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Characterization of rabbit embryo-derived cells grown under conditions promoting naïve mouse pluripotency*

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After the first derivation of Embryonic Stem Cells (ESCs) from the inner cell mass (ICM) of mouse blastocyst in 1981 by Evans, Kaufman and Martin, the race for derivation of ESCs from other species began. It took almost a decade to obtain human ESCs. Shortly, it became evident that both murine and human ESCs differ in their pluripotency status.

The discovery of factors responsible for reaching the naïve state of pluripotency in mouse ESCs resulted in boosted pluripotency manifesting with high homogeneity and clonogenicity of cell cultures and increased ability to produce chimeras. Mouse naïve pluripotency was established by application of inhibitors that block GSK3B, MEK FGF pathways, named 2i and 3i conditions.

Here, we present the characterization of rabbit embryo-derived cells grown under conditions promoting naïve mouse pluripotency. Blastocysts were isolated from Popielno White Breed rabbits, next ICMs were isolated by immunosurgery and plated on inactivated feeder layer and cultured under 2i or 3i conditions. The 2i culture of ICMs was conducted with N2B27 medium with PD0325901 as a MEK inhibitor and CHIR99021 as a GSK3β inhibitor. The 3i culture of ICMs was performed with 2i medium with the addition of FGFR inhibitor, SU5402.

Here, we show for the first time that 2i culture conditions led to the appearance of cells morphologically

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similar to naïve mouse ESCs, with the high and homogenous expression of pluripotency factors NANOG, SOX2, KLF4 and OCT4. However, 2i conditions were only sufficient for the induction of naïve-like phenotype, as after several passages the features were lost. On the other hand, 3i conditions appeared to be detrimental to the cell culture of ICM.

KEY WORDS: naïve pluripotency / rabbit embryonic stem cells / 2i-culture conditions / 3i-culture conditions

Embryonic stem cells (ESCs) are derived from the *in vitro* culture of isolated early blastocyst's inner cell masses. The network of transcription factors and epigenetic regulators hold the ESCs in a pluripotent state by repressing the genes required for differentiation and stimulating the genes that maintain the core pluripotent network [Ema et al. 2008; Pasini *et al.* 2010]. When mouse embryonic stem cells were isolated for the first time by Evans, Kaufman and Martin in 1981 [Evans and Kaufman 1981, Martin 1981] the race to obtain these cells from different species began.

After derivation of human ESCs in 1998 [Thomson *et al.* 1998] from blastocysts' ICMs, it was evident that although the ESCs from both species share similar features, human ESCs ability to fulfill stringent criteria for pluripotency is limited. When mouse ESCs self-renew in the naïve state of pluripotency, their capacity of single-cell clonogenicity, continuous replication and differentiation into lineages from all three embryonic germ layers, including germ cells is highly increased. The differences of ESCs derived from human and other primates are reflected in flattened colony appearance, low clonogenicity, requirements for bFGF growth factor rather than LIF and limited ability to participate in chimera formation after their injection into environment of the early embryo [see for review: Davidson *et al.* 2015]..

Since the definition of naïve culture conditions for mouse ESCs and culture in N2B27 serum-less conditions in the presence of two inhibitors (so-called 2i conditions) or three inhibitors (3i) blocking GSK3B, MEK and FGF pathways [Silva *et al.* 2008], many works have been dedicated to find suitable conditions for reaching naïve or naïve-like pluripotency state in ESCs derived from other species [Buehr *et al.* 2008, Osteil *et al.* 2016, Bogliotti *et al.* 2018].

However, after many years of research, naïve embryonic stem cells from non-rodent species have been evasive. Big differences exist between stem-like cell lines from different mammalian species [Manor *et al*. 2015, Nichols and Smith 2009] and the race for Holy Grail of naïve pluripotency continues.

Despite the continued attempts of many research groups to derive rabbit ESC lines, no truly naïve lines were obtained. Many rabbit cell lines exhibiting primed stem-like properties exist yet to date, none of them were able to successfully pass chimaera complementation assay and produce viable offspring with contribution to germline [Fang *et al*. 2006, Graves and Moreadith 1993, Honda *et al.* 2008, Honda *et al.* 2009, Honda 2013, Intawicha *et al.* 2009, Moreadith and Graves 1992, Osteil *et al*. 2016, Wang *et al.* 2007, Zakhartchenko *et al.* 2011]. Up to date there are no reports describing the derivation of rabbit ESCs with use of conditions promoting naïve mouse pluripotency.

The knowledge of the molecular pathways that govern pluripotency motivated us to apply it in the derivation of rabbit ESCs using 2- and 3- small molecule inhibitors (2i and 3i conditions, respectively) [Silva *et al.* 2008, Nichols *et al.* 2009, Buehr *et al.* 2008, Ying *et al.* 2008]. PD 0325901 inhibits MEK/Erk activation in fibroblast growth factor (FGF) signaling pathway, which is necessary for driving mouse ES cells into lineage commitment and its inhibition stabilizes the ESCs ground state. The complementary inhibition of Glycogen synthase kinase 3 beta (GSK3β) by CHIR99021 promotes and maintains the proliferation of rodent stem cells [Silva *et al*. 2008, Buehr *et al.* 2008], similarly to the effect of Wnt signaling [Willert *et al.* 2003]. Additionally, SU5402 (present in 3i but not in 2i conditions) is a potent antagonist of the FGF receptor. However, the role of Wnt and MEK/Erk pathways is not as well understood in other mammalian species, and the effect of their alteration may not be as beneficial. For example in bovine, although GSK3β inhibition in *in vitro* cultured embryos results in up-regulation of *OCT4* and *NANOG* [Madeja *et al.* 2015], longer exposure to GSK3i reduces their ability to form ESC-like colonies [Madeja *et al.* 2015], and culture in complete "naïve" media eventually results in down-regulation of these pluripotency factors [Brinkhof *et al.* 2017].

We believed that conditions that stabilize the mouse ESC could lead to the derivation of truly naïve rabbit ESCs.

Material and methods

Animals

Rabbits (*Oryctolagus cuniculus*, Popielno White Breed) were maintained under a 14-h light/10-h dark cycle in the facilities of The Institute of Genetics and Animal Breeding of the Polish Academy of Sciences (IGAB PAS) according to the institutional guidelines. Experimental procedures were approved by the Third Local Ethics Committee (Warsaw, Poland).

Blastocyst recovery

Blastocysts were recovered following natural matings. The embryos were flushed from uteri with pre-warmed M2 medium at day 3.5 to 6 after natural mating. Embryos with morphological abnormalities such as granular cytoplasm, irregular shape or excessively thick mucin layer were excluded from this study. Total of 320 properly developed blastocysts were used for this study.

Blastocyst immunosurgery

The mucin coat and zona pellucida were removed by 0.5% of pronase treatment (37°C for 3 min). Next, the trophectoderm was removed by immunosurgery. In brief, blastocysts were incubated for 2 hours at 37°C in 20% anti-rabbit whole serum (Sigma) in N2B27 medium (see Cell culture of ICMs, outgrowths and colonies for medium description), washed 3 times in N2B27 and incubated (without CO_2) at 37°C in 20% complement (Calbiochem) in N2B27 medium.

The lysed trophectoderm cells were removed by gentle pipetting and the isolated ICMs were plated separately (1 ICM/well) on 10μg/ml mitomycin C (Sigma) treated STO feeder cells (Sigma) either in 2i or 3i medium (see Cell culture of ICMs, outgrowths and colonies for medium description). Feeders were seeded the previous day in 2 cm^2 wells at a density of 2 x 10^4 cells/cm^2.

Cell culture of ICMs and outgrowths and colonies

ICMs and outgrowths were cultured in N2B27 medium, which was used to dilute all compounds and grow cells. It consisted of a 1:1 ratio of DMEM/F12-N2 medium (DMEM/F12, 1% of N2 supplement and 1% of L-Glutamine) and Neurobasal B27 medium (Neurobasal medium, with 1% B27 supplement and 1% L-Glutamine) (all the above components were purchased from Gibco), supplemented with 100mg/ml Pen/Strep (Sigma) and 1000U/ml LIF (Sigma). 2i medium consisted of the N2B27 medium with 1mM PD0325901 (Stemgent) MEK inhibitor and 1mM CHIR99021 (Stemgent)-GSK3β inhibitor. 3i medium consisted of 2i medium plus 2μM SU5402 (Calbiochem) – fibroblast growth factor receptor inhibitor.

For the first 48h after plating ICMs were left undisturbed. Next, the medium was changed daily, and the outgrowths were observed under light microscope for 6-10 days. Afterwards, outgrowths were passaged onto fresh feeder layer. The first 2 passages were done mechanically by picking up the outgrowths/colony-like cells and dissociating them mechanically into clumps by pipetting and plating onto fresh feeders. Subsequent passages were done by covering cells with TrypLeExpress (Gibco), incubating cells for 5 min at 37°C and mechanical dissociation.

After the first passage the colonies were grown in maintenance medium which consisted of: DMEM high glucose (Gibco) with 15% FCS (PAA), 2mM L-glutamine (Lonza), 0.1mM NEAA (Sigma), 0.1mM 2-mercaptoethanol (Gibco), 1000U/ml LIF (Sigma).

Primary rabbit fibroblasts derivation and culture

Primary rabbit fibroblasts were obtained from adult skin biopsies. Briefly, the skin pieces were washed quickly in 70% ethanol, and then twice with fresh media, dissected and cut into small pieces that were placed in gelatin-coated 35mm culture dishes with pre-warmed DMEM media (Sigma) containing 10% Foetal Calf Serum (FCS, PAA) and 100mg/ml Pen Strep (Sigma) and set in a 37°C, 5% CO_2 incubator. Media were changed at least every 5 days. When fibroblasts reached 50 percent confluence, skin explants were removed from the dish with sterile forceps. Passage of fibroblasts was done by aspirating the media, rinsing cells with PBS, adding Trypsin/EDTA until the cells were covered and incubation for 4 minutes at 37°C. Cells were split at 1:8 ratio and frozen when confluence reached 80%.

Immunocytochemistry staining

Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature (RT), washed with PBS and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 15 min. After washing with PBS, 10 min of epitope recovery was made with 50 mM ammonium chloride (Sigma), which was subsequently washed with PBS. Then, blocking buffer solution (20% FCS in PBS with 0.1% Tween-20) was added for 1 hour at RT. After removal of blocking solution, cells were incubated overnight at 4°C with the primary antibodies (indicated in Tab. 1) in blocking buffer. Next day, after washing, secondary antibodies were added diluted in blocking buffer (See table 1) for 90 minutes at room temperature. Finally, the secondary antibody solution was removed, cells were washed with PBS and mounted with Vectashield/ DAPI mounting medium (Vector). Samples were double-labeled for either NANOG and OCT4, or SOX2 and KLF4.

Table1. List of primary and secondary antibodies with used dilutions

Primary antibody	Dilution	Secondary antibody	Dilution
NANOG (Peprotech 500-P236)	1:500	Anti-rabbit (Abcam ab150063)	1:500
SOX2 (Abcam ab97959)	1:200	Anti-rabbit (Abcam ab150063)	1:500
OCT4 (SantaCruz sc-8629)	1:200	Anti-goat (Abcam ab150129)	1:500
KLF4 (R&D Systems AF3158)	1:100	Anti-goat (Thermo Scientific A11057)	1:500

Imaging

Images were acquired using Nikon Eclipse Ti inverted microscope, and Nikon A1R confocal microscope with 405,488, 546 and 647 lasers.

Results and discussions

A total of 320 rabbit embryos collected 3.5-5 days post mating were selected for inner cell masses (ICMs) isolation by immunosurgery as described in the Materials and Methods. Isolated ICMs were plated on inactivated STO feeder layer - 260 ICMs in 2i and 60 ICMs in 3i culture conditions.

Morphology of Inner Cell Mass outgrowths grown in 2i Medium

Isolated ICMs were plated separately in 2 cm^2 wells in 2i medium (Fig. 1A). After 48 hours, ICMs started forming outgrowths (Fig. 1B) and they were passaged by day 10 (Fig. 1C). The overall efficiency of outgrowth formation under 2i conditions was 17.7% (46/260).

Two to three days after the mechanical passage of outgrowths, the first colonies started to appear. They had dome-like morphology (defined edges, and high nucleus to

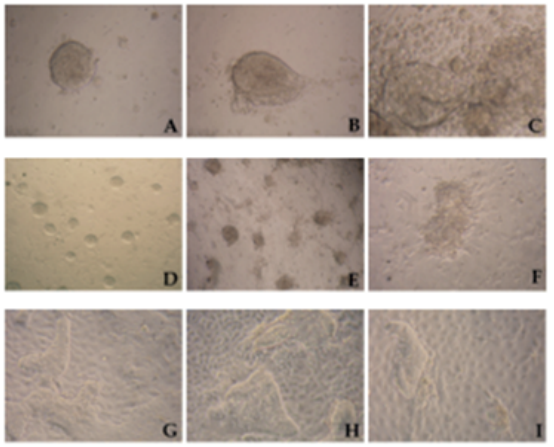

Fig. 1. Inner Cell Mass Outgrowths and cell colonies grown in 2i medium at passage 1 and passage 2. A. Inner cell mass (ICM) after removal of trophectoderm (TE) B. ICM two days after the immunosurgery C. ICM outgrowth five days after immunosurgery D. Appearing colonies three days after first passage E. Colonies loosing dome shape and defined edges at day four after passage F. Close up of colony, see edges loosing definition and cells starting to differentiate. G.H.I. Different cell lines, showing defined colonies and flattened cells originating from colonies.

cytoplasm ratio) characteristic of pluripotent stem cell colonies (Fig. 1D). However, after several days most of the colonies started to lose the dome shape, flattened and colonies lost the defined edges (Fig. 1 E, F). Ten colonies that maintained the pluripotent type morphology were selected, manually picked and passaged onto fresh feeders. However, it was not possible to expand them in an undifferentiated state for longer than six passages as the cells gradually differentiated (Fig. 1 G, H, I).

Morphology of Inner Cell Mass outgrowths grown in 3i Medium

Parallel to 2i conditions, another set of isolated ICMs was cultured in 3i media. However, differently to culture in 2i medium, ICMs plated in 3i medium were weakly attached. The efficiency of outgrowth formation under 3i conditions was only 3% (2/60). After several days outgrowths were severely disturbed and lost the typical characteristics of ESC colonies (Fig. 2 A, B, C). After mechanical passage, the two outgrowths that maintained dome-like morphology were loosely attached and cells were not proliferating. It was not possible to maintain the cell culture beyond passage one.

Fig. 2. Inner Cell Mass Outgrowths in 3i medium. A. Inner cell mass 3 days after immunosurgery, B. ICM outgrowth five days after immunosurgery C. ICM outgrowth ten days after immunosurgery.

Expression of pluripotency markers in outgrowths obtained in 2i and 3i culture conditions

The expression of the main pluripotency markers was verified by immunofluorescent staining of appearing colonies. Cells of early passage (p1) cultured in 2i medium were positive for NANOG, SOX2, KLF4 and OCT4 (Fig. 3 A, B), showing clearly the features of embryonic stem cells. The staining was rather uniform showing a high degree of homogeneity. Together with morphological features of small, dome-shaped colonies, it was indicating that phenotype of colonies at p1 in 2i medium was similar to naïve murine ESCs [Czechanski *et al*. 2014]. However, by passage 3 the expression of the pluripotency markers (Fig. 3 C, D) was no longer detected in the majority of the cells.

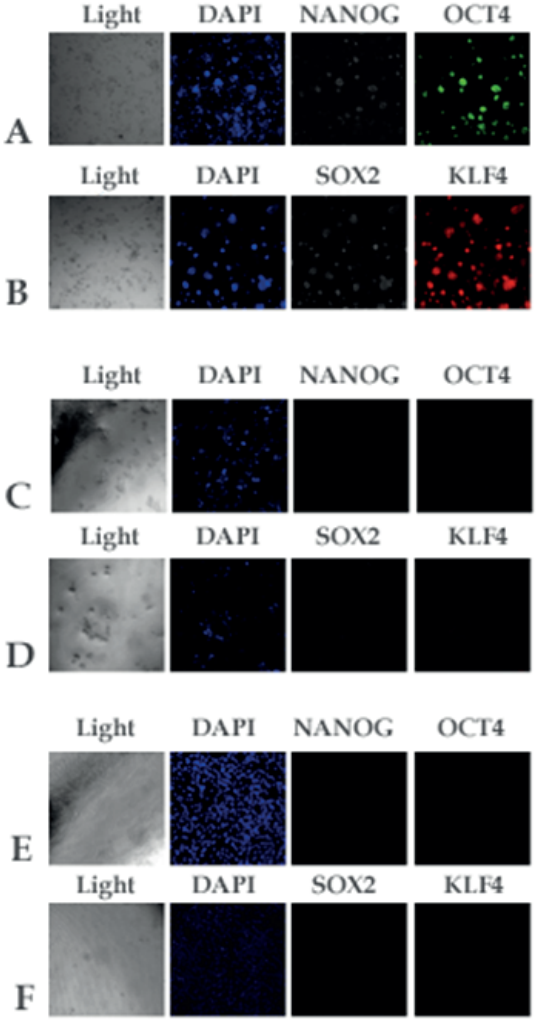

Fig. 3. Pluripotency markers immunostaining of ICM-derived cells grown in 2i medium at passage 1 and passage 3. A, B Rabbit ICM derived cells at passage 1. A: From left: bright field microscopy, nuclear staining with DAPI (blue), co-staining of NANOG (white) and OCT4 (green). B: From left: bright field microscopy, nuclear staining with DAPI (blue), co-staining of SOX2 (white) and KLF4 (red). C, D Rabbit ICM derived cells at passage 3. C: From left: bright field microscopy, nuclear staining with DAPI (blue), co-staining of NANOG (white) and OCT4 (green). D: From left: bright field microscopy, nuclear staining with DAPI (blue), co-staining of SOX2 (white) and KLF4 (red). E, F Rabbit fibroblasts. E: From left: bright field microscopy, nuclear staining with DAPI (blue), co-staining of NANOG (white) and OCT4 (green). F: From left: bright field microscopy, nuclear staining with DAPI (blue), co-staining of SOX2 (white) and KLF4 (red).

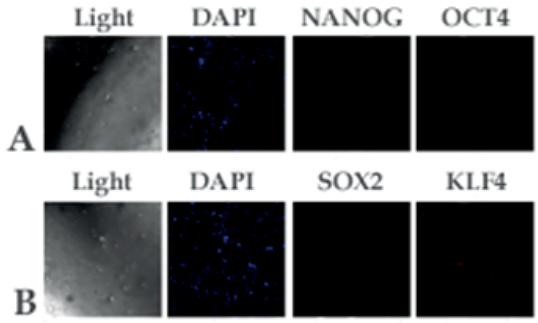

Fig. 4. Pluripotency markers immunostaining of ICM-derived outgrowths cultured in 3i medium. A. ICM-derived outgrowths. From left: bright field microscopy, nuclear staining with DAPI (blue), co-staining NANOG (white) and OCT4 (green). B. ICM-derived outgrowths. From left: bright field microscopy, nuclear staining with DAPI (blue), co-staining of with SOX2 (white) and KLF4 (red).

When outgrowths cultured in 3i medium were profiled for expression of key pluripotency markers only few, single cells appeared positive (Figure 4 A, B).

Rabbit skin fibroblasts used as negative control did not show any expression of pluripotency-related factors (Figure 3 E, F).

Here we describe the endeavour of rabbit embryonic stem cell line derivation, under conditions promoting naïve pluripotency in mouse ESCs. We were able to obtain cells that are morphologically similar to naïve mouse ESCs, when 2i medium was applied during the derivation. Additionally, obtained cell colonies expressed the main pluripotency markers OCT4, SOX2, NANOG and KLF4 in a uniform pattern. However, the high pluripotency status appeared to be rapidly lost, as we were unable to maintain the emerging colonies in the pluripotent state for longer than 3 passages in these conditions. We have also observed the change in morphology of colonies starting from passage 2 and dramatic decrease in pluripotency markers expression at passage 3. Rare colonies that continued proliferating were lost, most probably due to the apoptosis or differentiation, by passage 6. This suggests that 2i conditions, although initially lead to formation of naïve like colonies, are unfit for long term culture and maintenance of the naïve phenotype of rabbit embryonic stem cells. It is possible that although 2i conditions are sufficient for induction of naïve-like phenotype, other, yet unknown, factors are necessary to sustain this state and shield the cells from the differentiation stimuli.

Similarly to the work describing the derivation of bovine ESCs [Malaver-Ortega *et al.* 2012], the ESC markers were only highly expressed during the early stages of the rabbit ICM primary culture, and down-regulated at subsequent passages. All rabbit ESCs obtained until now by various laboratories [Fang *et al.* 2006, Honda *et al.* 2009, Hsieh *et al.* 2011, Intawicha *et al*. 2009] appear to be in primed state of pluripotency and they share similarities with the mouse post-implantation epiblast and with the primate and human ESCs. Unlike the mouse ESCs they rely on FGF2 and Activin/nodal/TGF-B but not on LIF signalling to maintain pluripotency [Ginis

et al. 2004, Honda *et al.* 2009, Hsieh *et al.* 2011, Osteil *et al.* 2016, Wang *et al.* 2008]. Recently Osteil *et al*. 2016 found that deriving and expanding rabbit ESCs with FCS supplementation and single-cell dissociation, in growth factor free conditions, placed the cells in an intermediate state between the primed and naïve pluripotency. These cells depended on activin signalling for self-renewal, similarly to the epiSCs in mice and to some extent were able to colonize developing embryo when injected into morula stage embryo. Another important difference is that the primate and the rabbit ESC lines share the ability of differentiation into trophectoderm cell lineage while the mouse ESCs do not [Ginis *et al.* 2004, Tan *et al.* 2011].

We were not able to establish the naïve rabbit ESC line with use of 3i culture conditions. We have observed much lower efficiency of outgrowth formation in 3i than in 2i (2% vs 17.7%) which suggests that inhibition of FGF receptor by SU5402 is detrimental for cell cultures at this stage. Not surprisingly, no rabbit ESC line could be obtained from rabbit blastocyst using 3i medium, as most of the cells were lost after first 2 passages.

It is known that human, primate, bovine, pig and rabbit embryos share several properties that are absent in the mouse, including their response to FGF/ERK pathway modulation [Kuijk *et al.* 2015, Malaver-Ortega *et al.* 2012, Piliszek *et al.* 2017]. For example in the rabbit embryos, unlike the mouse, blocking of MEK/Erk pathway with small molecule inhibitors is not sufficient to direct the cells towards the naïve epiblast fate [Piliszek *et al*. 2017]. Also in contrast to the murine embryo, in the rabbit, human, primate and bovine embryos, *OCT4*, *NANOG* and *GATA6* expression is not restricted to the ICM and it is maintained in the trophectoderm for a longer period when compared with the mouse [Malaver-Ortega *et al.* 2012, Boroviak *et al.* 2015, Roode *et al*. 2012, Piliszek *et al.* 2017].

Understanding how ESCs from various species select their fate and what stimulates them to leave the pluripotent state, selecting between mesoderm and neuroectoderm fate, could give us clues needed to recognize the switch in order to block it, and concomitantly to protect pluripotent state. It is believed that pluripotency maintenance and lineage choice are intricately linked [Thomson *et al.* 2011]. In the mouse, the pluripotency circuit acts as a unit and represses lineage-specific gene expression in ESCs. We believe that in order to obtain veritable rabbit ESCs, it is important firstly to understand how cell fates are determined during rabbit embryo development. Clearly, the mechanisms governing the fate specification during rabbit early embryogenesis differ from well-known mouse counterpart.

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