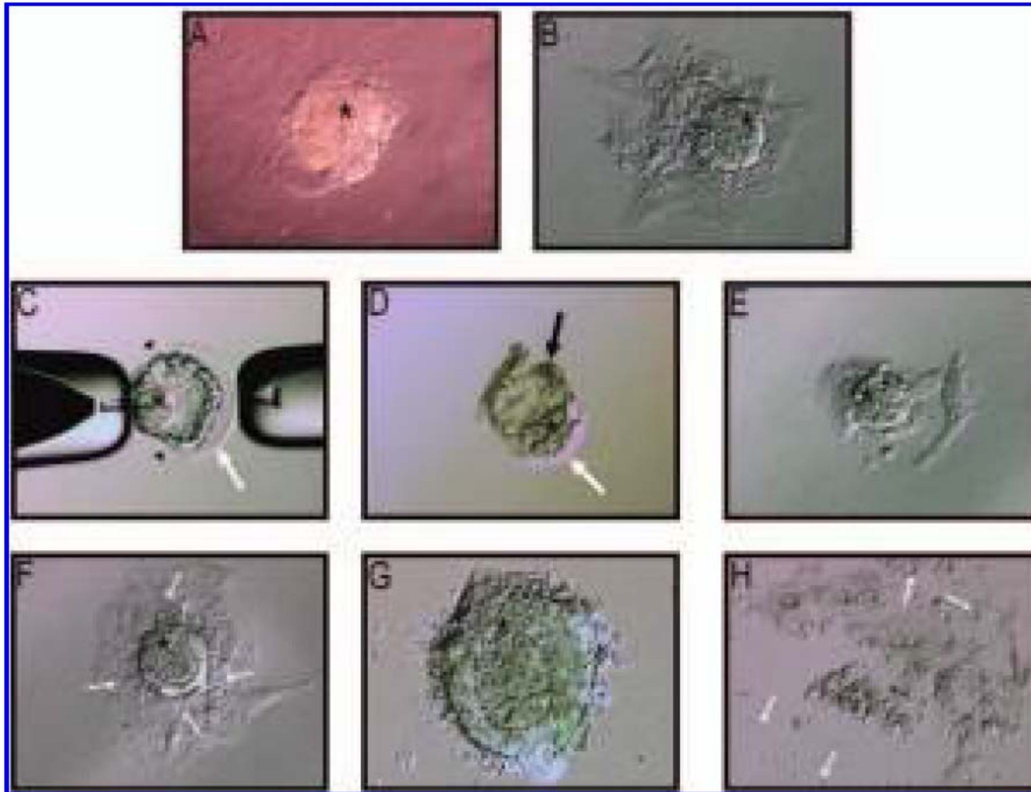


Figure 4. ESCs derived by whole-blastocyst culture plus laser drilling technology show a typical ESC morphology, express undifferentiated markers and retain three germ layer differentiation potential. A) Typical morphology (10X magnification) of an ESC line growing on MEFs established by whole-blastocyst culture and subsequent laser drilling of the trophoectoderm. **B)** 40X magnification of an ESC colony established by whole-blastocyst culture followed by laser drilling of the trophoectoderm. **C)** Expression of SSEA-1 (green). Nuclei counterstaining with DAPI (blue) .**D)** Expression of Oct3/4 (red). **E)** Representative pictures of EBs. **F)** Expression of alpha-fetoprotein (endoderm). **G)** Expression of smooth muscle actin (mesoderm). Black arrows depict actin positive cells **H)** Expression of nestin (ectoderm).

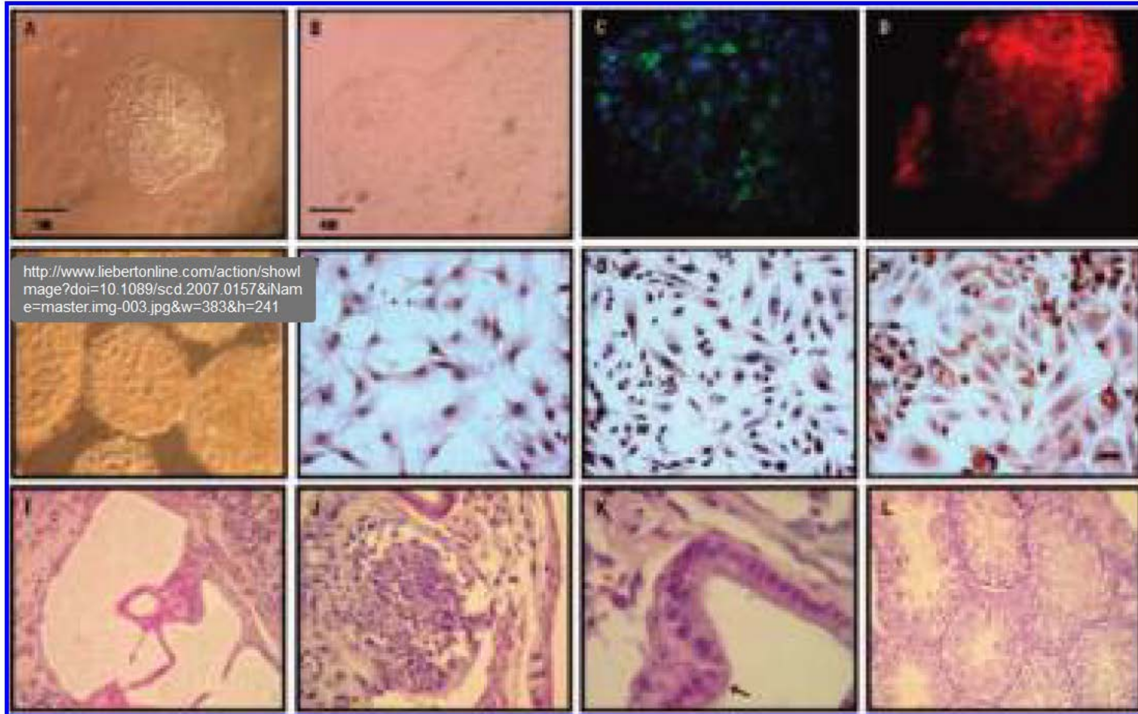
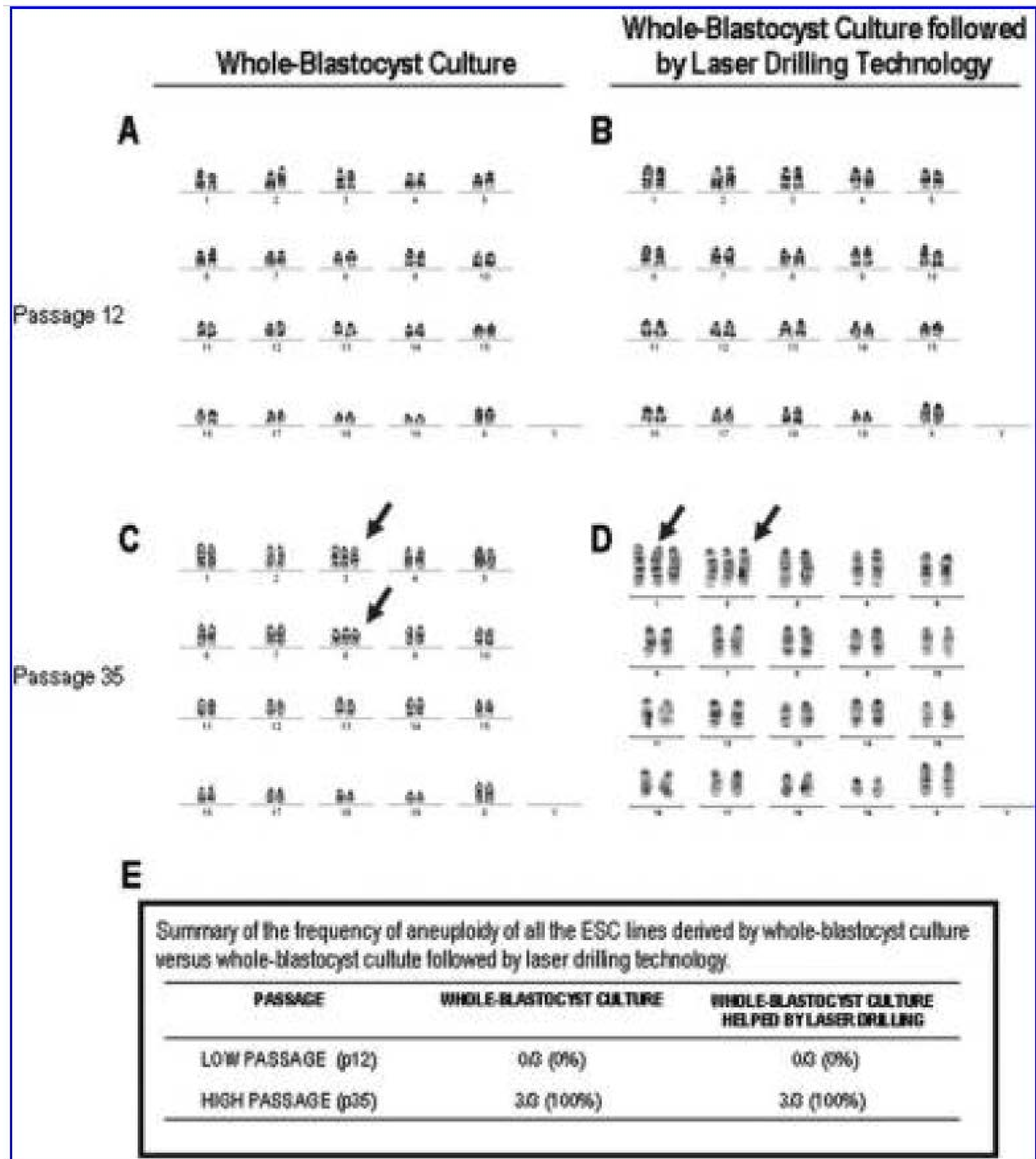


Figure 5. Cytogenetic characterization of established ESC lines. A) Representative diploid karyotype of an ESC line established by whole-blastocyst culture after 12 passages in *in vitro* culture. **B)** Representative diploid karyotype of an ESC line established by laser drilling technology after 12 passages in *in vitro* culture. **C)** Representative aneuploid karyotype of an ESC line established by whole-blastocyst culture after extended *in vitro* culture. **D)** Representative aneuploid karyotype of an ESC line established by laser drilling technology after extended *in vitro* culture. Blue arrows denote karyotypic changes (chromosome trisomies). **E)** Summary of the frequency of aneuploidy of all the ESC lines derived by whole-blastocyst culture versus whole-blastocyst cultute followed by laser drilling technology.



Summary of the frequency of aneuploidy of all the ESC lines derived by whole-blastocyst culture versus whole-blastocyst cultute followed by laser drilling technology.

PASSAGE	WHOLE-BLASTOCYST CULTURE	WHOLE-BLASTOCYST CULTURE HELPED BY LASER DRILLING
LOW PASSAGE (p12)	0/3 (0%)	0/3 (0%)
HIGH PASSAGE (p35)	3/3 (100%)	3/3 (100%)

Table 1. Efficiency of ICM Isolation Based on Blastocyst Quality and ICM Isolation Method Using either MEFs Versus Gelatin as Growth Surface

Growth surface	*ICM isolation method*	*Blastocyst quality*	
		Good	*Poor*
MEFs	Whole blastocyst culture	53%[a]	65%[c]
	Laser drilling	47%[b]	N.F.
	Whole blastocyst culture helped by laser drilling	100%	100%
Gelatin	Whole blastocyst culture	80%	60%
	Laser drilling	16%[d]	N.F.
	Whole blastocyst culture helped by laser drilling	100%	75%

MEFs, Inactive mouse embryonic fibroblasts; N.F., not feasible.
Statistical differences are shown between:
[a]Whole blastocyst culture helped by laser drilling vs. whole blastocyst culture for good-quality blastocysts in MEFs, $p = 0.005$.
[b]Whole blastocyst culture helped by laser drilling vs. laser drilling for good-quality blastocysts in MEFs, $p = 0.01$.
[c]Whole blastocyst culture helped by laser drilling vs. whole blastocyst culture for poor-quality blastocysts in MEFs, $p = 0.05$.
[d]Whole blastocyst culture helped by laser drilling vs. laser drilling for good-quality blastocysts in gelatin, $p = 0.00001$.

Table 2. Efficiency of ES Cell Establishment Based on Blastocyst Quality and ICM Isolation Method Using Either MEFs or Gelatin as Growth Surface

Growth surface	*ICM isolation method*	*Blastocyst quality*	
		Good	*Poor*
MEFs	Whole blastocyst culture	16%[a]	30%[c]
	Laser drilling	6%[b]	N.F.
	Whole blastocyst culture helped by laser drilling	33%	50%
Gelatin	Whole blastocyst culture	0%	0%
	Laser drilling	0%	N.F.
	Whole blastocyst culture helped by laser drilling	0%	0%

MEFs, Inactive mouse embryonic fibroblasts; N.F., not feasible.
Statistical differences between:
[a]Whole blastocyst culture helped by laser drilling vs. whole blastocyst culture for good-quality blastocysts, $p = 0.04$.
[b]Whole blastocyst culture helped by laser drilling vs. laser drilling for good-quality blastocysts, $p = 0.00001$.
[c]Whole blastocyst culture helped by laser drilling vs. whole blastocyst culture for poor-quality blastocysts, $p = 0.09$.