Stem Cell Rev and Rep (2011) 7:494–505
DOI 10.1007/s12015-010-9221-7

Influence of E-Cadherin-Mediated Cell Adhesion on Mouse Embryonic Stem Cells Derivation from Isolated Blastomeres

Sheyla González · Elena Ibáñez · Josep Santaló

Published online: 29 December 2010

Abstract Efforts to efficiently derive embryonic stem cells (ESC) from isolated blastomeres have been done to minimize ethical concerns about human embryo destruction. Previous studies in our laboratory indicated a poor derivation efficiency of mouse ESC lines from isolated blastomeres at the 8-cell stage (1/8 blastomeres) due, in part, to a low division rate of the single blastomeres in comparison to their counterparts with a higher number of blastomeres (2/8, 3/8 and 4/8 blastomeres). Communication and adhesion between blastomeres from which the derivation process begins could be important aspects to efficiently derive ESC lines. In the present study, an approach consisting in the adhesion of a chimeric E-cadherin (E-cad-Fc) to the blastomere surface was devised to recreate the signaling produced by native E-cadherin between neighboring blastomeres inside the embryo. By this approach, the division rate of 1/8 blastomeres increased from 44.6% to 88.8% and a short exposure of 24 h to the E-cad-Fc produced an ESC derivation efficiency of 33.6%, significantly higher than the 2.2% obtained from the control group without E-cad-Fc. By contrast, a longer exposure to the same chimeric protein resulted in higher proportions of trophoblastic vesicles. Thus, we establish an important role of E-cadherin-mediated adherens junctions in promoting both the division of single 1/8 blastomeres and the efficiency of the ESC derivation process.

S. González · E. Ibáñez · J. Santaló (✉)
Departament de Biologia Cel·lular, Fisiologia i Immunologia,
Facultat de Biociències, Universitat Autònoma de Barcelona,
08193 Bellaterra, Spain
e-mail: Josep.Santalo@uab.cat

S. González
e-mail: Sheyla.Gonzalez@uab.cat

E. Ibáñez
e-mail: Elena.Ibanez@uab.cat

Keywords Adherens junctions · ESC · Mouse embryos · Single blastomeres · TSC · Uvomorulin

Introduction

Embryonic stem cells (ESC) are traditionally derived from the inner cell mass (ICM) of blastocysts [1]. However, ethical issues associated with the destruction of human embryos during this process have lead to alternative methods, such as the derivation of ESC from single blastomeres. Different attempts have been done in order to efficiently derive ESC from isolated blastomeres [2–5] but, although a bulk of information has been obtained from these studies, there are still some aspects, such as events that occur during the preimplantational development, that need to be addressed in order to improve the derivation efficiency of the lines.

The mouse embryo undergoes the first differentiating event during the preimplantational development when the trophectoderm (TE) epithelium and the inner cell mass (ICM) are formed in the blastocyst. Morphological changes, produced until the blastocyst stage is reached, are dependent on the intercellular junctions between blastomeres. At early cleavage stages (2- and 4-cell) blastomeres are connected by simple membrane apposition while specialized intercellular junctions such as gap junctions, adherens junctions, tight junctions and desmosomes appear from the 8-cell stage onwards in order to form the blastocyst [6]. All of these junctions are involved in multiple morphoregulatory processes such as communication, adhesion, differentiation, signalling and proliferation [7], which are crucial in the differentiation of the ICM and TE in the blastocyst.

Adherens junctions are first structured at the blastomere membrane of embryos at the 8-cell stage and are mediated

by E-cadherin, the epithelial counterpart of a glycoprotein family (cadherins) which regulates cell-adhesion in a calcium-dependent manner [8]. E-cadherin has a signaling role complementary to its adhesion capacity, which is involved in multiple processes such as the establishment of tissue limits, metastasis, tissue reorganizations, cell migration, cell differentiation and proliferation [7]. In early mouse embryogenesis, E-cadherin is involved in the compaction at the morula stage and in the transition from morula to blastocyst stage [9]. Particularly, E-cadherin is crucial in inducing the polarization of the compacted morula in order to form a functional and fully polarized TE [10].

The importance of intercellular junctions in ESC derivation efficiency and maintenance is well established; it has been shown that spontaneous differentiation of mouse ESC is associated with a downregulation of the surface E-cadherin mediated by inhibitory molecules, gelatinase activity and cell motility [11–14]. Furthermore, E-cadherin has been demonstrated to be required for LIF-dependent self-renewal of mouse ESC [15]. Regulative mechanisms underlying the importance of E-cadherin-mediated junctions in the establishment and maintenance of human and mouse ESC have been recently described [16–18]. Signaling pathways controlling proliferation, cell fate and differentiation in mouse ESC and early embryos are less well known, but a clear connexion has been established between the adhesion-mediated signaling of E-cadherin and the Wnt pathway in controlling such processes through the adaptor protein β-catenin, a common factor between the two pathways [19, 20]. Therefore, different signaling pathways coexist in ESC and early embryos and their function depends on the cell environment.

Previous studies performed in our laboratory on the derivation of mouse ESC from isolated blastomeres at different developmental stages revealed very low establishment rates when a single blastomere from an 8-cell stage embryo (1/8) was used. Compared to its counterparts with a higher number of blastomeres (2/8, 3/8 and 4/8 groups), the 1/8 group exhibited low rates of blastomere division when seeded onto the feeder cell monolayer at the beginning of the derivation process [21]. In view of these results, the low division rate of 1/8 blastomeres could be related to the lack of direct contact with neighbouring blastomeres. As previously mentioned, E-cadherin is involved in modulating proliferation and, in addition, it is important in signaling at the 8-cell stage. Thus, it might be a good candidate to investigate in order to determine its relationship with the low division capacity of 1/8 blastomeres and therefore, the low ESC derivation rate.

The aim of the present study was to assess the importance of E-cadherin-mediated adherens junctions in the derivation efficiency of mouse ESC lines from single blastomeres. To this aim, an approach consisting in the adhesion of a chimeric E-cadherin to the surface of 1/8 blastomeres was devised, to recreate the signalling produced by native E-cadherin between neighbouring blastomeres inside the embryo. The effect of this approach on the rate of division of 1/8 blastomeres and on the efficiency of the ESC derivation process was determined.

Material and Methods

Experimental Design

To determine the influence of E-cadherin-mediated adherents junctions on ESC derivation from isolated 1/8 blastomeres, a chimeric E-cadherin (E-cad-Fc), consisting in the extracellular domain of mouse E-cadherin (amino acids 1-709) and the human IgG1 Fc domain, was used to recreate the signaling process exerted by native E-cadherin between neighbouring blastomeres. In preliminary experiments, the adhesion of E-cad-Fc to the blastomere surface was checked by immunofluorescence. Because E-cadherin-mediated cell adhesion is dependent on the presence of the calcium ion, the adhesion of E-cad-Fc was performed in a DMEM standard medium containing calcium. As a negative control, a calcium- and magnesium-free PBS solution was used. The specificity of the binding was also tested by removing E-cad-Fc from the blastomere surface by incubating the blastomeres in a calcium- and magnesium-free PBS solution. It is important to note that the use of the chimeric protein allowed us to distinguish it from the endogenous mouse E-cadherin by using antibodies against the Fc human IgG1 domain. To further confirm the specific interaction between E-cad-Fc and native E-cadherin, the distribution of native E-cadherin was assessed by direct labelling with an anti-mouse E-cadherin antibody.

ESC derivation experiments in the presence of E-cad-Fc were carried out in two series (Fig. 1). In the first series of experiments, 1/8 blastomeres (n=100) were cultured in ESC culture conditions in the presence of E-cad-Fc to assess its effect on the division capacity of the single blastomeres and on the ESC derivation efficiency. After 48 h, E-cad-Fc was passively removed by diluting it through successive changes of fresh medium every 2 days (long exposure). In the second set of experiments, 1/8 blastomeres (n=125) were cultured in ESC culture conditions but, in this case, E-cad-Fc was actively removed 24 h after blastomeres were seeded onto the feeder cell monolayer (short exposure). This second set of experiments was designed to determine whether the effect of E-cad-Fc could be restricted to the first 24 h and, hence, increase the

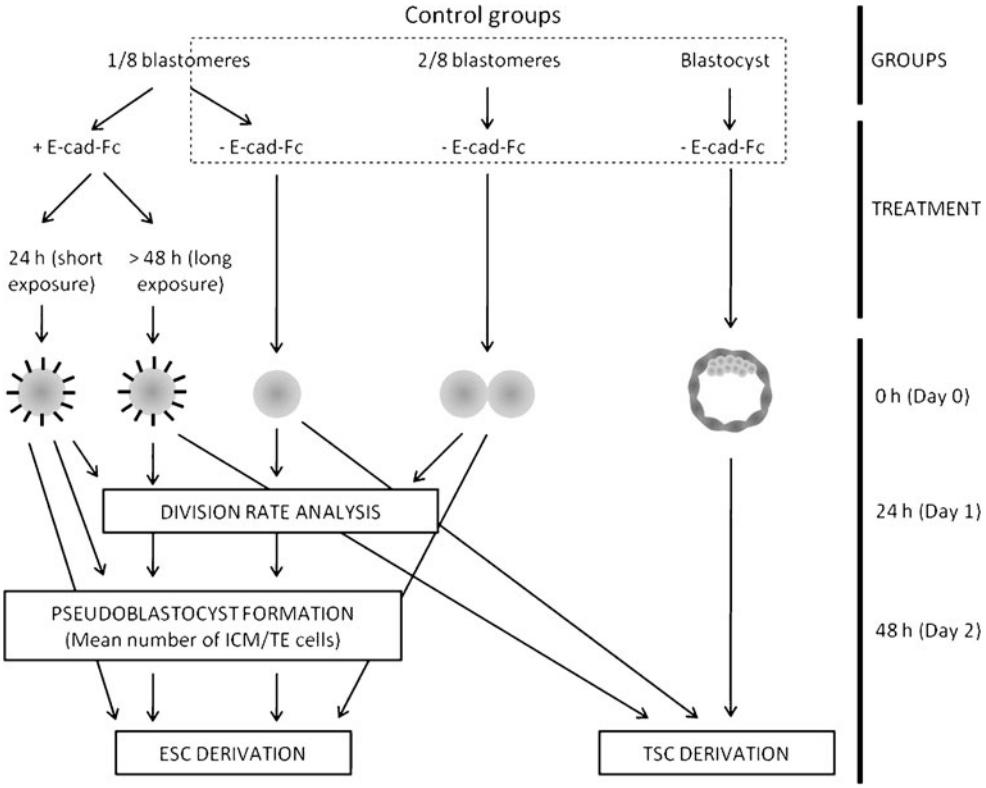

Fig. 1 Schematic representation of groups and treatments performed in the experiments

division rate while avoiding the differentiating event to TE observed in the first series of experiments with a long exposure to E-cad-Fc. As control groups for both sets of experiments, 1/8 blastomeres (n=222) and 2/8 blastomeres (n=30) without E-cad-Fc were used.

In all derivation experiments, structures resembling blastocysts (hereafter referred as pseudoblastocysts) were observed on the second day of culture. The mean number of ICM, TE and total cells in pseudoblastocysts derived from experiments with E-cad-Fc (n=25) and in control pseudoblastocysts (n=50) was estimated by immunofluorescence (Oct4 for ICM and Cdx2 for TE).

Finally, 1/8 blastomeres (n=30) subjected to a long exposure to E-cad-Fc were cultured in trophoblast stem cells (TSC) derivation conditions in order to confirm whether this long exposure to E-cad-Fc had a differentiating effect to TE. 1/8 blastomeres (n=25) and blastocysts (n=25) cultured in TSC conditions without E-cad-Fc were used as controls in this experiment.

ESC and TSC lines obtained in this study were characterized by immunofluorecence through the expression of pluripotency markers (Oct4, Sox2 and Nanog for ESC, and Cdx2 for TSC). Moreover, the in vitro differentiation potential of the ESC lines derived was confirmed by immunofluorescence through the expression of the neuroepithelial stem cell protein (Nestin, ectodermal layer), alpha-fetoprotein (AFP, endodermal layer) and α-smooth muscle actin (α-SMA, mesodermal layer).

Feeder Cells Culture

STO mouse embryo fibroblasts (ECACC) were inactivated by a treatment with mitomycin C (Invitrogen) during 3 h at a concentration of 10 μg/ml in order to produce feeder cells [22]. To plate the feeder cells, Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 15% fetal calf serum (FCS) (Invitrogen) was used.

Embryo Collection and Blastomere Isolation

Embryos collected from female 129/Sv mice mated with male C57Bl/6 mice were used [1]. Prior to mating, females were subjected to a superovulation process by intraperitoneal injection of 5 iu of pregnant mare serum gonadotrophin (PMSG) (Intervet) followed by 5 iu of human chorionic gonadotrophin (hCG) (Farma-Lepori) 48 h after the first injection. Embryos at the 2-cell stage were obtained 48 h after the hCG injection by flushing the oviducts with Hepes-buffered KSOM and were cultured in KSOM (EmbryoMax; Millipore) at 37°C under 5% CO_2 until 8-cell and blastocyst stages were reached.

Groups of 1 or 2 blastomeres from 8-cell stage embryos (1/8 and 2/8 groups, respectively) were isolated by micromanipulation in a PBS solution [23]. Briefly, the zona pellucida was disrupted with a 10 μm drilling micropipette containing Tyrode's acid solution and individual blastomeres were aspirated with a 20 μm diameter micropi-

pette. All blastomeres of the 8-cell stage embryos were used to avoid a bias on results due to possible differences in blastomere commitment at this stage.

Detection of E-cad-Fc Adhesion to the Blastomere Surface by Immunofluorescence

Groups of 1/8 blastomeres were incubated with 1.5 μg/ml of E-cad-Fc (Sigma-Aldrich) during 2 h in a 20 μl drop inside a non-adherent polypropylene plate (Nunc). E-cad-Fc was diluted in standard DMEM medium with calcium to facilitate its adhesion to the blastomere surface. Afterwards, blastomeres were washed 3 times in DMEM during 5 min, fixed in 4% paraformaldehyde (Sigma) during 15 min, washed again 3 more times and incubated with a 1:20 dilution of a monoclonal mouse anti-human IgG1 secondary antibody FITC-conjugated (Invitrogen) during 2 h. Finally, samples were washed again in DMEM and stained with Hoechst 33258 (Invitrogen) at 10 μg/ml as a nuclear counterstain.

The same protocol was used to analyse the presence of E-cad-Fc on the blastomere surface after the first change of medium performed at 48 h in the first set of experiments (long exposure).

To remove E-cad-Fc off the blastomere surface within the 24 h in the second set of experiments (short exposure), blastomeres were washed 3 times in a calcium- and magnesium-free PBS solution supplemented with 150 mM EDTA. Afterwards, blastomeres were incubated with the secondary antibody mentioned above to verify the complete withdrawal of E-cad-Fc.

Samples were examined with an epifluorescence microscope Olympus Bx60 and an image capture and analyzing system (Software Genus, 3.0 version).

Detection of Native E-Cadherin on the Blastomere Surface by Immunofluorescence

Native E-cadherin on the surface of blastomeres at the 8-cell stage (isolated or inside the embryo) was labelled using an anti-mouse E-cadherin antibody (Table 1). Isolated blastomeres or embryos were fixed in 4% paraformaldehyde during 15 min. Blocking and permeabilization was performed overnight in a PBS solution containing 0.5% Triton X-100 (Sigma), 3% goat serum (Sigma) and 0.2% sodium azide (Sigma). Incubation with the primary antibody was done overnight at 4°C and detection with the corresponding secondary antibody (Table 1) was performed during 2 h at room temperature. Samples were washed with block solution before and after incubation with antibodies. Finally, samples were stained with Hoechst 33258 (Invitrogen) at 10 μg/ml as a nuclear counterstain and examined with an epifluorescence microscope Olympus Bx60 and an image capture and analyzing system (Software Genus, 3.0 version).

Establishment of Stem Cell Lines and Exposure to E-cad-Fc

ESC lines were derived from 1/8 and 2/8 blastomeres using a defined culture medium [5, 21]. Briefly, isolated blastomeres were seeded onto a feeder cell monolayer cultured in 50 μl drops contained in a 60 mm Petri dish

Table 1 Primary and secondary antibodies used for cell characterization

Primary antibody	Dilution	Reference	Secondary antibody	Concentration	Reference	Cell type stained
Rabbit monoclonal anti-E-cadherin	1:500	Abcam ab40772	Goat anti-rabbit IgG Alexa Fluor 594	6 μg/ml	Molecular Probes A-11037	Blastomeres 8-cell stage
Mouse monoclonal anti-Oct4	1:50	Santa Cruz sc-5279	Chicken anti-mouse IgG Alexa Fluor 488	6 μg/ml	Molecular Probes A-21200	ESC colonies
Rabbit polyclonal anti-Oct4	1:1500	Santa Cruz sc-9081	Goat anti-rabbit IgG Alexa Fluor 594	6 μg/ml	Molecular Probes A-11037	ICM cells
Rabbit polyclonal anti-Nanog	1:200	Abcam ab21603	Goat anti-rabbit IgG Alexa Fluor 594	6 μg/ml	Molecular Probes A-11037	ESC colonies
Rabbit polyclonal anti-Sox2	1:200	Millipore AB5603	Goat anti-rabbit IgG Alexa Fluor 594	6 μg/ml	Molecular Probes A-11037	ESC colonies
Mouse monoclonal anti-Cdx2	1:100	BioGenex MU392A-UC	Chicken anti-mouse IgG Alexa Fluor 488	6 μg/ml	Molecular Probes A-21200	TSC colonies/ TE cells
Rabbit polyclonal anti-Nestin	1:250	Abcam ab5968	Goat anti-rabbit IgG Alexa Fluor 594	6 μg/ml	Molecular Probes A-11037	Differentiated ESC colonies
Rabbit polyclonal anti-AFP	1:400	Dako A0008	Goat anti-rabbit IgG Alexa Fluor 594	6 μg/ml	Molecular Probes A-11037	Differentiated ESC colonies
Mouse monoclonal anti-α-SMA	1:400	Sigma A5228	Chicken anti-mouse IgG Alexa Fluor 488	6 μg/ml	Molecular Probes A-21200	Differentiated ESC colonies

with ESC derivation DMEM medium supplemented with 100 μM 2-ß-mercaptoethanol (Invitrogen), 1 mM L-glutamine (Invitrogen), 1X non essential aminoacids (Invitrogen), 10^3 units/ml leukemia inhibitory factor (LIF; Millipore), 20% Knockout Serum Replacement (KSR; Invitrogen) and 0.1 mg/ml adrenocorticotropic hormone (ACTH; Sigma-Aldrich).

In the derivation of ESC lines from 1/8 blastomeres, E-cad-Fc at a concentration of 1.5 μg/ml was added to the culture medium before 1/8 blastomeres were seeded and, as previously mentioned, it was passively removed after the first 48 h in the first set of experiments (long E-cad-Fc exposure) or actively removed at 24 h in experiments with short exposure to E-cad-Fc by the aforementioned means. Control 1/8 and 2/8 blastomeres were cultured in E-cad-Fc-free ESC derivation medium.

TSC lines were established following the protocol described by Tanaka (2006) [24]. Briefly, 1/8 blastomeres or blastocysts were seeded onto a feeder cell monolayer in 50 μl drops in a 60 mm Petri dish with TSC establishment RPMI1640 medium (LabClinics) supplemented with 20% FCS, 2 mM L-glutamine, 100 μM 2-ß-mercaptoethanol and 1 mM sodium pyruvate (Sigma-Aldrich). Moreover, the TSC establishment culture medium contained variable amounts of heparin (1–1.8 μg/ml; Sigma-Aldrich) and fibroblast growth factor 4 (25–45 ng/ml; FGF4; Pepro-Tech) depending on the derivation step.

In the derivation of TSC lines from 1/8 blastomeres, E-cad-Fc at a concentration of 1.5 μg/ml was added to the culture medium before 1/8 blastomeres were seeded. E-cad-Fc was not passively removed until the third day of culture when all outgrowths were attached to the feeder cells and the culture medium was changed following the protocol of Tanaka (2006) [24]. TSC lines were also derived from blastocysts, as a positive control for the derivation in TSC conditions, and from 1/8 blastomeres in the absence of E-cad-Fc, as a negative control.

Stem Cell Lines Characterization and Immunostaining of Pseudoblastocysts

Stem cell colonies were first selected based on their morphology. ESC colonies present a defined morphology at the edge and have round cells. TSC colonies, by contrast, present a non-defined morphology at the edge and have flattened cells.

Later, pluripotency of the selected ESC- or TSC-like colonies was confirmed by immunofluorescence, using specific markers Oct4, Nanog and Sox2 for ESC, and Cdx2 for TSC (Table 1). Stem cell colonies were washed 3 times in PBS, fixed in 4% paraformaldehyde during 15 min and washed again 3 times in PBS. Blocking and permeabilization was performed in the same block solution used for native E-cadherin detection, during 30 min. Incubation with primary antibodies was done overnight at 4°C. Then they were washed 3 times in PBS and incubated with the corresponding secondary antibody (Table 1) during 2 h at room temperature. Finally, samples were washed again in PBS and stained with Hoechst 33258 at 10 μg/ml as a nuclear counterstain.

Pseudoblastocysts were harvested on day 2 and immunostained in order to determine the mean number of ICM and TE cells. ICM cells were detected by Oct4 marker and TE cells by Cdx2 marker. The immunostaining protocol followed was the same used for the stem cell colonies except that pseudoblastocysts were washed with block solution instead of PBS and that permeabilization was performed overnight.

Samples were examined with an epifluorescence microscope Olympus Bx60 and an image capture and analyzing system (Software Genus, 3.0 version).

In Vitro Differentiation of Embryonic Stem Cell Lines

To further confirm the pluripotency of the ESC lines derived, they were subjected to differentiating culture conditions to produce cells of the three germ layers: ectoderm, endoderm and mesoderm. First, ESC lines were subcultured to gelatin pre-coated dishes (Sigma) in the absence of feeder cells and LIF. Moreover, the KSR of the culture medium was replaced with FCS to favor differentiation. Culture medium was replaced every two days and after a period of 7 days, the lines were fixed for immunofluorescence analysis.

Characterization of Embryonic Stem Cell Lines Differentiated in Vitro

In vitro differentiated ESC lines were characterizated by immunofluorescence for the detection of specific markers of the three germ layers such as Nestin (ectoderm), AFP (endoderm) and α-SMA (mesoderm). Primary and secondary antibodies used are detailed in Table 1. ESC lines were also immunostained with Oct4 marker in addition to each marker of differentiation to detect not fully differentiated cells and, finally, stained with Hoechst 33258 as a nuclear counterstain. The immunofluorescence protocol was the same used for the characterization of the undifferentiated ESC lines.

Statistical Analysis

Results were statistically analysed by X^2 and Fisher exact test for proportions and Student's *t*-test for mean comparisons. Values with a $P<0.05$ were considered statistically significant.

Results

E-cad-Fc Adhesion to the Blastomere Surface

E-cad-Fc adhered to the blastomere surface when the incubation was performed in a non-adherent polypropylene plate since this avoids compound binding to the dish and the E-cad-Fc is free to adhere to the blastomere surface. This association was observed after 2 h of incubation with the blastomere, only when E-cad-Fc was diluted in calcium containing DMEM medium unlike to what happened when E-cad-Fc was diluted in a calcium- and magnesium-free PBS solution supplemented with EDTA (Fig. 2b and d).

The pattern observed after E-cad-Fc was adhered to the blastomere surface was a dotted, foci-like labelling pattern most frequently homogenously distributed throughout the surface (Fig. 2b), suggesting that native E-cadherin was uniformly redistributed along the entire surface of the blastomere after its isolation from the embryo. However, some blastomeres presented a localised area where E-cad-Fc foci were more concentrated, suggesting that it was the area where the initial neighbouring blastomeres established contact inside the embryo. This distribution was further confirmed by a direct labelling of the native E-cadherin (Fig. 2i and j).

Similarly, the presence of E-cad-Fc after the first 48 h, when the change of the medium is produced, was checked by immunofluorescence in the pseudoblastocysts formed on day 2. Some foci on the blastomeres surface were found indicating that the change of the medium did not produce a complete withdrawal of E-cad-Fc in the first set of experiments with a long exposure to E-cad-Fc (Fig. 2f).

Finally, incubation in a calcium- and magnesium-free PBS solution of those blastomeres exposed during 24 h to E-cad-Fc clearly removed the E-cad-Fc signal detected by immunostaining, confirming the complete withdrawal of the E-cad-Fc (Fig. 2h). All these results corroborate the specificity of E-cad-Fc binding to the blastomere surface.

Effect of a Long Exposure to E-cad-Fc on ESC Derivation Efficiency

The percentage of blastomere division (Fig. 3a) after 24 h of culture with E-cad-Fc was 67% (Table 2), a value significantly higher ($p=0.0003$) than that of 1/8 control groups cultured without E-cad-Fc (44.6%), although significantly lower ($p<0.0001$) than the one from 2/8 control group in which the two blastomeres connect through native E-cadherin-mediated junctions (100%). Despite the increased rate of division, only 1 ESC line (1%) was obtained from 1/8 blastomeres exposed to E-cad-Fc. This derivation efficiency is similar to that obtained for the 1/8 control group without E-cad-Fc in the culture medium, in which

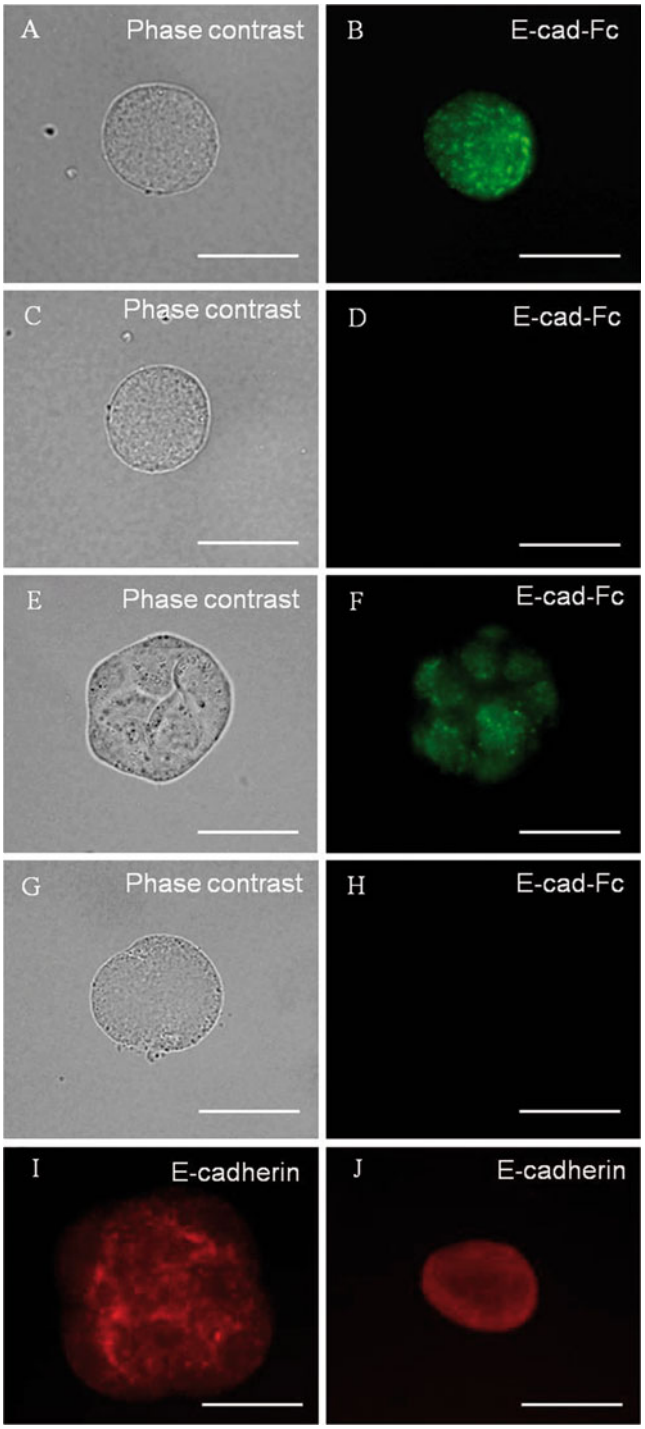

Fig. 2 Immunofluorescence detection of E-cad-Fc adhesion to the blastomere surface and native E-cadherin. **a**–**b** 1/8 blastomere incubated with E-cad-Fc for 2 h in calcium-containing DMEM medium. **c**–**d** 1/8 blastomere incubated with E-cad-Fc for 2 h in calcium- and magnesium-free PBS. **e**–**f** Pseudoblastocyst formed on day 2 of culture from 1/8 blastomeres subjected to a long exposure to E-cad-Fc. **g-h** 1/8 blastomere exposed to E-cad-Fc for 24 h and washed with calcium- and magnesium-free PBS supplemented with EDTA. **i** Native E-cadherin distributed throughout blastomeres surface and concentrated at blastomeres contact regions from an 8-cell stage embryo. **j** Native E-cadherin homogeneously distributed on the surface of an isolated blastomere from an 8-cell stage embryo. Scale bar: 30 μm

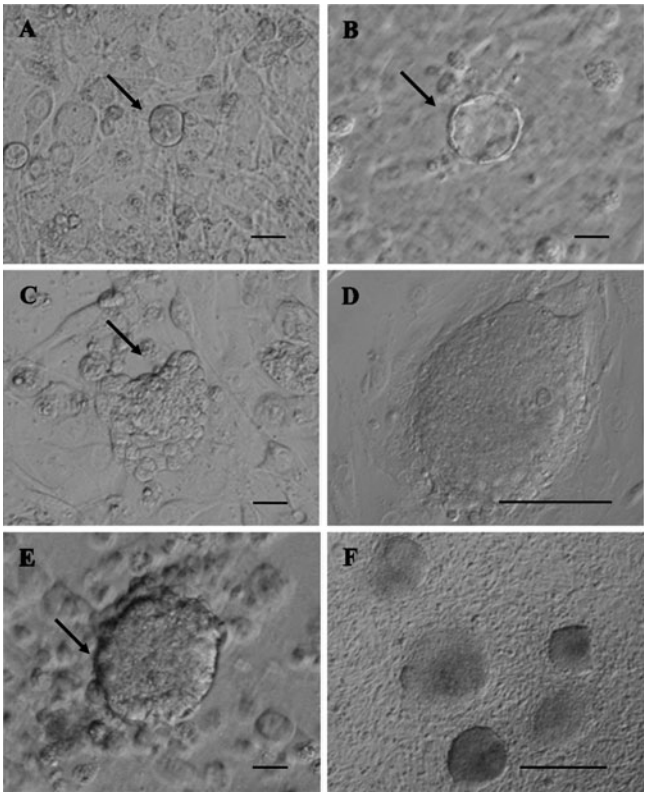

Fig. 3 Images of ESC and TSC lines establishment from 1/8 blastomeres. **a** First division of a 1/8 blastomere. **b** Pseudoblastocyst formed on the second day of culture. **c** TSC-like outgrowth. **d** TSC colony. **e** ESC-like outgrowth. **f** ESC colonies. Scale bar **a**–**c** and **e**: 30 μm; scale bar **d**: 100 μm; scale bar **f**: 200 μm

only 5 ESC lines (2.2%) could be derived, and significantly lower (p=0.0001) than that of the 2/8 control group (23.3%). Thus, the increase of the division rate in the group of 1/8 blastomeres in contact with E-cad-Fc for more than 48 h does not directly improve the efficiency of the ESC derivation process.

On day 2 of culture, pseudoblastocysts (Fig. 3b) formed from both 1/8 blastomeres cultured with E-cad-Fc and control groups. Morphologically, most pseudoblastocysts derived from 1/8 blastomeres in contact with E-cad-Fc did not seem to contain ICM cells, unlike to their counterparts in the control group. In order to confirm the lack of ICM cells, the mean number of ICM, TE and total cells of these pseudoblastocysts (Table 3) was determined by immunofluorescence (Fig. 4). When E-cad-Fc was passively removed from blastomeres surface, pseudoblastocysts showed a mean number of 2.1 ICM cells while 44% of them did not contain any ICM cell. The mean number of ICM cells in the control group without E-cad-Fc was 6.0 and just 10% of the pseudoblastocysts contained TE cells only. Comparisons between 1/8 blastomeres exposed to E-cad-Fc and the control group resulted to be significantly different (p<0.0001 for means comparison and p=0.0018 for percentage of pseudoblastocysts without ICM cells). Moreover, the mean number of total cells was significantly lower (p=0.0005) in those pseudoblastocysts obtained after exposure to E-cad-Fc when compared to controls, suggesting that a long exposure to E-cad-Fc has a negative effect on ICM cells proliferation.

On day 3 of culture, after pseudoblastocysts attached to the feeder cells, outgrowths with a TSC-like morphology were observed in the culture (Fig. 3c). In particular, blastomeres cultured in the presence of E-cad-Fc produced 22% of TSC-like outgrowths (Table 2) and although this value did not significantly differ from the production of ESC-like outgrowths in the same group (14%), it resulted to be significantly higher (p<0.0001) than the percentage of TSC-like outgrowths found in the control group without E-cad-Fc (3.6%). Moreover, TSC-like outgrowths were positive for Cdx2 marker as suspected from the morphology of the pseudoblastocysts they came from (those containing TE cells only or with few ICM cells) (Fig. 4). Thus, long exposure of blastomeres to E-cad-Fc produces an increased percentage of TSC-like outgrowths.

Effect of a Short Exposure to E-cad-Fc on ESC Derivation Efficiency

In view of the results obtained with a long exposure to E-cad-Fc, a second set of experiments was designed in order to determine whether the effect of E-cad-Fc could be restricted to the first 24 h and, hence, increase the division rate of 1/8 blastomeres while avoiding the inhibition of ICM proliferation and the bias towards TSC derivation.

Table 2 Rates of first division and of ESC lines derivation

Derivation group	n	% First division after 24 h (n)	% ESC-like outgrowths (n)	% TSC-like outgrowths (n)	% ESC lines (n)
Control (1/8)	222	44.6 (99)^{a}	17.1 (38)^{a}	3.6 (8)^{a}	2.2 (5)^{a}
Control (2/8)	30	100 (30)^{b}	60.0 (18)^{b}	0.0 (0)^{a}	23.3 (7)^{b}
E-cad-Fc long exposure (>48 h)	100	67.0 (67)^{c}	14.0 (14)^{a}	22.0 (22)^{b}	1.0 (1)^{a}
E-cad-Fc short exposure (24 h)	125	88.8 (111)^{bc}	50.4 (63)^{b}	4.0 (5)^{a}	33.6 (42)^{b}

$^{a–c}$Values with different superscripts within the same column differ significantly

Table 3 Number of ICM and TE cells in pseudoblastocysts

Derivation group	n	% Pseudoblastocysts without ICM cells (n)	Mean of ICM cells (±SEM)	Mean of TE cells (±SEM)	Mean of total cells (±SEM)
Control	50	10 (5)[b]	6.0±0.59[a]	14.0±0.52[a]	19.9±0.85[a]
E-cad-Fc long exposure (>48 h)	25	44 (11)[a]	2.1±0.58[b]	13.1±0.63[a]	15.2±0.76[b]
E-cad-Fc short exposure (24 h)	25	4 (1)[b]	6.4±0.66[a]	13.9±0.77[a]	20.4±0.94[a]

[a–b] Values with different superscripts within the same column differ significantly

As in previous experiments, short exposure (24 h) to E-cad-Fc increased the percentage of 1/8 blastomeres division (88.8%) so that significant differences ($p<0.0001$) were found compared to 1/8 control groups without E-cad-Fc (44.6%), but not to 2/8 control group (100%; Table 2).

The complete withdrawal of E-cad-Fc improved the production of ESC lines from 1/8 blastomeres and 42 ESC lines (33.6%) were derived (Figs. 3f and 5a). This ESC derivation rate was significantly higher ($p<0.0001$) than the one found after a longer culture with E-cad-Fc (1%) and in 1/8 control groups without E-cad-Fc (2.2%), while resembling the ESC derivation rate obtained from the 2/8 control group (23.3%; Table 2). The confirmation by immunofluorescence of the in vitro differentiation of an ESC line towards cells of the three germ layers is shown in Fig. 5b.

Most of the pseudoblastocysts (96%) formed on day 2 after an active removal of E-cad-Fc exhibited an evident ICM, similar to pseudoblastocysts from the control group without contact with E-cad-Fc (90%). As in previous experiments, pseudoblastocysts were immunostained to determine the proportion of ICM and TE cells (Fig. 4). In this sense, pseudoblastocysts from the group with E-cad-Fc showed a mean number of 6.4 ICM cells while just 4% of these structures presented TE cells only. No significant differences were found between these data and the one from the control group (Table 3). By contrast, comparisons with 1/8 blastomeres exposed to a passive withdrawal of E-cad-Fc revealed significant differences in both the percentage of pseudoblastocysts without ICM cells ($p=0.0019$) and the mean number of ICM cells ($p<0.0001$), indicating an increased number of ICM cells in pseudoblastocysts when E-cad-Fc was removed from the culture medium after 24 h. No significant differences between 1/8 blastomeres with E-cad-Fc in these experiments and the control group were found in the mean number of TE cells and in the mean number of total cells of the pseudoblastocysts (Table 3).

The production of ESC-like outgrowths in short exposure experiments was significantly higher ($p<0.0001$) than the one of TSC-like after pseudoblastocysts attachment (Table 2) indicating an improvement in the production of ESC-like outgrowths with regards to long exposure experiments in which there was a passive withdrawal of E-cad-Fc. Further, in short exposure experiments differences with 1/8 control blastomeres were only detected in the number of ESC-like outgrowths ($p<0.0001$) unlike what happened in previous experiments of long exposure to E-cad-Fc. Therefore, a short exposure to E-cad-Fc during 24 h increases the production of ESC-like outgrowths and, consequently, the efficiency of ESC lines derivation.

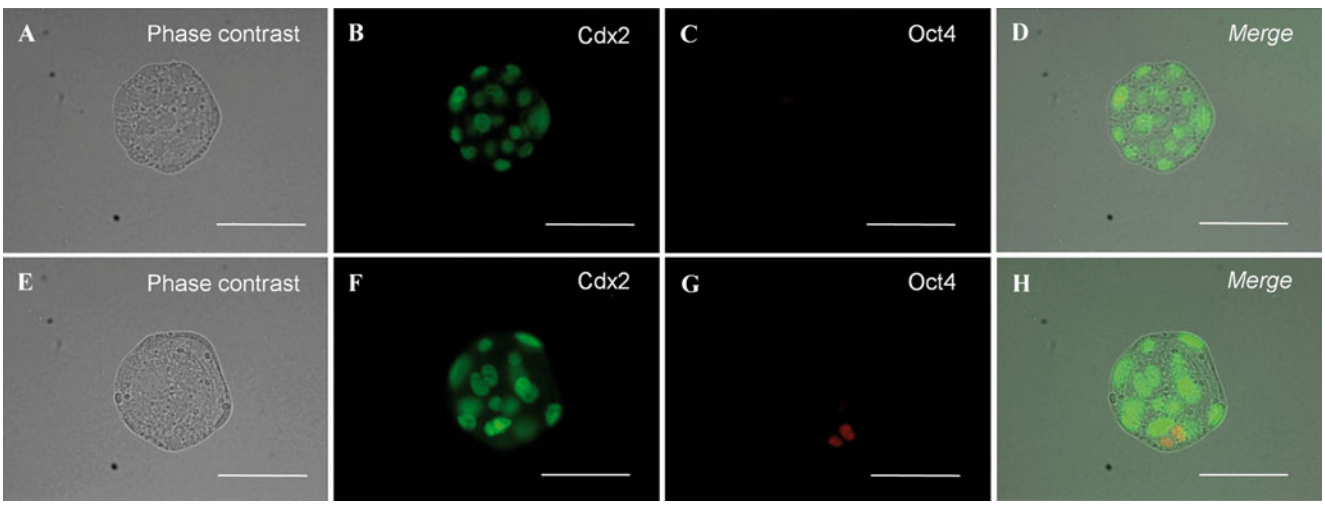

Fig. 4 Immunostaining of pseudoblastocysts without ICM cells (**a**–**d**) and containing ICM cells (**e**–**h**). Scale bar: 50 μm

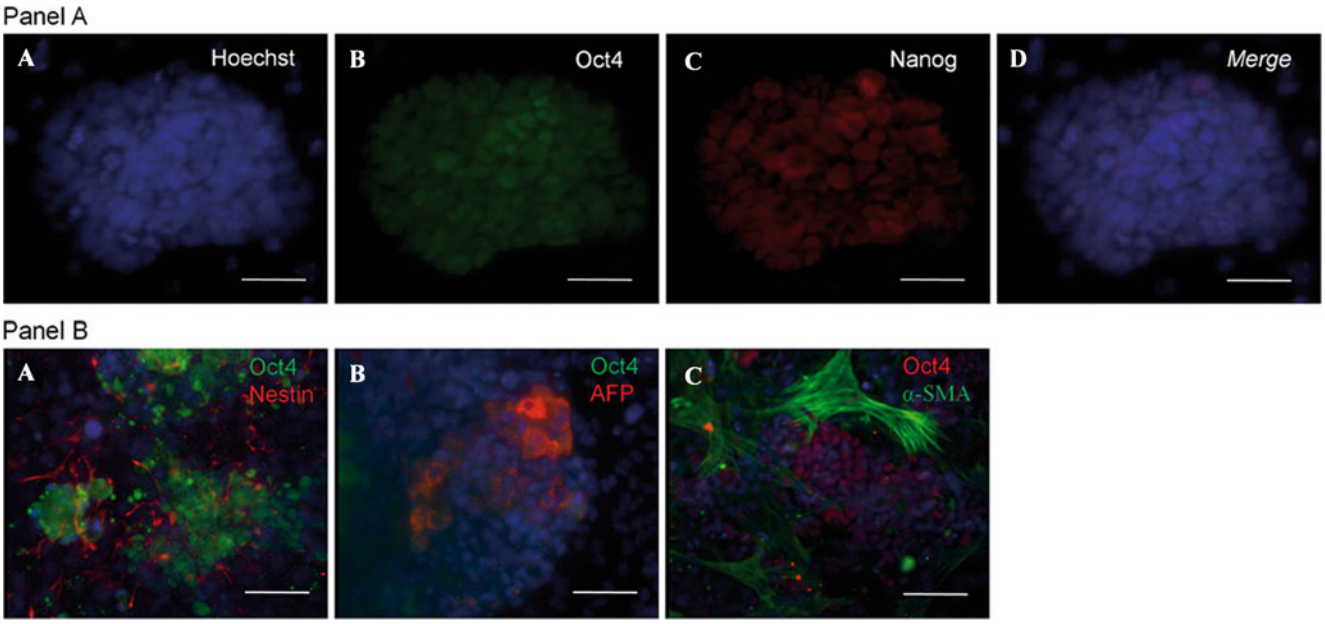

Fig. 5 **a** Immunostaining of an ESC colony showing expression of Oct4 (*B*, *green*) and Nanog (*C*, *red*) pluripotency markers. Scale bar: 50 μm. **b** Immunostaining of an in vitro differentiated ESC line showing the expression of Oct4 and Nestin (*A*), Oct4 and AFP (*B*) and Oct4 and α-SMA (*C*). Scale bar: 100 μm

Establishment of TSC Lines After Culture with E-cad-Fc

TSC culture conditions were introduced in 1/8 groups exposed to E-cad-Fc (long exposure) exhibiting a TSC-like morphology on the third day of culture and 4 TSC lines (13.3%; Table 4) were obtained (Fig. 3d). Control 1/8 groups not exposed to E-cad-Fc were also cultured in TSC conditions and only 1 TSC line (4%) was derived. These results, although not statistically significant, suggest an increased capacity to establish TSC lines after a long exposure of the 1/8 blastomeres to E-cad-Fc. Moreover, the production of TSC lines from the group with E-cad-Fc (13.3%; Table 4) was compared with their respective production of ESC lines (1%; Table 2) and results revealed significant differences ($p=0.0101$) suggesting that E-cad-Fc in contact with 1/8 blastomeres during more than 24 h favours the production of TSC colonies over that of ESC colonies.

Blastocysts were cultured under TSC conditions as a positive control for the derivation conditions of TSC lines (Fig. 6a–c) and an efficiency of 56% was obtained (Table 4), a value significantly higher ($p<0.0072$) than that obtained for 1/8 blastomeres with or without E-cad-Fc.

Therefore, 1/8 blastomeres exposed to E-cad-Fc during more than 24 h (long exposure) and cultured in TSC conditions produced TSC lines positive for Cdx2 marker more efficiently than ESC lines, although at lower rates than blastocysts under our conditions. It is important to note that TSC colonies formed from 1/8 blastomeres did not present a homogeneous expression of Cdx2 in the entire colony (Fig. 6d–f).

Discussion

The aim of the present study was to investigate the influence of the adhesion protein E-cadherin on the division capacity of single mouse blastomeres at the 8-cell stage. We wanted to elucidate whether an increased division potential could affect their ESC derivation efficiency.

Table 4 Derivation of TSC lines from the culture of 1/8 blastomeres with E-Cad-Fc

Derivation group	n	% First division after 24 h (n)	% ESC-like outgrowths (n)	% TSC-like outgrowths (n)	% TSC lines (n)
Control (1/8)	25	68 (17)[a]	0 (0)[a]	24.0 (6)[a]	4.0 (1)[a]
Control (blastocyst)	25	–	0 (0)[a]	92.0 (23)[b]	56.0 (14)[b]
E-cad-Fc long exposure (>48 h)	30	90 (27)[a]	0 (0)[a]	26.7 (8)[a]	13.3 (4)[a]

[a–b]Values with different superscripts within the same column differ significantly

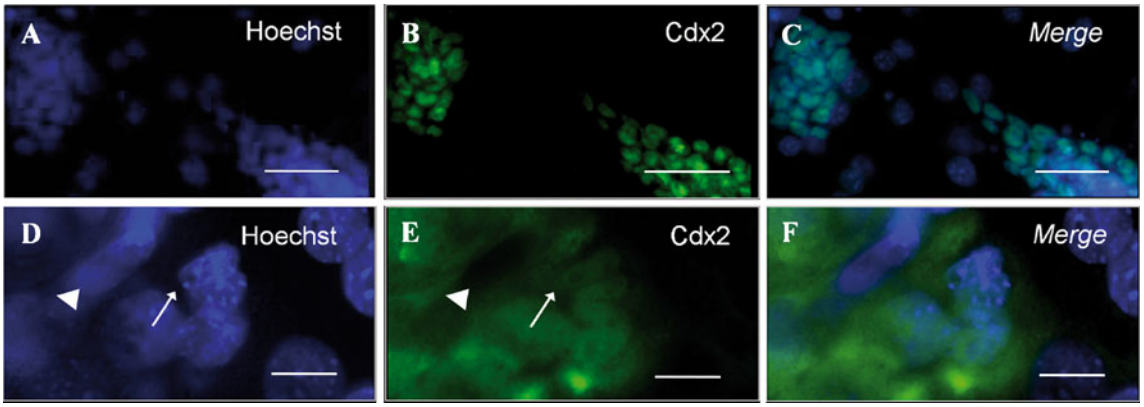

Fig. 6 Immunostaining of TSC colonies. **a**–**c** TSC lines derived from blastocysts showing a homogeneous expression of Cdx2 marker (**b**, *green*). Scale bar: 50 μm. **d**–**f** TSC lines derived from 1/8 blastomeres showing a variegated expression of Cdx2 marker (*arrow*: Cdx2 positive cells; arrowhead: Cdx2 negative cells). Scale bar: 10 μm

In our study, we adhered a chimeric E-cadherin (E-cad-Fc) to 1/8 blastomeres to simulate the signaling produced in vivo when two or more blastomeres connect by means of E-cadherin junctions at the 8-cell stage. The adhesion of E-cad-Fc to the blastomere surface was performed maintaining the E-cad-Fc during at least 2 h in contact with blastomeres, since there are consistent data indicating that cadherin-mediated adhesion proceeds from a weak to a strong state over a time of 1 h when it finally connects with the cytoskeleton [7]. The foci-like pattern found by immunofluorescence for the E-cad-Fc, the need of calcium to adhere E-cad-Fc, the complete withdrawal observed when these blastomeres were washed in a calcium- and magnesium-free PBS solution and the similar distribution of E-cad-Fc and native E-cadherin on blastomeres surface confirm the specificity of the adhesion of E-cad-Fc to the native E-cadherin of blastomeres in our experimental design.

E-cadherin is a cell-to-cell adhesion molecule with a central and crucial role during development [25]. Its ability to participate or to influence morphogenetic processes in the developing embryo such as cell proliferation, cell death, cell polarization and differentiation has been described. Particularly, with regards to proliferation, E-cadherin ensures that blastomeres find a partner to contact with and, hence, they proliferate and structure in tissues [7]. Our results from the culture of 1/8 blastomeres with E-cad-Fc during 24 h revealed an increased division capacity when compared to control groups cultured without E-cad-Fc under ESC derivation conditions. Moreover, we have succeeded in mimicking the division capacity of 2/8 blastomeres, which contact each other through native E-cadherin. Altogether, our data seem to indicate that E-cad-Fc increases the proliferating capacity of single blastomeres at the 8-cell stage during the first 24 h of culture, when blastomere cleavage occurs, probably by simulating adherens junctions and stimulating the signaling produced between neighbouring blastomeres at this stage.

Experiments maintaining the E-cad-Fc in the culture medium (long exposure) revealed a decrease in the number of ICM cells in pseudoblastocysts with regards to the control group without E-cad-Fc, indicating that E-cad-Fc reduces the proliferation capacity of ICM cells at this stage. Polarization induced by residual E-cad-Fc in the culture medium may inhibit the formation of ICM cells without affecting the proliferation of TE cells. After these pseudoblastocysts obtained from 1/8 blastomeres attached to the feeder cells during the next 24 h, they formed a higher number of TSC-like outgrowths than of ESC-like outgrowths, and the percentage of TSC-like outgrowths produced was higher than that observed in the control 1/8 group. It is well known that E-cadherin confers a unique adhesion capacity to ensure the formation of the TE, the first ephitelium in the embryo [10]. Free E-cad-Fc in the culture medium may adhere to the external and free surface of cells in the outgrowth thus inducing polarization and the formation of TSC-like cells, as occurs at the morula stage to form the TE. To confirm that TSC-like outgrowths were able to form TSC lines, they were cultured in TSC conditions and 4 TSC lines were derived. Again, the more efficient production of TSC lines with regards to the derivation of ESC lines in the same group suggests that a long exposure to E-cad-Fc is favouring the production of TE-like rather than ICM-like cells. The quality of the conditions used to derive TSC lines was confirmed by TSC derivation from blastocysts, and the efficiency obtained was similar to that previously described by other authors [26]. Thus, our experimental conditions for deriving TSC lines are good but the establishment of TSC lines from 1/8 blastomeres is less efficient than from blastocysts, as expected.

Experiments about a short exposure (24 h) to E-cad-Fc, by contrast, produced pseudoblastocysts with a mean number of ICM cells similar to control 1/8 blastomeres non-exposed to E-cad-Fc. Thus, a short exposure to E-cad-Fc does not result in a reduction in the proliferation capacity of ICM cells, as observed with a long exposure. Outgrowths formed in this group were mostly ESC-like, whereas the production of TSC-like outgrowths was almost eliminated. In this way, the ESC derivation efficiency increased to values similar to those obtained from 2/8 blastomeres and, resulted highly significant with regards to control 1/8 blastomeres without E-cad-Fc and with a long exposure to E-cad-Fc. Changes in the distribution of E-cad-Fc depending on the time E-cad-Fc is in contact with 1/8 blastomeres could explain differences in the ESC derivation efficiencies found in experiments about short and long exposure to E-cad-Fc. It is well known that E-cadherin is associated with the maintenance of the undifferentiated state in mouse and human ESC, so that downregulation of the surface E-cadherin leads to the differentiation of mouse ESC [11, 13, 14]. Particularly, E-cadherin distribution has been shown to be crucial in such processes. Li et al. (2010) showed that E-cadherin is highly and homogeneously expressed on the cells surface of the undifferentiated colonies in contrast to differentiated colonies, indicating that a uniform distribution of E-cadherin on the cells conforming the ESC colonies contributes to maintain pluripotency and avoids differentiation [12]. Combining these data with our results, suggests that early treatment with E-cad-Fc during only the first 24 h could enhance the probability of first dividing blastomeres to have an ICM-committed phenotype thus assuring that these blastomeres have a higher potential to form ESC-like cells during the process of ESC derivation. It is well known that in embryos from 8-cell stage onwards, inner blastomeres in the embryo present a homogeneous distribution of E-cadherin on their surface while outer blastomeres present E-cadherin restricted to areas of contact with other blastomeres. Hence, inner blastomeres are committed to become ICM cells while outer blastomers form TE cells at the blastocyst stage [27]. Thus, E-cad-Fc uniformly distributed thorough the surface of blastomeres during the first 24 h could be promoting the production of ICM-committed blastomeres which are more prone to produce ESC lines. By contrast, 1/8 control blastomeres despite producing pseudoblastocysts with the same mean number of ICM cells, could not be committed enough to produce ESC-like cells in the process of ESC derivation. Probably, long exposure to E-cad-Fc produced significantly lower percentages of ESC lines due to the fact that residual E-cad-Fc in the culture medium induces a continuous effect of polarization that is translated in a high proportion of TSC-like cells and, hence, in a low ESC derivation efficiency.

The association between the adhesive signaling of E-cadherin and Wnt signaling has been proposed through an intermediary protein, β-catenin, which can regulate both cell adhesion and gene expression [19, 28]. Wnt signaling regulates genes which are involved in proliferation and differentiation during development and in adult tissues. Willert et al. (1998) proposed that Wnt signaling has a direct effect on cell adhesion so that E-cadherin could inactivate Wnt signaling by recruiting the soluble cytoplasmic pool of available β-catenin [29]. Moreover, it is well known that Wnt signaling may suppress differentiation in early embryos and ESC and that β-catenin in Wnt signaling is also involved in the transcription of genes that control proliferation such as c-myc, which positively regulates G1/S transition [20]. Pooling all these data together with our results suggests that the adhesion of chimeric E-cad-Fc to the blastomere surface may promote the activation of the E-cadherin pathway by sequestering the β-catenin pool available for the Wnt pathway and therefore inducing the consequent downregulation of the Wnt signaling. Despite this, a low level of activation of Wnt signaling may persist since during the first 24 h in contact with E-cad-Fc blastomeres are induced to increase their percentage of division probably by a positive regulation of genes involved in G1/S transition.

Overall, we can conclude from our study that the division rate of single blastomeres at the 8-cell stage can be increased by culturing them with E-cad-Fc during 24 h at the beginning of the derivation process. Under these conditions, the efficiency of the derivation process from one single blastomere can be improved and resemble the one obtained when starting from two blastomeres, which are connected through native E-cadherin junctions. Therefore, establishment of ESC lines from isolated blastomeres not only depends on the embryo volume from which derivation has started but on the initial conditions of the signaling between neighbouring blastomeres as we previously suggested [21].

Finally, given that 1/8 single blastomeres incubated with E-cad-Fc during 24 h can yield an ESC derivation efficiency of 33.6%, it is tempting to speculate that by using this novel experimental approach, if confirmed by other authors, a decompacted 8-cell stage embryo could theoretically produce an ESC line with an efficiency that could reach 96% ($p=1-(0.664)^8$). This potential ESC derivation efficiency is higher than the best obtained from a single blastocyst (88%) described until now [5].

Acknowledgements This work received financial support from the Spanish *Ministerio de Educación y Ciencia* (MEC) projects BIO 2005-04341 and BIO2006-11792, the *Generalitat de Catalunya* DGR project #2009SGR-00282 and MEC FPU fellowship AP2006-02038. We thank Marc Puigcerver and Jonatan Lucas for their technical assistance.

Conflicts of interest The authors declare no potential conflicts of interest.

References

1. Brook, F. A., & Gardner, R. L. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. *Proceedings of the National Academy of Sciences, 94*(11), 5709–5712.
2. Chung, Y., Klimanskaya, I., Becker, S., et al. (2006). Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. *Nature*. doi:10.1038/nature04277.
3. Chung, Y., Klimanskaya, I., Becker, S., et al. (2008). Human embryonic stem cell lines generated without embryo destruction. *Cell Stem Cell*. doi:10.1016/j.stem.2007.12.013.
4. Klimanskaya, I., Chung, Y., Becker, S., Lu, S. J., & Lanza, R. (2006). Human embryonic stem cell lines derived from single blastomeres. *Nature*. doi:10.1038/nature05142.
5. Wakayama, S., Hikichi, T., Suetsugu, R., et al. (2007). Efficient establishment of mouse embryonic stem cell lines from single blastomeres and polar bodies. *Stem Cells*. doi:0.1634/stemcells.2006-0615.
6. Fleming, T. P., Javed, Q., Collins, J., & Hay, M. (1993). Biogenesis of structural intercellular junctions during cleavage in the mouse embryo. *Journal of Cell Science Supplement, 17*, 119–125.
7. Steinberg, M. S., & McNutt, P. M. (1999). Cadherins and their connections: adhesion junctions have broader functions. *Current Opinion in Cell Biology*. doi:10.1016/S0955-0674(99)00027-7.
8. Clayton, L., Stinchcombe, S. V., & Johnson, M. H. (1993). Cell surface localisation and stability of uvomorulin during early mouse development. *Zygote*. doi:10.1017/S0967199400001660.
9. Riethmacher, D., Brinkmann, V., & Birchmeier, C. (1995). A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *Proceedings of the National Academy of Sciences, 92*(3), 855–859.
10. Kan, N. G., Stemmler, M. P., Junghans, D., et al. (2007). Gene replacement reveals a specific role for E-cadherin in the formation of a functional trophectoderm. *Development*. doi:10.1242/dev.02722.
11. Chou, Y. F., Chen, H. H., Eijpe, M., et al. (2008). The growth factor environment defines distinct pluripotent ground states in novel blastocyst-derived stem cells. *Cell*. doi:10.1016/j.cell.2008.08.035.
12. Li, Z., Qiu, D., & Sridharan, I. (2010). Spatially resolved quantification of E-cadherin on target hES cells. *The Journal of Physical Chemistry B*. doi:10.1021/jp906737q.
13. Spencer, H. L., Eastham, A. M., Merry, C. L., et al. (2007). E-cadherin inhibits cell surface localization of the pro-migratory 5T4 oncofetal antigen in mouse embryonic stem cells. *Molecular Biology of the Cell*. doi:10.1091/mbc.E06-09-0875.
14. Xu, Y., Zhu, X., & Hahm, H. S. (2010). Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules. *Proceedings of the National Academy of Sciences*. doi:10.1073/pnas.1002024107.
15. Soncin, F., Mohamet, L., Eckardt, D., et al. (2009). Abrogation of E-cadherin-mediated cell-cell contact in mouse embryonic stem cells results in reversible LIF-independent self-renewal. *Stem Cells*. doi:10.1002/stem.134.
16. Xu, Y., Zhu, X., Hahm, H. S., et al. (2010). Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules. *Proceedings of the National Academy of Sciences*. doi:10.1073/pnas.1002024107.
17. Chen, G., Hou, Z., Gulbranson, D. R., & Thomson, J. A. (2010). Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. *Cell Stem Cell*. doi:10.1016/j.stem.2010.06.017.
18. Ohgushi, M., Matsumura, M., Eiraku, M., et al. (2010). Molecular pathway and cell state responsible for dissociation-induced apoptosis in human pluripotent stem cells. *Cell Stem Cell*. doi:10.1016/j.stem.2010.06.018.
19. Brembeck, F. H., Rosario, M., & Birchmeier, W. (2006). Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. *Current Opinion in Genetics & Development*. doi:10.1016/j.gde.2005.12.007.
20. Sineva, G. S., & Pospelov, V. A. (2010). Inhibition of GSK3beta enhances both adhesive and signaling activities of beta-catenin in mouse embryonic stem cells. *Biology of the Cell*. doi:10.1042/BC20100016.
21. González, S., Ibañez, E., & Santaló, J. (2010). Establishment of mouse embryonic stem cells from isolated blastomeres and whole embryos using three derivation methods. *Journal of Assisted Reproduction and Genetics*. doi:10.1007/s10815-010-9473-9.
22. Martin, G. R., & Evans, M. J. (1975). Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. *Proceedings of the National Academy of Sciences, 72*(4), 1441–1445.
23. Handyside, A. H., Pattinson, J. K., Penketh, R. J., Delhanty, J. D., Winston, R. M., & Tuddenham, E. G. (1989). Biopsy of human preimplantation embryos and sexing by DNA amplification. *Lancet*. doi:10.1016/S0140-6736(89)91723-6.
24. Tanaka, S. (2006). *Derivation and culture of mouse trophoblast stem cells in vitro*. *Methods in Molecular Biology. Embryonic stem cell protocols* (pp. 35–44). Humana Press.
25. Vleminckx, K., & Kemler, R. (1999). Cadherins and tissue formation: integrating adhesion and signaling. *Bioessays, 21*(3), 211–220.
26. Rossant, J. (2001). Stem cells from the Mammalian blastocyst. *Stem Cells*. doi:10.1634/stemcells.19-6-477.
27. Vestweber, D., Gossler, A., Boller, K., & Kemler, R. (1987). Expression and distribution of cell adhesion molecule uvomorulin in mouse preimplantation embryos. *Developmental Biology*. doi:10.1016/0012-1606(87)90498-2.
28. Bienz, M. (2005). Beta-Catenin: a pivot between cell adhesion and Wnt signalling. *Current Biology*. doi:10.1016/j.cub.2004.12.058.
29. Willert, K., & Nusse, R. (1998). Beta-catenin: a key mediator of Wnt signaling. *Current Opinion in Genetics & Development*. doi:10.1016/S0959-437X(98)80068-3.