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Efficient generation of embryonic stem cells from single blastomeres of cryopreserved mouse embryos in the presence of signaling modulators

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Abstract

Context: Derivation of embryonic stem cells (ESC) from single blastomeres is an interesting alternative to the use of whole blastocysts. But derivation rates are lower and the requirements for successful ESC obtention are still poorly defined.

Aims: To investigate the effects of embryo cryopreservation and of signaling modulators present during embryo culture and/or ESC establishment on ESC derivation efficiency from single 8-cell mouse blastomeres.

Method: Fresh and cryopreserved 2-cell embryos were cultured and biopsied at the 8-cell stage. Single blastomeres were cultured in the presence of 2i or R2i cocktails, with or without adrenocorticotropic hormone (ACTH). We analyzed ESC derivation efficiencies and characterized pluripotency genes expression and karyotype integrity of the resulting lines. We also evaluated the impact of embryo preculture with R2i on epiblast cell numbers and derivation rates.

Key results: ESC generation was not compromised by embryo cryopreservation and ACTH was dispensable under most of the conditions tested. While 2i and R2i were similarly effective for ESC derivation, R2i provided higher karyotype integrity. Embryo preculture with R2i yielded increased numbers of epiblast cells but did not lead to increased ESC generation.

Conclusions: Our findings help to define a simplified and efficient procedure for the establishment of mouse ESC from single 8-cell blastomeres.

Implications: This study will contribute to improve the potential of this experimental procedure, providing a tool to investigate the developmental potential of blastomeres isolated

from different embryonic stages and to reduce the number of embryos needed for ESC derivation.

Keywords

Embryonic stem cells, single blastomeres, mouse embryos, TGBβ inhibition, ERK inhibition, adrenocorticotropic hormone, embryo culture, embryo cryopreservation

Introduction

Embryonic stem cells (ESC) are pluripotent cells with infinite self-renewal ability *in vitro* and able to generate all derivates of the three embryonic germ layers. These fundamental features enable ESCs to be a powerful tool for basic and applied research in regenerative medicine, development biology, disease modeling and drug testing (Avior *et al*., 2016; Mahla, 2016; Weinberger *et al*., 2016).

Traditionally, ESC have been derived from the inner cell mass (ICM) of blastocysts (Bc-ESC) (Evans and Kaufman, 1981; Martin, 1981), but they can also be established from earlier embryonic stages (Strelchenko *et al.,* 2004; Tesar, 2005) and even from single blastomeres (Bm-ESC) (Delhaise *et al.*, 1996; Klimanskaya *et al*., 2006; Chung *et al*., 2006; González *et al.*, 2010). In humans, this latter approach could circumvent the ethical concerns associated with embryo destruction and could potentially allow the generation of autologous ESC lines for children born from embryos transferred in preimplantation genetic screening cycles (Klimanskaya *et al.*, 2006). However, the extremely low efficiencies of Bm-ESC derivation in humans have so far precluded the translation of this approach into the clinical practice. A more realistic application of Bm-ESC derivation is based on the separate use of all the blastomeres from an 8-cell embryo to initiate the derivation process. This, though leading to the destruction of the used embryos, could increase the probability of obtaining at least one ESC line from each embryo, thus reducing the total number of embryos needed to generate ESC lines both in clinical and research settings. On the other hand, the generation of Bm-ESC offers a powerful tool to investigate the development potential of blastomeres isolated from mammalian embryos at different developmental stages, and especially past the two-cell stage, when the ability of all the blastomeres to develop autonomously into blastocysts and liveborn animals is highly reduced (Boiani *et al.*, 2019). Although these applications are feasible in the mouse with the current efficiencies of Bm-ESC derivation, technical limitations still exist and improvements in Bm-ESC derivation, particularly from 8-cell stage embryos, should be implemented to achieve the full potential of this experimental approach.

In humans, hESC are typically derived from cryopreserved embryos, but this practice is uncommon when working with mice, so the effects of cryopreservation on mESC derivation efficiencies and on the characteristics of the lines produced are largely unknown. Nonetheless, the use of cryopreserved embryos as the source of mESC could help to simplify and optimize the procedure, particularly for the longer and more complex Bm-ESC derivation, involving an extra step of embryo biopsy. So far, only one study has investigated the effects of embryo

cryopreservation on mESC derivation, and the authors reported that it probably affected derivation efficiency, as well as the karyotype, pluripotency genes expression, and differentiation capacity of the mESC lines produced from whole blastocysts (Assadollahi *et al*., 2019). Whether cryopreservation also affects Bm-ESC derivation is presently unknown.

Indeed, requirements for successful Bm-ESC derivation are not exactly the same as for ESC derivation from whole embryos, particularly regarding culture conditions. For instance, the adrenocorticotropic protein hormone (ACTH), a peptide hormone produced by the pituitary gland and involved in the response to biological stress, is often used for Bm-ESC derivation, but unnecessary for ESC derivation from blastocysts. ACTH has been shown to promote ESC proliferation and to have a positive effect on single blastomere culture and Bm-ESC derivation when present in the derivation medium until outgrowth formation (Ogawa *et al.*, 2004; Wakayama *et al.*, 2007; Lorthongpanich *et al.*, 2008; Lee *et al.,* 2012). Nonetheless, some mouse Bm-ESC lines have been obtained without ACTH supplementation (Hassani *et al*., 2014a), so it is unclear whether ACTH has an essential role in efficient Bm-ESC derivation, especially from 8-cell stage embryos.

Other signaling modulators have been shown to promote both Bc-ESC and Bm-ESC derivation when added to the culture media. Notably, the introduction of the 2i cocktail to mouse ESC derivation medium (Ying *et al.*, 2008) represented a significant advance in the derivation and pluripotency maintenance of Bc-ESC and Bm-ESC. This cocktail consists of a combination of the mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor PD0325901 and the glycogen synthase kinase 3 (GSK3) inhibitor CHIR99021. In the case of Bm-ESC from 8-cell blastomeres, derivation efficiencies up to 25% can be obtained when 2i is added to culture medium containing Knockout Serum Replacement (KSR), Leukemia Inhibitory Factor (LIF) and ACTH (Lee *et al*., 2012; Vila-Cejudo *et al*., 2019) or N2B27 medium containing LIF (Hassani *et al.,* 2014a). Alternatively, the use of the R2i cocktail, composed of PD0325901 and the transforming growth factor β (TGFβ) inhibitor SB431542, has resulted in derivation rates of up to 50% from single blastomeres of 8-cell embryos from the NMRI and BALB/c non-permissive strains in N2B27/LIF (Hassani *et al*., 2014a). In addition to the increase in derivation rates, these two cocktails maintain ESC in a ground state of pluripotency, characterized by the expression of core (Oct4, Nanog, Sox2) and naïve (Rex1, Esrrb, Tfcp2l1, etc.) pluripotency markers. However, in the case of the R2i cocktail, the number of 8-cell embryos used in the study was low (n=18) and no other groups have replicated these experiments, so more research is needed to validate the higher efficacy of R2i versus the 2i cocktail in terms of Bm-ESC derivation and to

test its potential benefits also in permissive mouse strains. The effects of the two cocktails on Bm-ESC derivation from cryopreserved embryos also deserves investigation.

In this study, we aimed to simplify and improve Bm-ESC derivation by investigating the impact of embryo cryopreservation and culture media supplements (ACTH, 2i, R2i) on Bm-ESC derivation from single blastomeres biopsied from 8-cell mouse embryos from the permissive B6CBAF1 strain. We analyze derivation efficiencies and characterize the expression profile of pluripotency genes and the karyotype integrity of the resulting Bm-ESC lines. In addition, we also evaluate the effect of the preculture of embryos with the inhibitor cocktails prior to ESC derivation.

Materials and methods

Animal procedures

Mouse care and procedures were conducted according to the protocols approved by the Ethics Committee on Animal and Human Research of the Universitat Autònoma de Barcelona and by the Departament d'Agricultura, Ramaderia, Pesca i Alimentació of the Generalitat de Catalunya (permit numbers 4090 and 9995, respectively).

Embryo obtention and cryopreservation

Mouse embryos were obtained from 6-12 weeks old B6CBAF1/J females (Charles River Laboratories). Females were induced to superovulate by intraperitoneal injection of 5 IU Pregnant Mare's Serum Gonadotropin (Foligon) followed by the injection of 5 IU Human Chorionic Gonatropin (Divasa-Farmavic) 48 h later, and were mated with B6CBAF1/J males. Embryos were collected at the 2-cell stage by flushing the oviducts with HEPES-buffered KSOM medium (H-KSOM) (Biggers *et al.,* 2000) 48 h later, and were divided into two groups: fresh embryos, which were immediately transferred to culture dishes, and cryopreserved embryos, which were slow-frozen (Costa-Borges *et al.,* 2009) and stored in liquid N_2 at -196ºC for a minimum of 1 month before thawing and culture.

Embryo culture

Fresh and thawed 2-cell embryos were cultured in equilibrated EmbryoMax® KSOM Medium (Merck) covered with mineral oil (Sigma) at 37ºC and 5% CO_2 until the 8-cell stage, when the biopsy was performed.

In some experiments, embryo culture media was supplemented with either the 2i or the R2i cocktail (preculture groups). The 2i cocktail was a combination of PD0325901 (Axon Medchem; 1 µM) and CHIR99021 (Axon Medchem; 3 µM), whereas R2i was a combination of PD0325901 and SB 431542 (Sigma, 10 µM). These embryos were either biopsied when reaching the 8-cell stage, and blastomeres used for mESC derivation, or maintained in culture until they reached the blastocyst stage, to monitor their development and perform cell counts.

Blastomeres isolation and culture for stem cells derivation

When reaching the 8-cell stage, embryos were biopsied by micromanipulation in PBS (Sigma) supplemented with 1% bovine serum albumin (BSA; Sigma) to obtain single blastomeres. Using a double pipette holder, the zona pellucida was drilled with a 10 µm diameter pipette containing acidic Tyrode's solution and, immediately, all the blastomeres were individually

aspirated with a 25 µm diameter biopsy pipette containing PBS with 1% BSA. Within each experimental group, blastomeres isolated from different embryos were pooled and randomly distributed among the different treatments.

The isolated blastomeres were individually transferred to 50 µl drops of mESC derivation medium that contained a feeder layer of mitomycin C-inactivated human foreskin fibroblasts (HFF-1; ATCC®SCRC-1041™), in a 60 mm Petri dish covered with mineral oil (Sigma). Culture dishes were prepared prior to the biopsy by adding several 50 µl 0.2% gelatin (Sigma) drops on a 60 mm Petri dish. After a 10 min incubation at 37ºC, gelatin was pipetted out and HFF solution drops were deposited at the same place and were covered with mineral oil.

Bm-ESC lines were derived using either KSR medium (K-medium) or N2B27 medium (N-medium). The K-medium consisted of DMEM supplemented with 100 µM 2-β mercaptoethanol (Gibco), 1x non-essential amino acids (Gibco), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco), 20% KSR (Life Technologies) and 10^3 U/ml LIF (Merk Millipore). The N-medium was composed of a 1:1 mixture of DMEM-F12 (Gibco) and neurobasal medium plus 100x N2 (Gibco), 50x B27 (Gibco) and 1 mM L-glutamine (Gibco) and was supplemented with 50 µM 2-β mercaptoethanol, 1x non-essential amino acids, 10^3 U/ml LIF, 50 U/ml penicillin and 50 µg/ml streptomycin. Both media were also supplemented with the 2i or the R2i inhibitor cocktails. Moreover, when indicated, 0.1 mg/ml ACTH (Prospec) were added during the first week of culture. This resulted in four different treatment groups in terms of the signaling modulators used: 2i, R2i, 2i+ACTH and R2i+ACTH. At least three replicates per treatment were performed, with a total of 60-70 blastomeres seeded for each treatment in K-medium and around 50 blastomeres in N-medium.

Blastomeres were cultured at 37ºC and 5% CO_2, changing the medium every 48 h for 7-10 days until *outgrowth*s were observed. After the first passage, *outgrowth*s were cultured in 4-well plates without ACTH and putative mESC lines were subcultured, using trypsin-EDTA (BioWest), once or twice a week and maintained in culture for at least 6 passages.

Characterization of stem cell lines

At the seventh passage, the stemness and differentiation potential of all the putative mESC lines were assessed by Alkaline Phosphatase Activity (ALP) staining and immunodetection of pluripotency and differentiation markers. Lines that were positive for these analyses were considered genuine mESC lines.

ALP activity was determined using a commercial kit (Sigma AB0300). Cells were fixed in 4% paraformaldehyde (PFA; Sigma) for 1 min at room temperature (RT) and washed twice with

PBS. Then, samples were washed twice with a 1:1 mixture of 5-bromine-4-chloride-3-inodyl phosphate and nitroblue tetrazolium and incubated for 10 min at RT in the dark. The pluripotency of the putative mESC lines was determined by the blue color of the colonies.

For immunofluorescence, putative mESC lines cultured on HFFs on glass coverslips and lines subjected to spontaneous in vitro differentiation, by culturing them in DMEM supplemented with 10% fetal bovine serum (FBS; BioWest) in feeder-free conditions for 10 days, were fixed to analyze their pluripotency and differentiation potential, respectively. Fixation consisted of an incubation in 4% PFA during 20 min at RT and three washes with PBS for 5 min each at RT. Samples were blocked and permeabilized with a PBS solution containing 0.2% sodium azide (Sigma), 0.5% Triton X-100 (Sigma) and 3% goat serum (BioWest) for 30 min at 37ºC. Next, they were incubated with primary antibodies overnight at 4ºC, washed three times with PBS for 5 min and incubated with secondary antibodies for 2 h at RT. Samples were washed again three times with PBS for 5 min, and the nuclear material was counterstained with 10 µg/ml Hoechst 33258 (Molecular Probes, Invitrogen) diluted in Vectashield (Vector Laboratories). Finally, samples were mounted and analyzed with an epifluorescence microscope (Olympus BX61) and the Cytovision software (Applied Imaging, Inc).

Mouse monoclonal anti-Oct4 (Santa Cruz Sc-5279, 1:50 dilution), rabbit polyclonal anti-Nanog (Abcam, ab80892, 1:100 dilution) and rabbit polyclonal anti-Sox2 (Merck Millipore, ab5603, 1:200 dilution) antibodies were used to detect pluripotency markers. Mouse monoclonal anti-Tubulin β3 (Tuj1; Biolegend 801201, 1:500 dilution), mouse monoclonal anti-α smooth muscle actin (αSMA; Sigma A5228, 1:200 dilution) and mouse monoclonal anti-alpha-fetoprotein (AFP; R&D Systems MAB1368, 1:50 dilution) antibodies were used to detect ectoderm, mesoderm and endoderm differentiation markers, respectively. Secondary antibodies were anti-mouse IgG Alexa Fluor 488 (Molecular Probes - Invitrogen A-21200, 1:500 dilution) for Oct4, Tuj1, αSMA and AFP, and anti-rabbit IgG Alexa Fluor 594 (Molecular Probes - Invitrogen A-11037, 1:500 dilution) for Sox2 and Nanog. All the antibodies were diluted in a PBS-based solution containing 0.2% sodium azide, 0.1% Triton X-100 and 3% goat serum.

Chromosome number analysis

Mouse ESC growing in a 35 mm Petri dish were treated for 7 h at 37ºC with 10 µg/ml Karyomax® Colcemid Solution (Gibco 15210-040). After trypsinization, resuspended cells were centrifuged for 5 min at 340 g, washed with PBS and centrifuged again for 5 min. Then, the samples were exposed to a 0.075M KCl hypotonic solution for 8 min at 37ºC, centrifuged for 10 min at 620 g and fixed in 3 methanol: 1 acetic acid solution. The cells suspension was

dropped on degreased slides with absolute methanol previously dried with a tissue for chromosome spreading, aged during 1.5-2 h at 120ºC and then stained with 10 µg/ml Hoechst 33258 diluted in Vectashield.

Three mESC lines were analyzed for each treatment. Images of 30-40 metaphases per line were taken in an epifluorescence microscope (Olympus BX61) with the Cytovision software (Applied Imaging, Inc) and the number of chromosomes was counted using ImageJ software. Counts of 40 chromosomes were considered as a normal euploid karyotype.

RNA extraction and qPCR

The expression of pluripotency genes (*Oct4*, *Nanog*, *Rex1*, *Esrrb* and *Tfcp2l1*) was assessed by real time quantitative PCR (qPCR) on some randomly selected blastomere-derived mESC lines (two lines per treatment). STO mouse fibroblast were used as a negative control.

Total RNA was extracted from trypsinized ESC cultures using Maxwell RSC SimplyRNA Tissue Kit (Promega) and its concentration and quality were assessed using a Nanodrop spectrophotometer (ThermoFisher). One microgram of the extracted RNA was used as a template for the reverse transcriptase reaction using the iScript cDNA Synthesis Kit (Bio-Rad).

The qPCR reactions were performed using iTaq Universal SYBR Green Supermix (Bio-Rad), on a CFX96 Real-Time System thermocycler (Bio-Rad). The amplification program consisted in a first denaturation step of 3 min at 95°C followed by 40 cycles of 10 s at 95°C (denaturing) and 30 s at 60°C (annealing and extension). Finally, to assess the specificity of the amplification product, an additional thermal denaturizing cycle was performed to obtain the melt curve of the qPCR products.

Validated commercial PrimePCR SYBR Green Assays (BioRad) for *Oct4* (Pou5f1, qMmuCED0046525), *Rex1* (*Zfp42*, qMmuCID0008767), *Nanog* (qMmuCID0005399), *Esrrb* (qMmuCED0039638) and *Tfcp2l1* (qMmuCID0013329) were used to assess pluripotency. A no template control (NTC) was added for each primer, all samples were tested in triplicate and the results were normalized to *Gapdh* (qMmuCED0027497) expression. The cycle quantification value (Cq-value) was determined for each sample with the BioRad CFX Maestro^{TM} 195 Software and relative expression was calculated using the ΔΔCq method.

Blastocysts cell counts

Some of the embryos precultured in the presence of 2i or R2i were maintained in culture until they reached the blastocyst stage (72 h). Blastocysts were fixed in 4% PFA during 15 min at RT and blocked with a PBS solution containing 1% BSA, 0.05% Tween 20 and 0.2% sodium azide

overnight at 4ºC. Fixed embryos were incubated with a mixture of mouse monoclonal anti-Oct4 (Santa Cruz Sc-5279, 1:50 dilution) and rabbit polyclonal anti-Nanog (Abcam ab80892, 1:200 dilution) primary antibodies diluted in block solution during 1.5 h at RT. Then, embryos were washed three times with block solution for 10 min and incubated 30 min at RT in the dark with a mixture of anti-mouse IgG-AF488 (Molecular Probes A21200, 6 µg/ml) and anti-rabbit IgG-AF594 (Molecular Probes A11037, 6 µg/ml) secondary antibodies. Finally, they were washed again thrice with block solution for 10 min, counterstained for 10 min with 10 µg/ml Hoechst 33528 diluted in block solution and mounted in 10 µg/ml Hoechst 33258 diluted in Vectashield. The number of total cells (Hoechst staining), ICM cells (positive for Oct4) and epiblast cells (positive for Nanog) were counted on an epifluorescence microscope (Olympus BX61) with the Cytovision and ImageJ software.

In these experiments, around 70 two-cell embryos were cultured per treatment group, with at least four replicates, and around 40 blastocysts per group were used for cell counts.

Statistical analysis

Embryo development, outgrowth formation and mESC derivation rates were analyzed with $\chi 2$ and Fisher exact test, using GraphPad Prism software.

Relative expression results from qPCR analyses were compared with an ANOVA test with the Bonferroni correction using the CFX Maestro software (Bio-Rad).

The number of ICM, epiblast and total cells were compared by One-Way ANOVA with post hoc Tukey test, using GraphPad Prism software.

In all statistical analyses, $p < 0.05$ was considered statistically significant.

Results

Effect of embryo cryopreservation, signaling modulators and culture medium on mESC derivation from single blastomeres

In the first set of experiments, a total of 546 blastomeres isolated from 8-cell embryos (n=70) were allocated to 8 different experimental groups, depending on the source of embryos (fresh or cryopreserved) and the signaling modulators added to K-medium during mESC derivation (2i or R2i with or without ACTH). In all the experimental groups, most of the outgrowths produced progressed in culture to form mESC lines (Table 1), with no remarkable differences in colonies morphology among them. All 144 mESC lines generated showed stem-cell like morphology (rounded colonies with defined edges), ALP activity, were positive for pluripotency markers (ALP, OCT4 and NANOG) and, after spontaneous differentiation, were positive for markers of three germ layers (TUJ1, α-SMA and AFP) (Figure 1).

In blastomeres biopsied from fresh embryos, the inhibitor cocktails 2i and R2i resulted in similar mESC derivation efficiencies, but addition of ACTH during the first week of culture decreased efficiencies, especially with the R2i treatment (Table 1). In particular, a significant reduction of more than 50% was observed in the R2i group when comparing derivation in the presence or absence of ACTH. Surprisingly, the reverse was observed in blastomeres obtained from cryopreserved embryos, where addition of ACTH during the first week of culture in the presence of R2i significantly increased derivation efficiencies by more than 50% and yielded the highest derivation rates of all experimental groups (38.9%). No effect of ACTH was observed in the case of 2i treatment. As in blastomeres of fresh embryos, no significant differences between 2i and R2i groups were detected in mESC derivation efficiencies from blastomeres of cryopreserved embryos (Table 1).

The source of blastomeres did not have a remarkable effect on mESC derivation efficiencies either. When comparing results for the same treatment groups between fresh and cryopreserved blastomeres, significant differences were only detected in the case of R2i, both in the presence or absence of ACTH. As mentioned, ACTH had a deleterious effect on mESC derivation from R2i-treated blastomeres isolated from fresh embryos, but a beneficial effect on their cryopreserved counterparts.

In a second set of experiments, mESC derivation was attempted using the same treatments but in N-medium. Given the lack of significant differences between the two embryo sources, only blastomeres obtained from cryopreserved embryos were used in these experiments. Although the percentage of outgrowths formed was high, without significant differences among

299 treatments, massive differentiation occurred at low passages resulting in extremely low rates
300 of mESC derivation in this medium (Table 2).

301 *Effect of embryo cryopreservation and signaling modulators on pluripotency gene expression*
302 *and karyotype integrity of blastomere-derived mESC lines*

303 Aside from the analyses used to validate the stemness of all the mESC lines obtained, some
304 lines were subjected to further characterization to assess the effect of embryo
305 cryopreservation and signaling modulators used during derivation and culture on the
306 expression of pluripotency markers (Figure 2.A) and karyotype integrity (Figure 2.B). To
307 determine the effect of signaling modulators, we randomly selected two (for qPCR) or three
308 (for chromosome counts) mESC lines derived from single blastomeres of cryopreserved
309 embryos from all the treatment groups in K-medium (2i, R2i, 2i+ACTH and R2i+ACTH). An
310 equivalent number of lines derived from fresh embryos of the 2i+ACTH group in K-medium was
311 also included to assess the effect of embryo cryopreservation.

312 Equivalent levels of *Esrrb*, *Nanog* and *Tfcp21l* gene expression were found by qPCR among the
313 different groups of mESC lines (Figure 2.A). Although lines derived from fresh embryos under
314 2i+ACTH treatment showed higher levels of expression of *Oct4* and *Rex1*, differences were only
315 significant when compared with lines obtained from cryopreserved embryos under R2i (for
316 *Oct4* expression) and R2i+ACTH (for *Rex1* expression). The lack of significant differences
317 between 2i and R2i groups, with or without ACTH, and between groups with or without ACTH,
318 either in the presence of 2i or R2i, for any of the genes indicates that signaling modulators
319 used during derivation and/or culture have no significant effect on pluripotency gene
320 expression of the mESC lines generated. Nor does embryo cryopreservation, as no differences
321 were detected for any of the genes between lines derived in 2i+ACTH conditions from either
322 fresh or cryopreserved embryos. For all the genes analyzed, STO feeder cells, used as a
323 negative control, displayed barely undetectable expression levels (data not shown).

324 Chromosome counts in mESC lines derived from cryopreserved embryos under R2i treatments,
325 with or without ACTH, showed a normal modal karyotype (40 acrocentric chromosomes) and
326 high percentages (58.3-71.0%) of euploid cells (Figure 2.B). By contrast, mESC lines derived
327 from cryopreserved embryos under 2i treatments presented more chromosomal
328 abnormalities. In particular, in the absence of ACTH one polyploid line and one line showing a
329 chromosomal fusion were observed, and in the presence of ACTH the lack of one chromosome
330 was detected in almost 50% of the cells in one of the three lines studied. These abnormalities
331 were not observed in the 2i +ACTH group derived from fresh embryos, since the three lines

analyzed presented a normal modal karyotype and high percentages of euploid cells (>60%). To elucidate a potential effect of embryo cryopreservation on the karyotype integrity of the resulting mESC lines, we decided to include a new experimental group: three lines derived from fresh embryos under R2i + ACTH treatment. The results showed that while two lines retained an euploid modal karyotype of 40 acrocentric chromosomes (with >60% of euploid cells), one line suffered the loss of one chromosome.

Effect of signaling modulators in embryo culture medium on blastocyst development, cell counts and mESC derivation

Two-cell embryos were thawed and cultured in the continuous presence of 2i or R2i, in parallel to a control group, and fixed at the blastocyst stage. No significant differences in 8-cell embryo formation or blastocyst development were observed among the three groups (Table 3). However, immunodetection analyses (Figure 3.A) revealed a significantly decreased number of total cells of the blastocysts in the 2i group (77.6 ± 2.0) when compared with the control and the R2i groups (85.4 ± 2.3 and 85.6 ± 1.8, respectively) (Figure 3.B). While R2i had no effect on total cell numbers, it significantly increased the numbers of both ICM (23.0 ± 0.8) and epiblast cells (14.9 ± 0.7) when compared with the control (19.6 ± 0.6 and 10.3 ± 0.5, respectively) and the 2i groups (20.1 ± 0.7 and 11.4 ± 0.5, respectively) (Figure 3.B).

To assess whether the increase in epiblast cells observed with R2i could translate into higher mESC derivation rates from the treated embryos, thawed 2-cell embryos precultured in the presence of R2i were biopsied at the 8-cell stage and individual blastomeres were seeded in K-medium supplemented with LIF, R2i and ACTH. No significant differences in mESC derivation rates from control and R2i precultured embryos were detected (Table 4).

Discussion

With the aim to simplify and improve Bm-ESC derivation from 8-cell stage mouse embryos, in the present study we evaluated the effect of embryo cryopreservation, derivation medium and signaling modulators on derivation efficiency, pluripotency gene expression and karyotype integrity of Bm-ESC lines. Furthermore, blastocyst formation, blastocyst cell number and Bm-ESC derivation were analyzed after embryo culture in the presence of signaling modulators.

Embryo cryopreservation is a common and well-established procedure in mice, as well as in other species. Nonetheless, several studies have shown that it can induce alterations in cytoskeletal organization, mitochondrial function, gene expression patterns, blastocyst cell numbers and allocation of cells to the ICM and trophectoderm (TE), among others, thus compromising the developmental potential of the thawed embryos (Sohn *et al.*, 2002; Larman *et al*., 2011; *Shin et al*., 2011; Dasiman *et al.*, 2013). Because of this, embryo cryopreservation could potentially impact subsequent ESC derivation from the thawed embryos and the characteristics of the ESC lines established, an issue that has been scarcely investigated. In the present study, blastomeres obtained from frozen-thawed embryos produced Bm-ESC lines with similar efficiencies than blastomeres obtained from fresh embryos, and no significant differences in terms of pluripotency gene expression or karyotype integrity were observed between the Bm-ESC lines derived from both embryo sources. These results enable us to uncouple ESC derivation from embryo collection, thus shortening and simplifying Bm-ESC derivation experiments and optimizing the use of the embryo donor females. Moreover, by using cryopreserved embryos, donor embryos are available for biopsy and Bm-ESC derivation at any time and derivation experiments are not subjected to the availability of female mice, superovulation schedules or superovulation success.

Our results differ from those observed in a previous work by Assadollahi *et al* (2019) that, to the best of our knowledge, is the only other study on the effects of embryo cryopreservation on mouse ESC derivation. In that study, C57BL/6 mouse embryos vitrified at the 8-cell stage were used for Bc-ESC derivation in 3i + LIF medium. Despite the limitations of the study (only 5 cryopreserved embryos used and no fresh control group), the authors concluded that cryopreservation seems to affect ESC derivation efficiency as well as the characteristics of the generated lines, such as karyotype or expression of pluripotency genes. Dissimilarities in the mouse strains, embryo stage at the time of cryopreservation, cryopreservation method, number of embryos used, source of the ESC (whole blastocysts vs. single 8-cell blastomeres) and derivation medium employed could explain the different outcomes of the two studies.

A recent study by Vila-Cejudo *et al* (2019) showed that the original classification of mouse strains into permissive and non-permissive for Bc-ESC derivation (Kawase *et al.*, 1994; Brook and Gardner, 1997) also stands when ESC are derived from single blastomeres, and this study further identified the hybrid B6CBAF1 strain as permissive for both Bc-ESC and Bm-ESC derivation. But they also demonstrated that, contrary to what occurs during Bc-ESC derivation, the effect of the embryos' genetic background on Bm-ESC derivation is not abolished by the addition of the 2i cocktail. R2i has been proposed as an alternative to the widely used 2i and proved to be as efficient as 2i in the derivation of Bc-ESC from several refractory strains and in the maintenance of these cells in a ground state of pluripotency (Hassani *et al.,* 2012, 2014b). R2i has also been used for ESC derivation from single blastomeres of mouse embryos at the 2-, 4- and 8-cell stages, but only from two non-permissive strains, resulting in approximately 2-fold higher efficiencies of Bm-ESC derivation than when 2i was used (Hassani *et al.*, 2014a).

Our results corroborate that R2i produces high ESC derivation efficiencies from single blastomeres from 8-cell embryos also in a permissive strain, but we failed to detect significant differences between 2i and R2i. Bm-ESC derivation rates in 2i (22.5%-31.3%) were similar or slightly higher than those previously obtained by our group (22.9%; Vila-Cejudo *et al.*, 2019) in the same mouse strain and culture conditions (KSR+ACTH+LIF) and than those reported by Hassani *et al* (2014a) in the non-permissive strains cultured in N2B27+LIF (23%-25%). Nonetheless, the derivation efficiency in R2i was slightly lower in our study (38.9% in the most efficient conditions) than the 46-50% obtained by Hassani *et al* (2014a). We tried to replicate the culture conditions from this other study by using N2B27 medium but, in agreement with previous results in our laboratory (Vila-Cejudo *et al.,* 2019, 2020), we did not succeed in establishing Bm-ESC lines in this medium or did so but with extremely low efficiencies (<4%) both when using 2i as when using R2i. Thus, in our hands, ESC lines can only be efficiently established from single blastomeres of 8-cell embryos in KSR+LIF+2i or KSR+LIF+R2i.

Bm-ESC lines derived under 2i and R2i displayed similar expression levels of core and naïve pluripotency genes, but R2i resulted in a higher number of lines with an euploid karyotype. These results are in agreement with those previously reported in Bc-ESC lines (Hassani *et al.*, 2014b), suggesting that the effects of these inhibitor cocktails on pluripotency gene expression and karyotype integrity is equivalent in Bc-Esc and Bm-ESC lines and across strains. The higher number of euploid lines under the R2i treatment may be attributed to the absence of CHIR99021 in this cocktail, as it has been reported that prolonged inhibition of GSK3 induces chromosomal instability in cultured cells (Tighe *et al*., 2007).

On the other hand, since the report that ACTH supports proliferation of single mouse ESC cells in KSR+LIF without loss of pluripotency (Ogawa *et al.*, 2004), ACTH has been regularly added to the culture medium for the derivation of mouse ESC from single blastomeres, both in the absence or presence of 2i (Wakayama *et al.*, 2007; Lorthongpanich *et al.*, 2008; González *et al.*, 2010; Lee *et al.,* 2012; Vila-Cejudo *et al.*, 2019). Some of these studies have proved that single blastomeres from 2- and 4-cell stage mouse embryos produce higher rates of Bm-ESC in the presence than in the absence of ACTH (Lee *et al.*, 2012; Lorthongpanich *et al.,* 2008). But no true comparisons have been made with blastomeres from embryos at the 8-cell stage. Our results demonstrate that, under 2i conditions, generation of ESC from single blastomeres of 8-cell embryos is not significantly enhanced by the presence of ACTH. These results agree with those obtained by Gonzalez *et al* (2010), though in that study the derivation medium lacked 2i and culture conditions slightly differed between blastomeres cultured in the presence or absence of ACTH. Concerning R2i, there are no reports on the combined effects of this inhibitor cocktail and ACTH on ESC derivation. Our observation that ACTH induced a significant decrease in Bm-ESC derivation rates in the R2i group when blastomeres were obtained from fresh embryos suggests a deleterious effect of the R2i+ACTH combination. Interestingly, such a deleterious effect was also observed in blastocyst formation from 2-cell mouse embryos cultured in N2B27+LIF+R2i in the presence of ACTH (Hassani *et al.*, 2014a). Surprisingly, when blastomeres were obtained from cryopreserved embryos, we observed the opposite effect, as Bm-ESC derivation in R2i conditions was significantly improved by the presence of ACTH. We cannot provide an explanation for these apparently incoherent effects of ACTH in fresh and cryopreserved embryos, and this issue deserves further investigation. In any case, our results indicate that ACTH is dispensable for Bm-ESC generation from blastomeres of 8-cell embryos, except when cryopreserved embryos are used as the source of blastomeres and derivation is performed under R2i conditions. As expected, because ACTH was only present during the first week of culture, no long-term effects of ACTH (either positive or negative) were detected in pluripotency gene expression or karyotype integrity of the established Bm-ESC lines.

Last, to try to further improve Bm-ESC derivation rates, we precultured the embryos in the presence of 2i or R2i cocktails from the 2-cell stage. Signaling pathways play a critical role in the first two differentiation events during early embryo development: the segregation of the ICM and TE and, subsequently, the segregation of the epiblast and hypoblast from the ICM. Thus, modulation of the endogenous signaling pathways can alter these differentiation events and shift the balance of cells allocated to each lineage, as demonstrated by increased epiblast cells numbers in mouse and human embryos cultured in the presence of 2i (Nichols *et al.*,

2009; Van Der Jeught *et al.*, 2013). Contrary to these previous results, we did not observe a significant increase in epiblast (Nanog positive) cells in the 2i-treated blastocysts. But embryo preculture in R2i significantly increased both ICM (Oct positive) and epiblast cell numbers, in line with the reported effects of SB431542 in human embryos (Van der Jeught *et al.,* 2014). Although epiblast cells are considered the progenitors of ESC (Boroviak *et al.,* 2014) and increased numbers of epiblast cells have been correlated with higher rates of Bc-ESC derivation in some studies (Campbell *et al.*, 2013), we did not find enhanced Bm-ESC establishment from embryos precultured in R2i. Unlike what occurs when whole embryos are used, successful derivation of Bm-ESC lines requires that individual blastomeres are able to generate epiblast cells. So it is possible that some blastomeres do not generate epiblast cells despite the R2i treatment or that they generate an insufficient number of epiblast cells to support the establishment of an ESC line.

In conclusion, we have provided evidence that embryo cryopreservation does not compromise the potential of single blastomeres for Bm-ESC generation, which allows to simplify and optimize Bm-ESC derivation procedures by uncoupling embryo collection from ESC derivation. Moreover, we have confirmed and extended previous results on the effectiveness of the R2i cocktail for the successful derivation and karyotype integrity of Bm-ESC lines, by using embryos from a permissive strain and a KSR-based medium. Finally, we have demonstrated that ACTH is dispensable for Bm-ESC generation (except when cryopreserved embryos and R2i conditions are used) and that embryo preculture with R2i results in increased numbers of epiblast cells but does not lead to increased Bm-ESC generation. Altogether, the findings of the present study help to define a simplified and efficient procedure for the establishment of mouse ESC lines from single blastomeres of 8-cell embryos. This will contribute to achieve the full potential of this experimental procedure.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of funding

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Data availability

Available upon request.

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616

Tables

Table 1. Outgrowth formation and derivation efficiencies of mESC lines established under different treatments with signaling modulators in K-medium from single blastomeres of 8-cell embryos obtained from fresh or cryopreserved 2-cell embryos.

Embryo source	Treatment group	Blastomeres (n)	% Outgrowth formation (n)	% mESC lines (n)
Fresh	2i	67	32.8 (22)[a]	31.3 (21)[a]
	R2i	71	38.0 (27)[a]	33.8 (24)[a]
	2i + ACTH	71	28.2 (20)[a,b]	22.5 (16)[a,b]
	R2i + ACTH	71	16.9 (12)[b]	14.1 (10)[b]
Cryopreserved	2i	61	24.6 (15)[a,b]	24.6 (15)[a,b]
	R2i	61	19.7 (12)[b]	16.4 (10)[b]
	2i + ACTH	72	27.8 (20)[a,b]	26.4 (19)[a,b]
	R2i + ACTH	72	38.9 (28)[a]	38.9 (28)[a]

[a-b] Different superscripts indicate statistically significant differences ($p<0.05$) among treatment groups within the same embryo source group ($\chi 2$ and Fisher exact test).

Table 2. Outgrowth formation and derivation efficiencies of mESC lines established under different treatments with signaling modulators in N-medium from single blastomeres of 8-cell embryos obtained from cryopreserved 2-cell embryos.

Treatment group	Blastomeres (n)	% Outgrowth formation (n)	% mESC lines (n)
2i	46	28.3 (13)	2.2 (1)
R2i	46	32.6 (15)	0
2i + ACTH	54	38.9 (21)	0
R2i + ACTH	54	40.7 (22)	3.7 (2)

No significant differences were found among groups.

Table 3. In vitro development of two-cell embryos cultured in the presence or absence of signaling modulators.

Treatment group	Embryos (n)	% 8-cell embryos (n)	% blastocysts (n)
Control	69	82.6 (57)	79.7 (55)
2i	70	77.1 (54)	72.9 (51)
R2i	68	85.3 (58)	83.8 (57)

No significant differences were found among groups.

633 **Table 4.** Outgrowth formation and derivation efficiencies of mESC lines established from single
634 blastomeres of 8-cell stage embryos obtained from cryopreserved 2-cell embryos cultured
635 with R2i in K-medium.

Treatment group	Blastomeres (n)	% Outgrowth formation (n)	% mESC lines (n)
Control	347	25.4 (88)	24.8 (86)
R2i	344	29.4 (101)	29.1 (100)

636 No significant differences between groups.

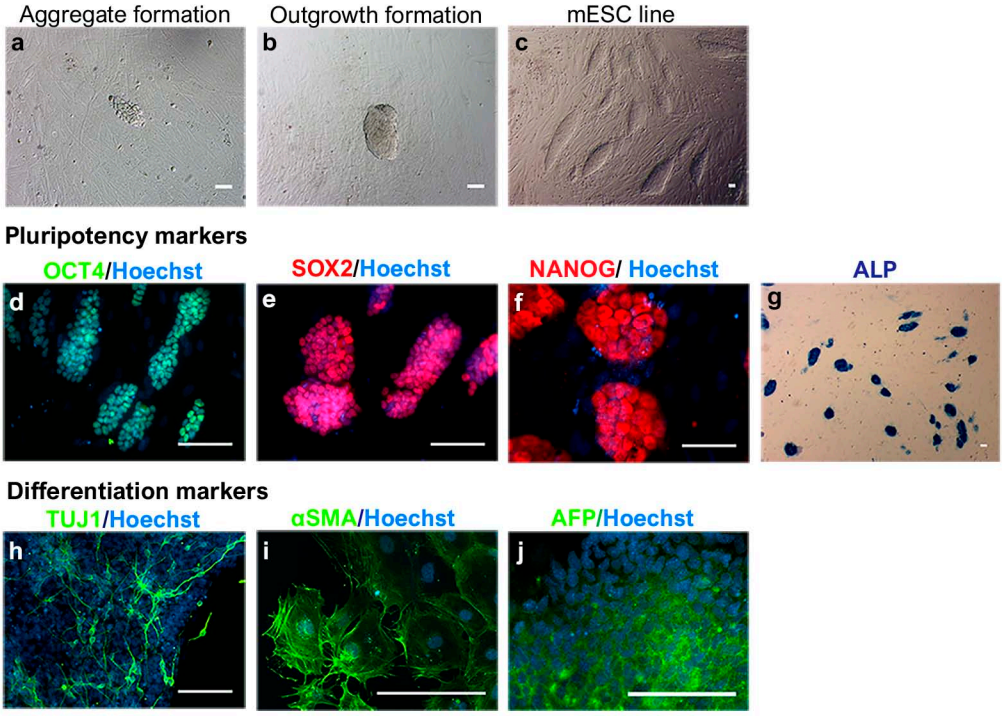
637

Figure Legends

Figure 1. Morphology of blastomere-derived mESC lines and immunodetection of pluripotency and differentiation markers. (**a-c**) Morphology of aggregate formation at day 3 of blastomere seeding (**a**), outgrowth obtained at day 9 (**b**), and colonies of a mESC line cultured for 6 passages (**c**), showing stem cell-like morphology with defined edges. (**d-f**) mESC colonies expressing pluripotency markers OCT4 (green, **d**), SOX2 (red, **e**) and NANOG (red, **f**). (**g**) mESC colonies showing alkaline phosphatase (ALP) activity (blue staining). (**h-j**) mESC colonies expressing ectoderm marker TUJ1 (green, **h**), mesoderm marker αSMA (green, **i**) and endoderm marker AFP (green, **j**) after spontaneous differentiation. In all the immunofluorescence images, nuclear material is counterstained with Hoechst (blue). All the scale bars represent 50 µm.

Figure 2. Expression of pluripotency markers and karyotype integrity in mESC lines established under different treatments with signaling modulators in K-medium from single blastomeres of 8-cell embryos derived from fresh or cryopreserved 2-cell embryos (A) Expression of pluripotency markers measured by qPCR. *Gapdh* was used for normalization of cDNA amount. Data represents the mean of two biological and three technical replicates ± SEM for each treatment group. Significant differences are denoted with *p≤ 0.05 (One-way ANOVA with Bonferroni correction)**. (B**) Stacked bar chart showing the percentage of cells with a normal (black) or abnormal (grey) chromosome number for each treatment group. Three mESC lines were analyzed for each treatment. Numbers on top of the bars indicate the modal karyotype of each line. F, fresh embryos. C, cryopreserved embryos.

Figure 3. Total, ICM and epiblast cells counts in blastocysts obtained from two-cell embryos cultured in the presence or absence of signaling modulators. (**A**). Epifluorescence raw and merge images of blastocysts immunostained for OCT4 and NANOG and counterstained with Hoechst. All the scale bars represent 60 µm. (**B**) Mean number ± SEM of total cells (Hoechst positive), ICM cells (OCT4 positive) and epiblast cells (NANOG positive) in blastocysts. Each grey dot represents the number of cells of a single blastocyst. $^{a\text{-}b}$Different superscripts indicate statistically significant differences among treatments ($p<0.05$; ANOVA).


Morphology
Aggregate formation
Outgrowth formation
mESC line
a
b
c
Pluripotency markers
OCT4/Hoechst
SOX2/Hoechst
NANOG/ Hoechst
ALP
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Differentiation markers
TUJ1/Hoechst
αSMA/Hoechst
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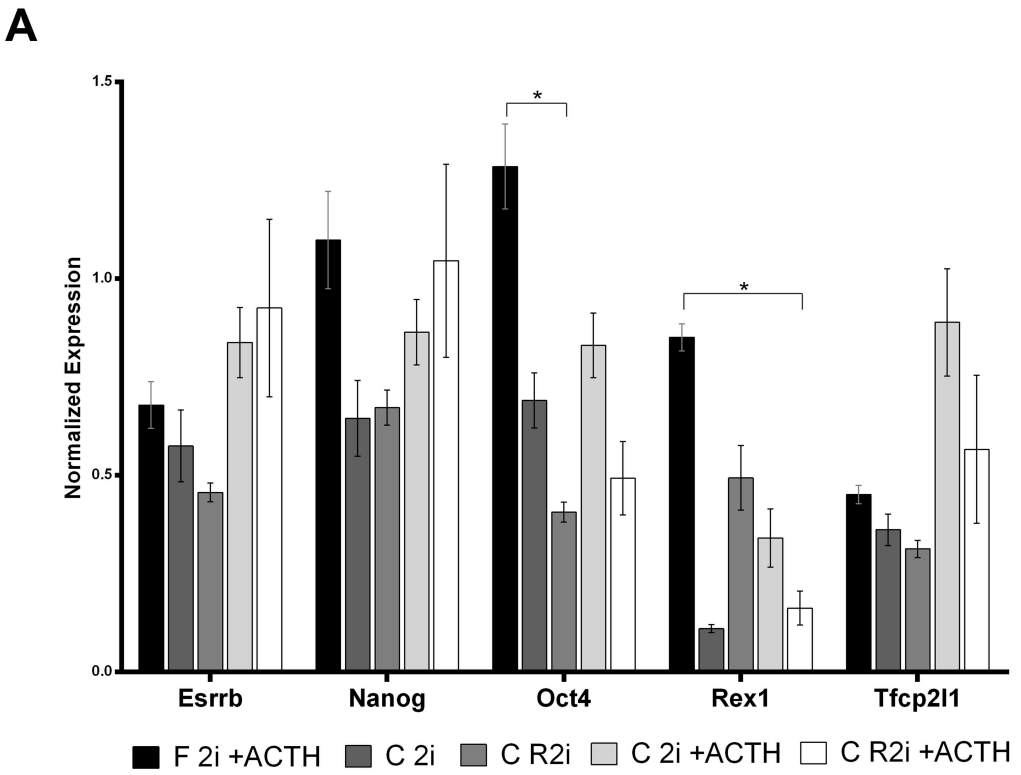
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Normalized Expression
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Esrrb
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Rex1
Tfcp2l1
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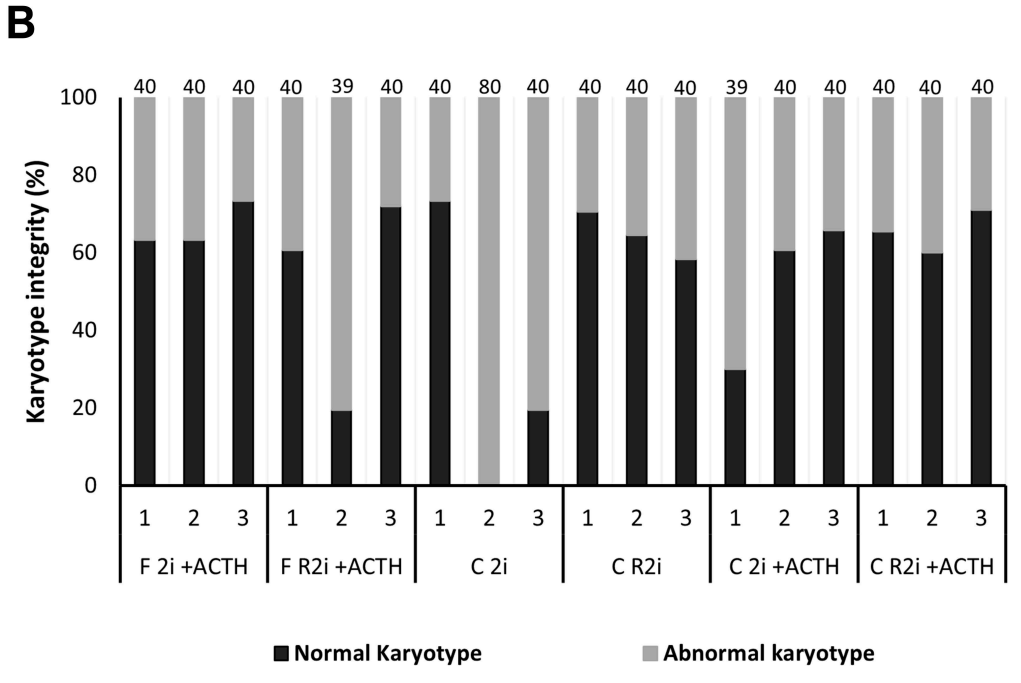
C 2i
C R2i
C 2i +ACTH
C R2i +ACTH


B
Karyotype integrity (%)
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F 2i +ACTH
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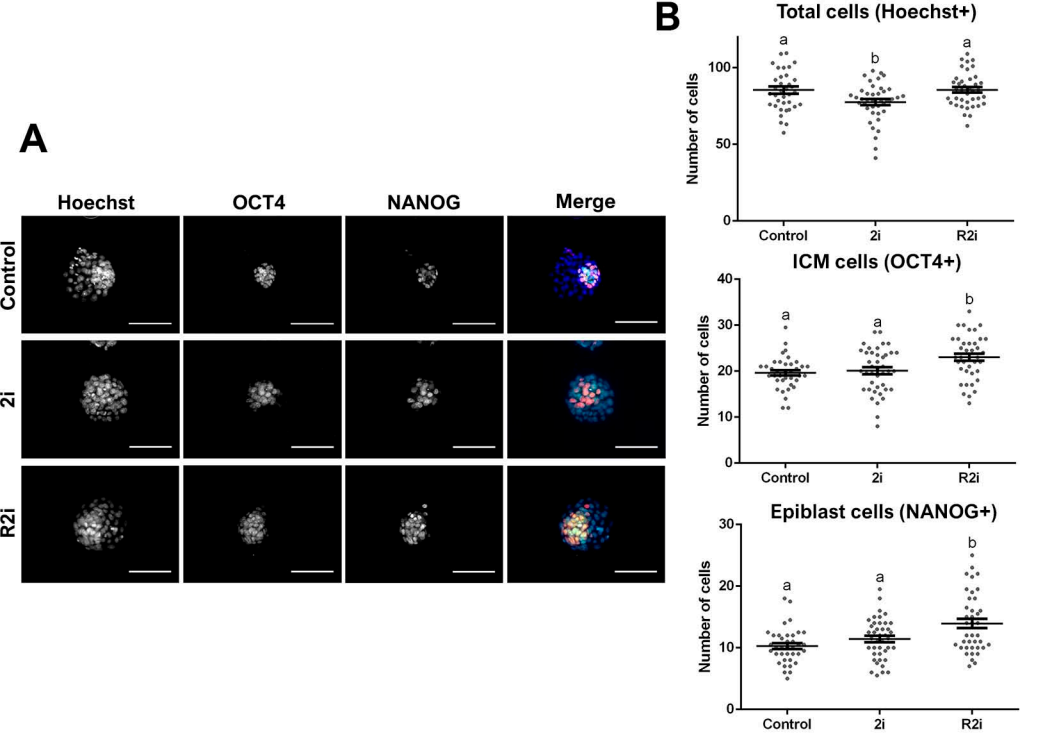
C 2i
C R2i
C 2i +ACTH
C R2i +ACTH
Normal Karyotype
Abnormal karyotype


A
Hoechst
OCT4
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Control
2i
R2i
B
Total cells (Hoechst+)
Number of cells
100
50
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2i
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Number of cells
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