



**Examining the effect of combined inhibition of the
differentiation pathways on development of in vitro cultured
rabbit embryos and attached inner cell mass cells**

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2 1 **Examining the effect of combined inhibition of the differentiation pathways on development of *in***
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4 2 ***vitro* cultured rabbit embryos and attached inner cell mass cells**
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44 19 **Running head:** Effect of combined inhibition on development of rabbit embryos
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Abbreviations: 2i, dual inhibition; iPSCs, induced Pluripotent Stem Cells; LIF, Leukemia Inhibitory Factor; FBS, Fetal Bovine Serum; BMP4, Bone Morphogenic Protein 4; hESCs, human ESCs; bFGF (FGF2), basic Fibroblast Growth Factor; mESCs, mouse ESCs; ICM, Inner Cell Mass; FGF4, Fibroblast Growth Factor 4; hiPSCs, human induced Pluripotent Stem Cells; riPSCs, rat induced Pluripotent Stem Cells; mEpiSCs, mouse Epiblast Stem Cells; TGF, Transforming Growth Factor; EB, Embryoblast; TE, Trophoblast; M, Mitochondria; ER, Endoplasmic Reticulum; GC, Golgi Complex; REM, Rabbit ES cell culture Medium; V, Vacuoles; RB, Residual Bodies; AB, Apoptotic Bodies; N, Nucleus;

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Abstracts

Dual inhibition (2i) of Ras-MEK-ERK and GSK3 β pathways enables derivation of ESCs from refractory mouse strains, and in case of permissive strains, derivation with no external protein factor stimuli involvement is possible. In addition, 2i condition is implicated to facilitate reprogramming of somatic cells into induced pluripotent stem cells (iPSCs). Furthermore, blocking of ERK signalling in mouse 8-cell stage embryos leads to ablation of GATA4/6 expressing hypoblast suggesting FGF dependence of hypoblast formation in mouse; while in human, bovine or porcine embryos, hypoblast remains unaffected or displays slight to moderate reduction in the cell number. In this study, we demonstrate that in rabbit embryos, segregation of hypoblast and epiblast is FGF independent and 2i treatment elicits only a limited reinforcement in favour of OCT4-positive epiblast population against GATA4/6-positive hypoblast population. It has been previously shown that TGF β / Activin A inhibition overcomes pervasive differentiation and inhomogeneity of rat iPSCs and ESCs and human iPSCs while prompting them to acquire naïve properties. However, TGF β / Activin A inhibition, alone or together with ROCK inhibition, was not compatible with the viability of rabbit embryos since electron microscopy analysis revealed compromised ultrastructure of cells in the embryo. Our data show that stages of rabbit embryonic development pertaining to emergence of pluripotent stem cells in the pre-implantation embryos might be governed by mechanisms specific to this species. Identifying these mechanisms would facilitate stable ESC line derivation and thus utilization of the rabbit as a model animal in different fields of medicine harnessing novel cell-based therapies.

Introduction

The technology of derivation of mouse embryonic stem cells has traditionally involved a layer of fibroblast cells called “feeders” supposed to provide mechanical and nutritional support but also factors promoting pluripotency, notably, the leukemia inhibitory factor (LIF); and fetal bovine serum (FBS) as a culture medium component. Transferring the technology over to primate (Thomson et al. 1995) and human system (Thomson et al. 1998) does not yield authentic embryonic stem cell lines but does produce cells with different morphology driven by different signalling pathways. LIF and bone morphogenic protein (BMP4) are required for mouse ESCs to maintain their pluripotent state (Hao et al. 2006; Qi et al. 2004; Shen and Leder 1992). However, BMP4 induces differentiation of human ESCs (hESCs) (Xu et al. 2002), while basic fibroblast growth factor (bFGF, FGF2) and Activin A promote hESC self-renewal (Vallier et al. 2005; Xu et al. 2005). Mouse ESCs (mESCs) likely represent pluripotent stem cells of preimplantation inner cell mass (ICM) origin, whereas hESCs correspond to a later epiblast stage (Brons et al. 2007; Tesar et al. 2007).

Fibroblast growth factor 4 (FGF4) is an autocrine protein produced by undifferentiated embryonic stem cells (Rathjen et al. 1999). Downstream activation of Ras-ERK signalling by FGFs provides essential stimuli for proliferation and differentiation in many cell types (Thisse and Thisse 2005). By the FGF-ERK1/2 circuitry deactivation, the ES cells do not progress to either neural or mesodermal lineage commitment but instead they retain the expression of key pluripotency markers *Oct4*, *Nanog* and *Rex1* (Kunath et al. 2007). The propagation of ES cells is also enhanced by inhibition of GSK3 (Sato et al. 2004; Ying et al. 2008). A potent inhibitor of *MEK*, PD0325901 (Bain et al. 2007), can act to achieve suppression of ERK signalling pathway by preventing its phosphorylation. In combination with an inhibitor of GSK3, CHIR99021 (collectively known as “2i”), ES cells can be propagated efficiently and also derived directly from blastocysts of both the permissive 129 mouse strains and the refractory CBA

1 strains (Ying et al. 2008). Ying *et al.* also reported no activation of STAT3, which was previously
2 considered to be central for ES cell self-renewal, suggesting that extrinsic stimuli such as LIF lying
3 upstream are dispensable for the so-called “ground state” of ESC self-renewal (Ying et al. 2008). By
4 applying the same inhibitors, mouse brain-derived neural stem cells can be reprogrammed into induced
5 pluripotent stem cells (iPSCs) only by *Oct4* and *Klf4* transfection. Derived iPSCs display stable up-
6 regulation of pluripotency markers *Oct4* and *Nanog*, reactivation of X chromosome, and somatic and
7 germline chimerism competence. The authors also reported promoting the cells that have previously
8 been dismissed as partially reprogrammed to full pluripotent state (Silva et al. 2008). First germ line-
9 competent, authentic embryonic stem cell lines expressing OCT4, NANOG, SOX2 and KLF4 from rat
10 blastocysts have also been established using 2i condition (Buehr et al. 2008). Later on, the inhibitors
11 that have been used to supplement standard mESC culture conditions have also been applied to
12 generate and maintain rat as well as human induced pluripotent stem cells (riPSCs and hiPSCs
13 respectively) (Liskovych et al. 2011). While riPSCs could be maintained under such conditions, the
14 authors still observed pervasive spontaneous differentiation; however they managed to significantly
15 mitigate it after supplying another small molecule, A-83-01, an inhibitor of type 1 transforming growth
16 factor beta (TGFβ) receptor linked to the Activin A/Nodal signalling axis, which allowed the cells to
17 grow as relatively homogeneous colonies, presented more mouse ESCs “dome”-shaped morphology
18 and contributed extensively to chimerism. Applying the same culture conditions to hiPSCs, also
19 represented colonies with atypical “dome”-shaped morphology resembling those of mESCs; although
20 their molecular signature remained more similar to typical human iPSC. The rationale behind this is the
21 fact that Activin A/Nodal signalling pathway is required to sustain the pluripotency of mouse epiblast
22 stem cells (mEpiSCs) but dispensable for mESCs pluripotency (Li et al. 2009). A Rho-associated
23 kinase inhibitor, Y-27632, has been found to impede apoptosis and increase proliferation, thus
24 reinforcing survival of hESCs following enzymatic dissociation of colonies into single cells (Watanabe
25 et al. 2007). Based on the above-mentioned findings, Kawamata and Ochiya combined Y-27632 with

CHIR99021, PD0325901 and A-83-01 (termed “YPAC”) and successfully established stable and uniform naïve rat ES cells which efficiently contributed to germline chimeras (Kawamata and Ochiya 2010). Latterly, another group succeeded in derivation of a novel germline competent rat ES cell line from Fischer344 transgenic rat by using CHIR99021 and PD0325901 (Men and Bryda 2013).

Rabbit (*Oryctolagus cuniculus*) is potentially a relevant animal model for studying human diseases, such as hypertrophic cardiomyopathy, myocardial infarction, hypertension or atherosclerosis (Bosze et al. 2003). Deriving naïve pluripotent embryonic stem cell lines from rabbit would enable gene targeting which has not been achieved yet. To this aim, many efforts have been made (Fang et al. 2006; Honda et al. 2008; Osteil et al. 2013; Wang et al. 2007), but the morphology of the colonies and the molecular signature of these cell lines render them more similar to those of primed EpiSCs rather than naïve ESCs typical for mice. Indeed, Honda *et al.* reported that bFGF governs the undifferentiated status of rabbit ES cells engaging Activin/Nodal-Smad2/3 signalling pathway and that they are insensitive to LIF (Honda et al. 2009). Furthermore, rabbit ICM and epiblast represented differential expression of genes associated with naïve versus primed pluripotency in mice proposing differences between mouse and rabbit (Schmaltz-Panneau et al. 2014).

Stable cultivation of naïve ESCs has not yet been reported in non-rodent species. However, among the rodent species naïve ESC can be isolated relatively easily only from certain inbred mouse strains, (Nichols and Smith 2011) as opposed to non-permissive mouse strains (Batlle-Morera et al. 2008) and to other mammals (du Puy et al. 2011). It has been suggested that the link between the physiological diapause and the naïve state is the reason why stable naïve ESCs has not been achieved yet (Nichols and Smith 2011). It has been also proposed that the naïve state is either absent or very transient in these species (Rodriguez et al. 2012).

Segregation of NANOG positive epiblast and GATA6/4 positive hypoblast (primitive endoderm) is a process that occurs in the blastocyst stage of the mouse embryonic development (Plusa et al. 2008), but regulation of this process appears to be species-specific. As it was mentioned before, small-molecule

1 inhibitors of FGF/ERK and GSK3 pathways enable derivation and propagation of ESCs from multiple
2 mouse strains and rat. Besides, inhibition of ERK signalling in 8-cell stage mouse embryos lead to
3 ablation of hypoblast development, but did not hinder the blastocyst formation. The ICM cells express
4 NANOG; their paternal X chromosome in XX embryos is not epigenetically silenced and contributes to
5 chimaeras with germline transmission. This implies the FGF/ERK signalling dependence of hypoblast
6 segregation from the bipotent ICM and without this signal, the entire ICM can preserve the pluripotent
7 state with no requirement for a paracrine support from the hypoblast (Nichols et al. 2009; Yamanaka et
8 al. 2010). Similar effect has also been observed in rat embryos (Roode et al. 2012).

9 In human embryos, GATA4 and SOX17 expressing hypoblast is segregated from the NANOG-
10 expressing epiblast by day 7. The effect of *FGF* inhibition on human embryos is different in that the
11 GATA4 positive hypoblast still forms indicating that the formation of human hypoblast is independent
12 of FGF signalling (Kuijk et al. 2012; Roode et al. 2012). The mechanism by which hypoblast
13 segregation in human embryos occurs is not clear yet, however, possible candidates include the TGF β
14 family proteins or the mitogen-activated protein kinase P38 α (Roode et al. 2012). Another recent report
15 showed that in response to 2i condition, cells in the ICM of human 6-day embryos express more OCT4
16 and NANOG but in agreement with the previous report, GATA6 positive hypoblast remains unaffected.
17 The increase in OCT4 and NANOG expression in the ICM is not reflected in the efficiency of the
18 establishment of embryonic stem cell lines from these embryos, as the efficiencies are not significantly
19 different in the control versus the 2i-treated embryos (Van der Jeught et al. 2013).

20 Bovine embryos stimulated with FGF4 and heparin form ICM composed entirely of hypoblast cells and
21 no epiblast cells while MEK inhibition increases the epiblast precursors and decreases hypoblast
22 precursor numbers but does not ablate them completely. These results demonstrate that GATA6
23 expression is not dependent on FGF signalling. The authors propose that FGF4-induced expression of
24 GATA6 in bovine embryos is an indirect effect caused by the repressive effect of activated MEK on
25 *NANOG* (Kuijk et al. 2012). Others confirmed species-specific differences in maintaining the

1 trophoblast lineage via FGF signalling (Harris et al. 2013).

2 In porcine embryos, NANOG can be first detected in the late blastocyst stage (from day 7.5) (du Puy et
3 al. 2011). In non-hatched blastocysts, GATA4 is never detected, although NANOG can be detected in a
4 few isolated cells suggesting that NANOG expression precedes GATA4 expression. MEK inhibition
5 results in significant reduction of GATA4 positive cell numbers and increase in NANOG positive cell
6 numbers; however, in agreement with observations in human and bovine embryos, MEK inhibition
7 does not eliminate the population of GATA4 positive cells. Moreover, the number of ICM cells is not
8 affected implying that the reduction of GATA4 positive cell number is due to the partial interference
9 with hypoblast segregation. MEK and GSK3 β inhibition and LIF supplementation which has been used
10 in a number of studies to impose the naïve state of pluripotency cannot be used to capture NANOG
11 positive ICM cells in porcine embryos, thus additional signals may be needed to prevent hypoblast
12 formation (Rodriguez et al. 2012).

13 In present study, we aimed to investigate how different inhibitors targeting differentiation pathways in
14 ESC, affect viability and the expression of embryonic stem cell-specific genes. Moreover, regarding the
15 efforts to establish stable and reproducible embryonic stem cell lines in rabbit, we also intended to
16 study the effect of these inhibitors on the embryonic outgrowths cultivated following an ESC line
17 establishment protocol.

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Results

Pluripotency and early differentiation marker expression in 4-day, 5-day, 6-day old embryos

To investigate the baseline expression of different embryonic stem cell-specific markers, 4-, 5- and 6-day old rabbit embryos, recovered from the oviducts, were immunostained with antibodies specific to the pluripotent cell marker OCT4 and an early differentiation/hypoblast marker GATA6. In 4-day embryos, we detected OCT4 expression only in a few nuclei; however, low OCT4 expression was found throughout the cytoplasm of all cells. In 5-day embryos, OCT4 expression started to localize in the nuclei, even though a relatively strong signal in the cytoplasm was still detectable. On day 6, OCT4 expression became exclusive to the cells of the embryoblast (EB) and no signal in the cytoplasm was observed (Fig. 1A). GATA6 expression in 4-day embryos was limited to the trophoblast (TE) with varying intensities among the individual cells. The same expression pattern for GATA6 was observed in 5-day embryos. In 6-day embryos, GATA6 was expressed in the embryoblast as well as in trophoblast with varying intensities. However, based on GATA6 expression in 6-day embryos, epiblast and hypoblast were indistinguishable from each other upon analysing the confocal images and boundaries between the ICM and trophoblast (Fig. 1B).

We further analysed GATA6 and CDX2 co-expression in 6-day embryos (Fig. 1C1) (Fig. 1C2). In the trophoblast layer, most of the cells were CDX2 positive (green circle), but we also detected CDX2 and GATA6 double positive (yellow circles) and double negative (blue circles) cells. In the embryoblast we found both GATA6 positive (red circles) and GATA6 negative cells.

To delineate the transcription level of *OCT4*, *GATA6* and *CDX2*, we performed quantitative real-time PCR analysis on day 4 (Fig. 1D) and 6 day (Fig. 1E) *in vivo* developed rabbit embryos. We detected high expression level of *OCT4*, *GATA6* and *CDX2* transcripts on day 4. On day 6 we detected significantly lower level of *OCT4* expression than *GATA6* or *OCT4*.

Effect of 1i, 2i, 3i and 4i conditions on *in vitro* development of rabbit embryos

Next, we investigated the effect of the inhibitors on different pathways in rabbit embryo development and how they preserve their viability over the culturing period. The used inhibitors were as follows: PD0325901 for MEK/ERK, CHIR99021 for GSK3 β , A-83-01 for TGF β receptor and Y27632 for ROCK. The following treatment groups based on the combinations of these inhibitors were used: PD0325901 (1i); PD0325901 and CHIR99021 (2i); PD0325901 plus CHIR99021 and Y27632 (3i); and PD0325901 plus CHIR99021 plus A8301 and Y27632 (4i).

We started by observing how the inhibition of the respective pathways affects viability of the embryos. The percentage of embryos (devoid of zona pellucida) *in vitro* cultured from the 8-cell stage that successfully develop to the early blastocyst stage on the day 4 *post coitum* did not differ across the treatment groups 2i (82.2%), 3i (82.5%), 4i (81.4%), compared to the control RDH-cultured embryos (87.5%), but there were significant differences between control and 1i treated group (78.5%) (Fig. 2A). Continued culture of the same embryos up to day 6 *post coitum* expanded blastocyst revealed tolerance of the embryos to 1i (45%) and 2i (47.5%) conditions as the percentage of embryos that successfully developed to the expanded blastocyst was comparable to the percentage observed in the control RDH-cultured embryos (42.5%); however, 3i (10%) and 4i (0%) treatments resulted in death of the embryos (Fig. 2A). Derived blastocysts were immunostained with antibody against GATA6. Most of the obtained blastocysts in 1i condition were retarded in their development and their epiblasts were smaller as it is shown on Fig. 2B.

Moreover, we performed real-time quantitative PCR analysis to investigate the effect of inhibitors on expression of pluripotency associated marker *OCT4*, and an early differentiation marker *GATA6* in 6-day rabbit embryos cultured from the 8-cell stage in inhibitor-supplemented media (Fig. 2C) compared to the control embryos cultured in RDH medium with no inhibitors. The best performing condition appeared to be 2i, since the transcription level of pluripotent marker, *OCT4*, was 2.4-fold higher

1 compared to the control embryos cultured in RDH with no inhibitors (Fig. 2C). In contrast,
2 transcription of *GATA6* in embryos treated with 2i did not display substantial increase compared to the
3 control embryos in RDH, but its transcription was slightly high in 1i condition (1.94-fold) (Fig. 2C).
4 Afterwards, in order to analyse the effect of the double inhibition on the expression of the OCT4 and
5 GATA6 on protein level in embryos cultured *in vitro* from the 8-cell stage, we carried out
6 immunohistochemical analysis of 6-day embryos (Fig. 2D) and then performed quantitation of the
7 fluorescent signal intensities pertaining to fluorescently labelled antibodies specific to the two protein
8 markers. We did not observe significant differences in OCT4 expression in 2i-treated embryos versus
9 the control embryos cultured in RDH (89.45% and 80.76%, respectively); likewise, GATA6
10 expression in embryos cultured in 2i condition did not display substantial difference compared to
11 control embryos in RDH culture medium (55.86% and 72.13%, respectively) (Fig. 2E).
12 To scrutinize the integrity of the cells and their organelles in the ICM of 6-day rabbit embryos cultured
13 under 2i, 3i and 4i conditions, we performed transmission electron microscopy analysis (Fig. 2F) (Fig.
14 2G). We measured the relative volume (%) of the cell organelles of the 6-day embryos cultured under
15 the individual inhibition culture conditions (Table 1). We found the mitochondria (M, 7.5%), the
16 endoplasmic reticulum (ER, 9.7%) and the Golgi complex (GC, 3.4%) significantly larger in the
17 control group (Table 1; Fig. 2F) compared to 3i condition (M, 5.2%; ER, 4.7% and GC, 2.8%; Table 1;
18 Fig. 2G) and 4i condition (M, 1.9%; ER, 4.5% and GC, 1.7%; Table1). The difference in relative
19 volume of these organelles between the control and 2i was not significant, indicating that 2i preserved
20 the organelle integrity (Table 1). *In vitro* culture of embryos in media supplemented with 4i resulted in
21 a significant increase in the size of the vacuoles (7.2% versus 4.9% in the control; Table 1). The
22 relative volume of the residual bodies in the cells of the embryos was significantly higher in all of the
23 inhibitor-supplemented media: 9.7% in 2i, 14.1% in 3i, 16.7% in 4i versus 5.1% in control (Table 1).
24 The volume of apoptotic bodies was significantly higher in 3i-supplemented media (2.2% versus 0.4%

1 in the control) (Table 1). The relative volume of apoptotic bodies in 4i was not reduced (0.6% versus
2 0.4% in the control).

3 In accordance to the results obtained from confocal microscopy, quantitative real-time PCR analysis
4 and electron microscopy morphometry, we proposed 2i to be best condition to keep the
5 undifferentiated state of the pluripotent cells in the rabbit embryo.

6 ***Rabbit early embryonic development in RDH and 2i condition by time-lapse microscopy***

7 We subsequently tracked the embryonic development in the control culture condition (RDH) against
8 2i-supplemented culture medium using time-lapse video technology to measure the time between the
9 different embryonic developmental stages, from 2-cell stage up to early blastocyst stage (75 hours).
10 The embryonic development was slightly faster in 2i medium; embryos reached the morula stage
11 somewhat earlier than in the control RDH medium; however, the differences were not significant (Fig.
12 3A). Similar effect was observed in another time-lapse video experiment comparing the development
13 of the embryo in control in contrast to the 2i culture condition from the morula up to the blastocyst
14 stage over the span of 48 hours. Embryos cultured in 2i were slightly smaller in size at each time point
15 but the difference in developmental dynamics of embryos cultured in control and 2i conditions was not
16 significant (Fig. 3B). Therefore, 2i treatment did not significantly affect the development of the rabbit
17 embryos from 2-cell stage up to early blastocyst stage (*Supplementary Material*; Sup.Mov. 1).

18 ***Effect of 2i treatment on rabbit embryonic outgrowths***

19 Subsequent to the analysis of 2i treatment on early rabbit embryonic development, we next
20 investigated its effect on attached rabbit blastocysts. We cultured the rabbit blastocysts from 8-cell
21 stage to early blastocyst stage in 2i and control medium (RDH). There was no significant difference in
22 the developmental rate between 2i treated and non-treated embryos (Fig. 4A). Developed blastocysts
23 were placed to culture plate and allowed to attach onto the mouse feeder layer in rabbit ES cell culture

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2 1 medium (REM) and 2i-supplemented REM. We performed a quantitative real-time PCR analysis after
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4 2 four days cultivation. Supplementation of culture medium with 2i did not exhibit distinct effects on the
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6 3 culture of rabbit ICM outgrowths. *GATA6* expression was reduced in 2i condition, however the same
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8 4 was true for *OCT4* expression which might suggest either more advanced differentiation of the cells in
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10 5 the condition of combined MEK/ERK and GSK3 β inhibition (2i) or compromised viability of the cells
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12 6 (Fig. 4B). To further analyse the expression of OCT4 and GATA6 at protein level we preformed
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14 7 immunostaining analysis. Colonies of embryonic outgrowth stained positive for OCT4 with varying
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16 8 degrees in both control (Fig. 4C1C2C3) (Fig. 4C4) and 2i condition (Fig. 4G1G2G3) (Fig. 4G4).
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18 9 Compact GATA6 negative colonies were surrounded by GATA6 positive cells (Fig. 4D1D2D3); (Fig.
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20 10 4D4); (Fig. 4E1E2E3) (Fig. 4E4); (Fig. 4F1F2F3) (Fig. 4F4). This was visualized clearly by 3D
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22 11 reconstruction of the respective confocal images.
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24 12 In the control condition, we found compact colonies with bright OCT4 signal, but their presence was
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26 13 transient, since after a few passages they disappeared from cell culture. Under 2i-supplemented culture
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28 14 condition, we did not find compact colonies with bright OCT4 signal and there was a low GATA6
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30 15 signal. Colonies with primed ESC morphology with dimmer OCT4 signal and positive staining for
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32 16 GATA6 were the only type of colonies presented in 2i condition (Fig. 4G1G2G3) (Fig. 4G4) and (Fig.
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34 17 4H1H2H3) (Fig. 4H4). Colonies that displayed morphology which is typical for rabbit ES-like cells,
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36 18 represented primed ESC morphology and were predominant in both conditions. The OCT4 expression
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38 19 did not seem to differ between control and 2i-treated cultures according to the confocal images.
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40 20 GATA6 expression under the effect of 2i did not disappear which confirmed the observations in rabbit
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42 21 embryos.
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44 22 Under control condition compact dome-shaped colonies of small cells with high nucleus-to-cytoplasm
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46 23 ratio produced a bright OCT4 signal, while they were negative for GATA6. This might indicate a
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48 24 phenotype of more “naïve” ESC-like cells. On the other hand, flat colonies of cells with lower nucleus-
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1 to-cytoplasm ratio emerged under 2i condition displayed a dimmer OCT4 signal, which might be more
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1 to-cytoplasm ratio emerged under 2i condition displayed a dimmer OCT4 signal, which might be more
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2 indicative of “primed” state of the ESC-like cells (Fig. 4G4).
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1 **Discussion**

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3 ESCs are sustained by a flexible signalling and transcription factor network, which appears to be
4 closely related to network existing in the embryo. The notion of naïve pluripotency implies that early
5 epiblast cells and their counterpart ESC derivatives have equal unrestricted access to all somatic
6 lineages and the germ line (Nichols and Smith 2011).

7 In order to examine the expression of transcriptions factors in rabbit embryos, embryoblast and
8 trophoblast, we first analysed the patterns of OCT4, GATA6 and CDX2 protein expression and their
9 gene transcription in rabbit embryos recovered at consecutive developmental stages.

10 We investigated how OCT4 and GATA6 are normally expressed in *in vivo* developed embryos
11 recovered from the oviducts at 4, 5 and 6 days *post coitum*. Expression of the pluripotency marker
12 OCT4 was first detected exclusively in the nuclei of cells of the embryoblast in 6-day embryos. GATA6
13 expression in 4- and 5-day embryos was limited to non-ICM cells but in 6-day embryos, its expression
14 observed in all cells of the embryo. Embryoblast was comprised of GATA6-positive and negative cells
15 while some cells of the trophoblast exhibited GATA6 signal; however, the majority of the trophoblast
16 cells were only CDX2-positive.

17 In our following experiments, we tested how different combinations of inhibitors of early
18 differentiation pathways affect the development, transcription and expression of OCT4 and GATA6.

19 The effect of the Ras-MEK-ERK1/2 blockade on development of preimplantation embryos has been
20 investigated in different species. PD0325901 is a very selective inhibitor of MEK/ERK pathway
21 downstream of FGF receptor engaged by the autocrine FGF4 that cells use to self-differentiate and
22 progress in their developmental program. CHIR99021 as an inhibitor of GSK3 pathway, has pleiotropic
23 effects, restricts neural differentiation, restores viability lost due to MEK/ERK inhibition; the 2
24 inhibitors are collectively named “2i”, (Ying et al. 2008). 2i was shown to enable derivation of
25 embryonic stem cell lines from refractory mouse strains as well as rat (Buehr et al. 2008).

1 Quantitation of fluorescent signal intensity from the confocal pictures in 6-day rabbit embryos cultured
2 in 2i-supplemented media as well as quantitative real-time PCR analysis of embryos cultured in 2i from
3 the 8-cell stage up until the stage of blastocyst (4.5 days) suggested that 2i kept the transcription of
4 *GATA6* to the minimum possible level while preserving the transcription of *OCT4*.

5 Differences in the mechanisms regulating the segregation of epiblast and hypoblast have been reported
6 in many species. In species other than mice or rat, MEK inhibition does not result in ablation of
7 hypoblast cells in the inner cell mass of the blastocysts, and *GATA6* expression does not disappear. In
8 mouse 8-cell stage embryos, the blockade of ERK signalling completely abolishes development of
9 hypoblast cells (Nichols et al. 2009). However, human embryos do not follow the same principle but
10 instead, *GATA4* positive hypoblast still forms even under MEK inhibition (Kuijk et al. 2012; Roode et
11 al. 2012). In bovine embryos the MEK inhibition does not ablate hypoblast precursor cells (Kuijk et al.
12 2012). Signal inhibition increases transcription of putative epiblast markers *NANOG* and *SOX2* specific
13 to ICM, while repressing putative hypoblast marker *GATA4*. Expression of other pluripotency-related
14 (*OCT4*, *KLF4*, *DPPA3*) or trophoblast enriched (*CDX2*) genes is not affected. Blocking MEK
15 signalling enhances the proportion of *NANOG* expressing cells in the porcine ICM, but does not
16 prevent the segregation of *GATA4* cells; inhibition of FGF signalling does not alter the segregation of
17 *NANOG* and *GATA4* cells, but affects the number of ICM cells (Rodriguez et al. 2012). Other
18 combinations of inhibitors did not produce significant reduction in *GATA6*, lowered the *OCT4*
19 expression or compromised the overall viability of the embryos.

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21 In rabbit, elevated transcription of *OCT4* under the effect of PD0325901 (1i) indicates that MEK/ERK
22 inhibition is responsible for this elevation; however, *GATA6* transcription was also elevated, suggesting
23 that GSK3 β inhibition is needed in addition to MEK/ERK inhibition to achieve any reduction in
24 *GATA6* transcription. Furthermore, the viability of the embryos was poor under the effect of
25 PD0325901 while lacking CHIR99021. This data suggest that GSK3 β inhibition is needed to maintain

cellular viability. This is consistent with previous findings that higher doses of PD0325901 alone lead to substantial growth arrest in hESCs and blockade of the ERK pathway, but it is largely cytostatic rather than cytotoxic (Hoshino et al. 2001). Blockade of the ERK signalling pathway by ERK kinase (MEK) inhibitors selectively enhances the induction of apoptosis by microtubule inhibitors in human tumour cells in which this pathway is constitutively activated (Kawabata et al. 2012). The addition of CHIR99021 restores viability. According to our time-lapse video analysis, the embryos in 2i-supplemented culture condition developed normally. When ERK signalling is not present down-modulation of GSK3 β becomes increasingly crucial to maintain metabolic activity, biosynthetic capacity and overall viability (Ying et al. 2008).

To investigate whether reduction in cell death rate by inhibition of ROCK could help to balance the potential negative effects of PD0325901, we included the combination of 3 inhibitors, “3i” (PD0325901+CHIR99021+Y27632) targeting MEK/ERK, GSK3 β and ROCK signalling pathways. Y27632, as a ROCK inhibitor, inhibits apoptosis caused by enzymatic dissociation of cells during passaging, as demonstrated by Watanabe (Watanabe et al. 2007). Y27632 has also been used to culture hESC and hiPSC in suspension (Zweigerdt et al. 2011). Another inhibitor, the A83-01, is an inhibitor of TGF β receptor upstream of Activin A/Nodal. TGF β receptor activity is needed to maintain primed EpiSC. All four inhibitors are collectively termed YPAC when used together. In our experiments, we termed this combination as “4i”.

To recognise the integrity of the cells and their organelles in the ICM of 6-day rabbit embryos cultured under 2i, 3i and 4i conditions, we performed transmission electron microscopy analysis. Investigating the impact of the inhibitors on the cellular ultrastructure of the embryo revealed afflicted mitochondria, endoplasmic reticulum and Golgi complex while the volume of vacuoles as well as residual and apoptotic bodies increased in 4i- and 3i-treated embryos. Despite ROCK inhibition by means of Y27632 in 4i formulation, the relative volume of apoptotic bodies was not reduced. Treatment with 2i did not result in significant disturbance of the organelles. 2i maintained integrity of mitochondria,

1 endoplasmic reticulum and Golgi complex in the ICM cells of the rabbit embryos while maintaining
2 the volumes of the structures, indicating minimum damage.
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4 Quantitative real-time PCR analysis of the attached ICM outgrowth 4 days following plating of the
5 rabbit embryos on the feeder layer in control versus 2i supplemented culture revealed lower expression
6 of both *OCT4* and *GATA6*. Confocal images of attached rabbit ICM in control or 2i condition did not
7 reveal any apparent differences in OCT4 and GATA6 expression displaying “primed” epiblast stem cell
8 colony-like morphology. In a few cases, and only in the control condition, compact dome-shaped
9 colonies displaying bright OCT4 signal and no GATA6 signal emerged, which could potentially
10 represent a “naïve” pluripotency. This finding suggests that FGF4/RAS/MEK/ERK and GSK3 β
11 signalling inhibition is not permissive for the maintenance of this type of colonies in rabbit. In contrast
12 the TGF β receptor inhibition together with 2i (Li et al. 2009) and also with Y27632 (Kawamata and
13 Ochiya 2010) have been used to generate rat ESC and iPSC displaying naïve “dome”-shaped colonies
14 and capable of contributing to chimaeras. Studies reveal a novel function of TGF β signalling inhibition
15 in the generation and maintenance of pluripotent stem cells (Tan et al. 2015). Rabbit embryonic stem
16 cells likely tend to assume a state that according to their molecular signature corresponds to the
17 primed/epiblast stem cell state (Honda et al. 2009; Osteil et al. 2013). Rabbit ICM and epiblast suggests
18 differences between mouse and rabbit regarding the expression of the few classical markers for naïve
19 versus primed pluripotency (Schmaltz-Panneau et al. 2014).

20 Conclusion

21 Inhibition the factors involved in Ras-MEK-ERK, GSK3 β and TGF β signalling pathways have been
22 implicated to alter the ratio of OCT4 positive epiblast and GATA6 positive hypoblast. As a result of
23 MEK inhibition, the number of hypoblast cells is reduced in mouse and rat embryos but in human,
24 porcine and bovine embryos the amount of the hypoblast cells is only moderately reduced, suggesting
25 that hypoblast formation is FGF-independent. Our results demonstrate that rabbit embryos respond in a

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2 1 similar way to non-rodent embryos because the hypoblast still forms even under combined MEK and
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4 2 GSK3 β inhibition. In rat and human ESC/iPSC, TGF β inhibition was previously shown to help
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6 3 maintain the naïve pluripotent stem cell phenotype in culture, whereas the inhibition the TGF β receptor
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8 4 was not compatible with rabbit embryo viability, suggesting that TGF β plays a crucial role in rabbit
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For Peer Review

Materials and Methods

Embryo collection

The animals used were Hycole rabbits handled in compliance with the Hungarian Code of Practice for the Care and Use of Animals for Scientific Purposes and the experiments were approved by the Hungarian Animal Care and Ethics Committee (Ref No. 767/001/2003). Superovulation and embryo collection was performed as previously published (Besenfelder et al. 1997). 8-cell stage embryos were collected 36 hours following insemination.

Embryo culture

The RDH medium was composed of 1:1:1 of RPMI 1640, DMEM and Ham's F10 medium (Invitrogen) supplemented with 5 mM taurine (Sigma) and 0.3% BSA (Sigma). Rabbit 8-cell stage embryos were cultured in small drop of RDH medium covered by embryo-tested light paraffin oil (Sigma) at 38.5°C under 5% CO₂ in humidified air (Jin et al. 2000).

RDH medium was used to prepare 1i, 2i, 3i and 4i supplemented media. The concentrations of inhibitors in RDH medium were as follows:

1i: 1 μ M of PD0325901 (04-0006, StemGent), **2i:** 1 μ M of PD0325901, 3 μ M of CHIR99021 (04-0004, StemGent), **3i:** 1 μ M of PD0325901, 3 μ M of CHIR99021, 10 μ M of Y-27632 (04-0012, StemGent). **4i:** 1 μ M of PD0325901, 3 μ M of CHIR99021, 10 μ M of Y-27632, 0.5 μ M of A-83-01 (04-0014, StemGent).

All rabbit embryos were cultured in RDH, 1i, 2i, 3i and 4i media covered by embryo-tested light paraffin oil (Sigma) at 38.5°C under 5% CO₂ in humidified air. (Jin et al. 2000).

Derivation of attached embryonic outgrowths

Rabbit 8-cell stage embryos collected at 1.5 days *post coitum* were cultured *in vitro* for 2 days in RDH

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2 1 and 2i supplemented media. Zona pellucid of 3.5-day old blastocysts was removed by exposing them to
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4 2 0.5% pronase (Sigma –Aldrich) in PBS for 10 minutes. Blastocysts were cultured on mitomycin C (10
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6 3 mg/ml; Sigma–Aldrich) inactivated mouse embryonic fibroblasts in rabbit ES cell culture medium
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8 (REM) and 2i-supplemented REM. The blastocysts were attached to the feeder layer and formed
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10 4 primary embryonic outgrowths. The following REM culture medium was used: Knock-out DMEM,
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12 5 Iscoves’s MDM, 4.5 g/L glucose; Invitrogen, UK) (1:1) supplemented with 15% Knock-out™ Serum
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14 6 Replacement (Life Technologies) and basic additives containing 1% nonessential amino acids
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16 7 (Invitrogen), 1% GlutaMAX supplement (Invitrogen), 1% nucleosides (adenosine, guanosine, cytidine,
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18 8 uridine and thymidine (all from Sigma), 450 mM alpha-monothioglycerol (Sigma),
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20 9 penicillin/streptomycin (50 unit/50 mg, Sigma), 20 ng/mL rat LIF (ESG2206, Millipore, Chemicon
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22 10 International, Temecula, CA), and 40 ng/mL human bFGF (Invitrogen) (Catunda et al. 2008;
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24 11 Maraghechi et al. 2013).
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26 12 Concentrations of the individual inhibitors in the REM culture medium were the same as the
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28 13 concentrations used at embryo culture medium.
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37 16 **Immunohistochemistry**

40 17 Immunostaining of embryos and embryonic outgrowths was performed using primary antibodies
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42 18 against OCT4 (ab27985, 1:100; Abcam, Cambridge, UK), CDX2 (ab15258, 1:100; Abcam), GATA6
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44 19 (AF1700, 1:10; R&D Systems Europe, UK). Secondary-fluorochrome-conjugated antibodies (Jackson
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46 20 ImmunoResearch Laboratories, West Grove, PA, USA) were diluted 1:400 in blocking buffer. Nuclear
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48 21 staining was performed by embedding the samples into DAPI containing Vectashield mounting
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50 22 medium (H-1200; VectorLaboratories, Burlingame, CA, USA). Embryos and embryo outgrowths were
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52 23 fixed in 4% paraformaldehyde, for 10 minutes at room temperature. The cells were permeabilized and
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54 24 blocked in one step using PBS with 0.5% Triton X and 3% BSA. Cells were incubated with the primary
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56 25 antibodies overnight at 4°C, thoroughly washed and then incubated with the secondary antibodies at
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37°C for 1 hour before final washing and mounting in Vectashield mounting medium (H-1200, Vector Laboratories) with DAPI onto the micro slides. Samples were visualized using the Carl Zeiss Axio Imager 2 fluorescent microscope and Carl Zeiss LSM 700 confocal microscope.

Transmission Electron Microscopy

Rabbit embryos were fixed in aldehyde mixture (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M cacodylate buffer, pH 7.1 – 7.3) for 1h at 4°C, and washed twice in cacodylate buffer, 5min each. The individual embryos were embedded into 4% agar and post-fixed in 1% osmic acid in cacodylate for 1h. The samples were dehydrated in series of progressively concentrated ethanol solutions and mounted into Durcupan ACM (Fluka). Semithin sections (1-2µm) were stained with methylene blue. Ultrathin sections (75-90nm) placed on copper or nickel meshes were contrasted with lead uranylacetate and lead citrate. Electron micrographs for morphometric measurements were acquired using transmission electron microscope JEM 100 CX II, Jeol (Japan) at an acceleration voltage of 80kV. 10-11 electron micrographs were made out of each embryo and analysed at a magnification of 7200x.

Relative volume (in %) of cell organelles was determined using a method published earlier (Bolender and Weibel 1973). Line segments with 150 test-points were placed on each micrograph. The number of test-points, within the area of the analysed organelle, was divided by the total number of points in the line segments, and the resulting value was expressed in percent. The volume of the individual organelles per sample was calculated based on the sum of all values measured from the individual micrographs. The organelles analysed in this way included: mitochondria (M), endoplasmic reticulum (ER), Golgi complex (GC), vacuoles (V), residual bodies (RB) and apoptotic bodies (AB).

Statistical significance of the difference in volume was determined using the chi-square (χ^2) test. The difference was considered to be significant at $p=0.05$. Statistical analyses of the individual organelle volumes were carried out using the Student *t*-test.

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Real-time quantitative PCR

Total RNA was extracted from rabbit embryos and growing stem cell colonies using TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer’s instructions. RNA was then reverse transcribed into single-stranded cDNA, using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, USA). Synthesized cDNA templates were subjected to quantitative real-time PCR analysis using SYBR® Green PCR Master Mix, with rabbit specific primer sets (Maraghechi et al. 2013). All reactions were performed in Eppendorf Mastercycler Realplex⁴. Relative mRNA levels were calculated with the comparative CT Method ($\Delta\Delta CT$ Method) and normalized to Gapdh mRNA level. GenEx qPCR data analysis software by MultiD (<http://www.multid.se>) was used to analysis the data. Results are expressed as mean $\pm$ S.D. A one-way ANOVA test (GenEx software) followed by the Tukey–Kramer multiple comparison test was used to document any statistically significant difference between multiple groups. Probability levels of $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ were considered to be significant.

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1
2 1 **Figure legends**

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6 3 **Figure 1. Expression of pluripotency and early differentiation markers in rabbit blastocysts**

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9 4 **A:** Baseline expression of OCT4 in rabbit embryos after their recovery from the oviducts on days 4, 5
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11 5 and 6 *post coitum*. OCT4 expression fully developed on day 6. **B:** Baseline expression of GATA6 in
12
13 6 embryos after their recovery from the oviducts on days 4, 5 and 6 *post coitum*. GATA6 expression was
14
15 7 limited to non-ICM cells in 4-day and 5-day embryos. In 6-day embryos, GATA6 expression was
16
17 8 observed in both ICM and non-ICM cells at varying intensities (Scale bars: 200µm, 100 µm). **C:**
18
19 9 GATA6 and CDX2 co-expression in 6-day embryos showing most cells of the trophoblast (top row) to
20
21 10 be CDX2-positive (green circle), CDX2/GATA6 double-positive cells (yellow circles) and
22
23 11 CDX2/GATA6 double-negative cells (blue circles) were also detected; the embryoblast (bottom row)
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25 12 was comprised GATA6 positive cells (red circles) and GATA6 negative cells (blue circles)(Scale bars:
26
27 13 100µm). C2 is confocal 3D reconstructions of C1 images showing the interface of trophoblast (TE) and
28
29 14 embryoblast (EB). **D and E:** Quantitative expression of *OCT4*, *GATA6* and *CDX2* mRNAs in 4-day (D)
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31 15 and 6-day (E) rabbit embryos. High level of expression of *OCT4*, *GATA6* and *CDX2* transcripts was
32
33 16 detected by day 4 of embryonic development, while the expression of *OCT4* transcript was down-
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35 17 regulated at 6-day embryos (TE, trophoblast; EB, embryoblast). Expression levels were calculated
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37 18 relative to *GAPDH* transcript level and normalized to the 7-days mRNA level. Error bars indicate ± SD
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39 19 (**p ≤ 0.001).

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49 21 **Figure 2. In vitro development of rabbit embryos in 1i, 2i, 3i and 4i conditions**

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51 22 **A:** The percentage of embryos (devoid of zona pellucida) in vitro cultured from the 8-cell stage that
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53 23 successfully develop to the compact morula stage on the day 4 *post coitum* compared to the control
54
55 24 RDH-cultured embryos (dark-grey lines). The percentage of the same embryos, after continued culture,
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57 25 that successfully develops to the expanded blastocyst stage on the day 6 *post coitum* (light-grey lines).
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3i and 4i treatments resulted in death of the embryos. Error bars indicate $\pm$ SD (* $p \leq 0.05$). **B:** Immunostaining of 6 day blastocysts cultured under different condition (Scale bars: 50 μ m). **C:** Quantitative real-time PCR analysis showing the expression of OCT4 and GATA6 in 6-day embryos cultured from the 8-cell stage in 1i, 2i and the control RDH medium. The highest expression of OCT4 transcript was detected in 2i condition, while the expression of GATA6 transcript was slightly higher in 1i condition. **D:** Immunohistochemical analysis of OCT4 and GATA6 expression in 6-day embryos cultured from the 8-cell stage in control RDH medium (first two rows and 2i last two rows) (Scale bars: 100 μ m). **E:** Quantitation of the fluorescent signal intensities pertaining to fluorescently labelled antibodies specific to OCT4 and GATA6 calculated as a percentage of fluorescent signals together with DAPI signal out of total DAPI signal. The percentage represents a mean value calculated from the individual confocal image slices of 6-day embryos. **F-G:** Transmission electron micrographs of the ICM of the 6-day embryos cultured in the control RDH and 3i condition. The figures depict healthy organelles (RDH) and damage arising from the culture condition that does not support viability of the embryos (3i). The electron micrograph shows three cells of the inner cell mass from the control group of embryos in RDH (F). One of them was localized in the centre of the image with oval nucleus (N) and nucleoli localized in the centre of cytoplasm. Intercellular spaces are partially extended (*). The electron micrograph of two cells of the inner cell mass of rabbit embryos treated with 3i shows numerous large oval vacuoles (V), residual bodies (RB) and apoptotic bodies (AB) located in the vicinity of an elongated nucleus (N) of heterochromatic type. The presence of mitochondria (M) and granular endoplasmic reticulum (ER) is very rare. Individual structures: Mitochondria (M) (dark and oval), granular endoplasmic reticulum (ER), small residual bodies ($\rightarrow$) and small vacuoles ($\blacktriangleright$) around the ESC. Magnification: 7200X.

Figure 3. Tracking of the embryonic development in the control culture condition (RDH) against 2i-supplemented culture medium using time lapse video technology

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2 1 **A:** measuring the time between the different embryonic developmental stages, from 2-cell stage up to
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4 2 early blastocyst stage (75 hours). **B:** measuring the time from morula stage up to the blastocyst stage
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6 3 over the span of 48 hours. Embryos cultured in 2i condition displayed slightly faster development and
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8 4 smaller size compared to the control embryos in RDH.
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13
14 6 **Figure 4. 2i treatment did not exhibit distinct effects on the culture of rabbit ICM outgrowths**

16 7 **A:** Rabbit embryos were cultured from 8-cell stage to early blastocyst stage in 2i and control medium.
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18 8 No significant difference in the developmental rate was observed. **B:** Quantitative real-time PCR
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20 9 analysis of *OCT4* and *GATA6* mRNA expression in attached ICM outgrowths displayed significant
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22 10 depletion of both *OCT4* and *GATA6* transcription in 2i supplemented medium compared to control ones
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24 11 REM, Rabbit ES cells culture medium. Expression levels were calculated relative to *GAPDH* mRNA
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26 12 level and normalized to the 7-days mRNA level. Error bars indicate $\pm$ SD. (** $p \leq 0.01$). **C-H:**
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28 13 Expression of OCT4 (C, G) and GATA6 (D, E, F, H) in attached ICM outgrowths cultured control
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30 14 (RDH) versus 2i-supplemented medium. **C** shows colonies with a high OCT4 expression that are also
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32 15 dome-shaped with cells displaying high nucleus-to-cytoplasm ratio. **D** and **E** show colonies of compact
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34 16 dome-shaped cells that were GATA6-negative, surrounded by GATA6-positive cells, in RDH medium.
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36 17 In 2i, dome-shaped colonies with bright OCT4 signal and GATA6-negative were not observed. The
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38 18 corresponding 3D reconstructions assembled from the individual confocal image slices are displayed as
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40 19 **C4** (OCT4), **D4**, **E4**, **F4** (GATA6) in embryos cultured in RDH medium, and **G4** (OCT4) and **H4**
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42 20 (GATA6) in embryos cultured in 2i medium. (Scale bars: 100 μ m).
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52 22 **Table 1. Relative volume (%) of organelles in the cells of ICM of 6-day rabbit embryos cultured**
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54 23 **in inhibitor supplemented media.** The individual treatments were the following: Dual inhibition (2i)
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56 24 preserved the integrity of mitochondria, endoplasmic reticulum and Golgi complex, but only the
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58 25 volume of residual bodies significantly increased. The integrity of organelles was impaired in 3i and 4i,
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which proved the viability data. ^avs. ^b significant difference at $p < 0.05$, ^avs. ^c

Supplementary Material

Movie 1. Time-lapse movies of rabbit embryos developing in control RDH and 2i supplemented RDH medium. We subsequently tracked the embryonic development in the control culture condition (RDH) against 2i-supplemented culture medium using time-lapse video technology to measure the time between the different embryonic developmental stages, from 2-cell stage up to early blastocyst stage (75 hours) and from the morula up to the blastocyst stage over the span of 48 hours using Primo Vision time-lapse embryo monitoring system kindly provided by C. Pribenszky.

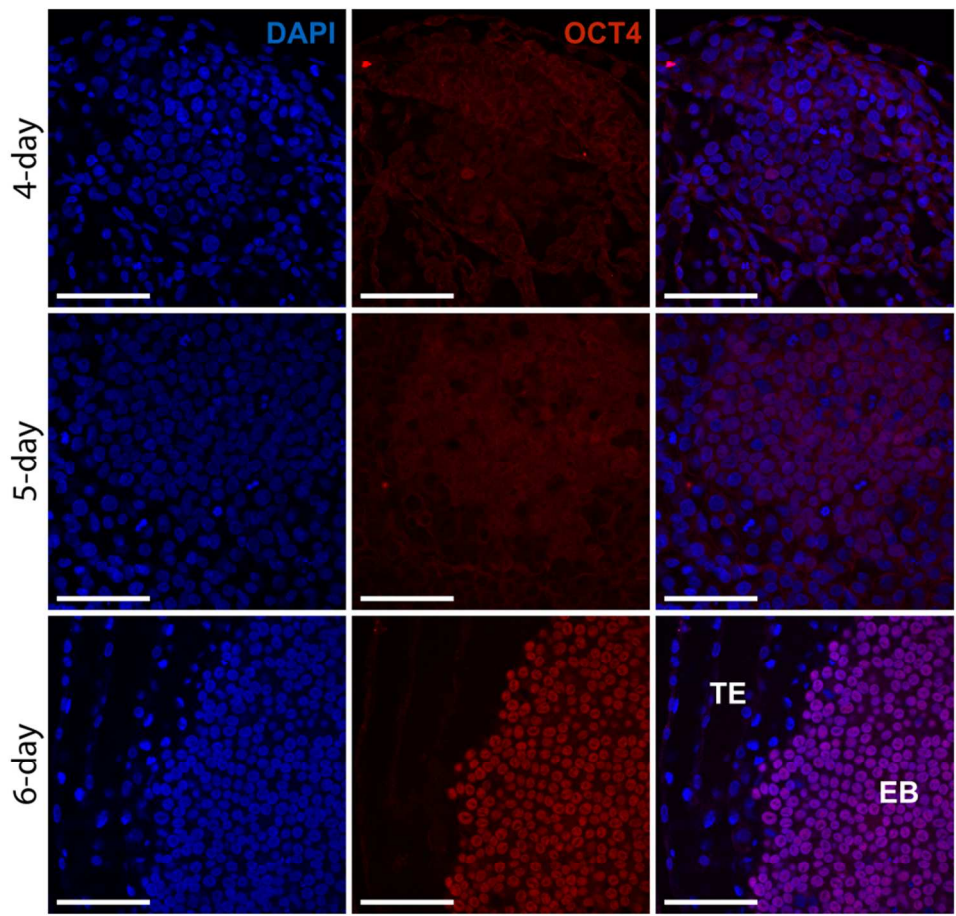


Figure 1A
Baseline expression of OCT4 in rabbit embryos after their recovery from the oviducts on days 4, 5 and 6 post coitum. OCT4 expression was fully developed only on day 6.
(Scale bars: 200µm, 100 µm).
361x361mm (72 x 72 DPI)

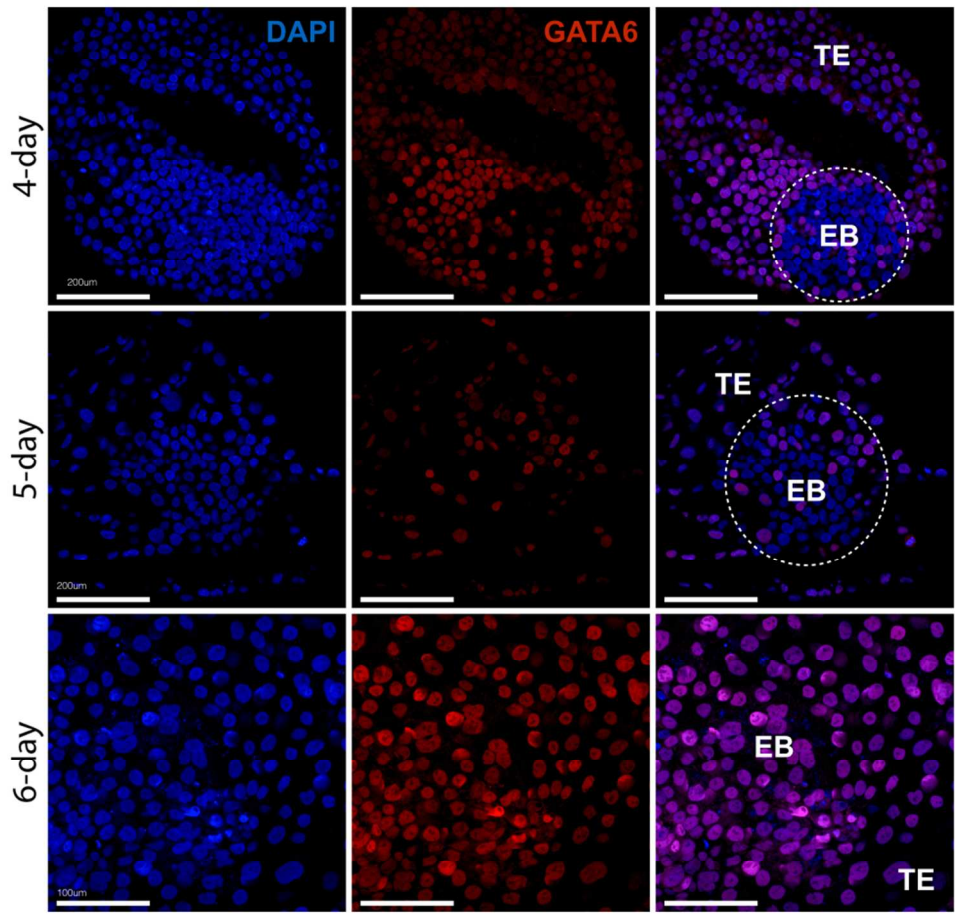


Figure 1B

Baseline expression of GATA6 in embryos after their recovery from the oviducts on days 4, 5 and 6 post coitum. GATA6 expression was limited to non-ICM cells in 4-day and 5-day embryos. In 6-day embryos, GATA6 expression was observed both in ICM and non-ICM cells at varying intensities.

(Scale bars: 200µm, 100 µm).
361x361mm (72 x 72 DPI)

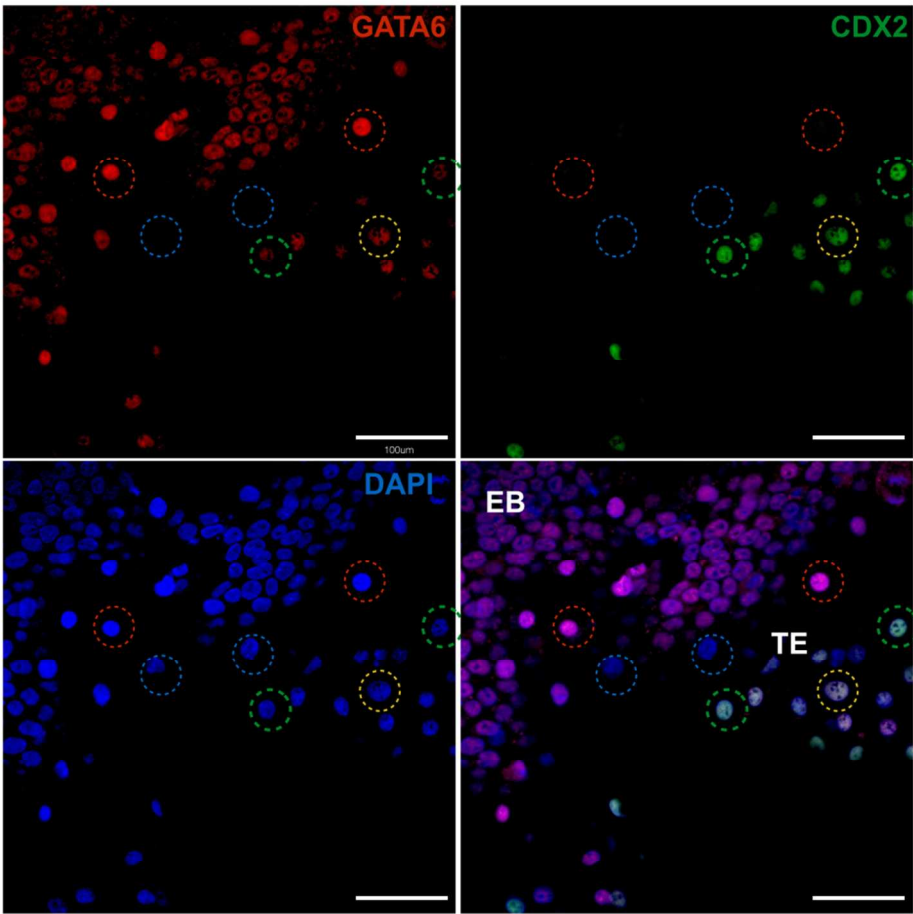


Figure 1C1
GATA6 and CDX2 co-expression in 6-day embryos. Most cells of the trophoblast (top row) are CDX2-positive (green circle). CDX2/GATA6 double-positive cells (yellow circles) and CDX2/GATA6 double-negative cells (blue circles) were also detected. The embryoblast (bottom row) comprised GATA6 positive cells (red circles) and GATA6 negative cells (blue circles) trophoblast (TE); embryoblast (EB). Scale bars: 100µm
361x361mm (72 x 72 DPI)

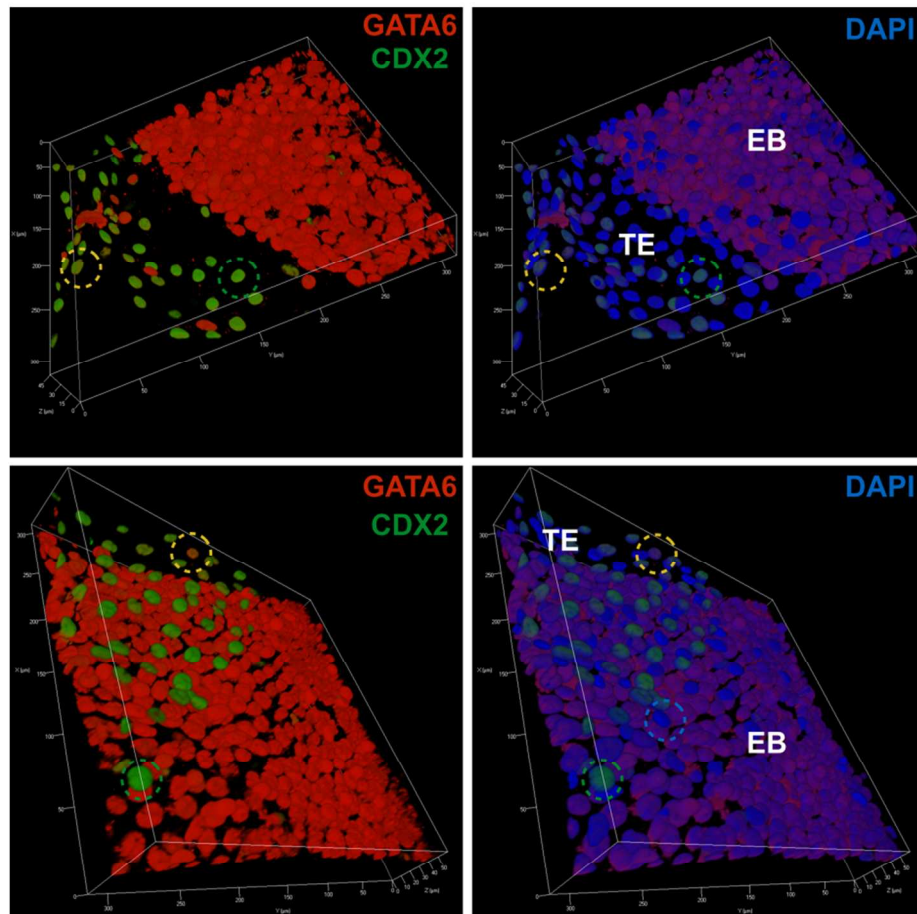


Figure 1C2

C2 is a confocal 3D reconstructions of C1 images showing the interface of trophoblast (TE) and embryoblast (EB). CDX2-positive (green circle), CDX2/GATA6 double-positive cells (yellow circles) and CDX2/GATA6 double-negative cells (blue circles) were also detected; the embryoblast (bottom row) was comprised GATA6 positive cells (red circles) and GATA6 negative cells (blue circles)(Scale bars: 100 μ m). 361x361mm (72 x 72 DPI)

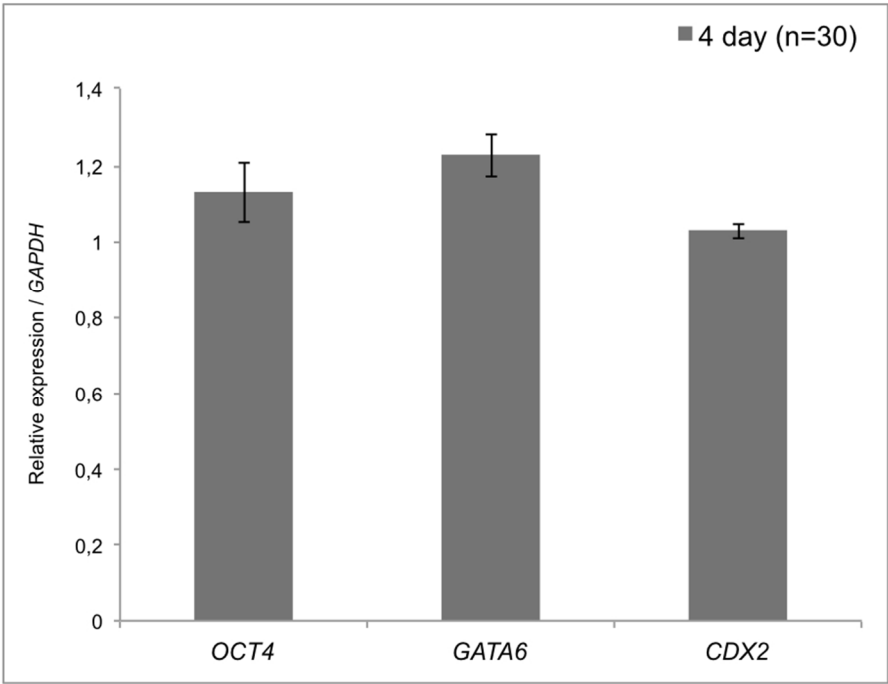


Figure 1D
Quantitative expression of OCT4, GATA6 and CDX2 in 4-day rabbit embryos. High level of expression of OCT4, GATA6 and CDX2 transcripts was detected by day 4 of embryonic development. Expression levels were calculated relative to GAPDH transcript level and normalized to the 7-days mRNA level. Error bars indicate SD (***p* ≤ 0.001).
361x270mm (72 x 72 DPI)

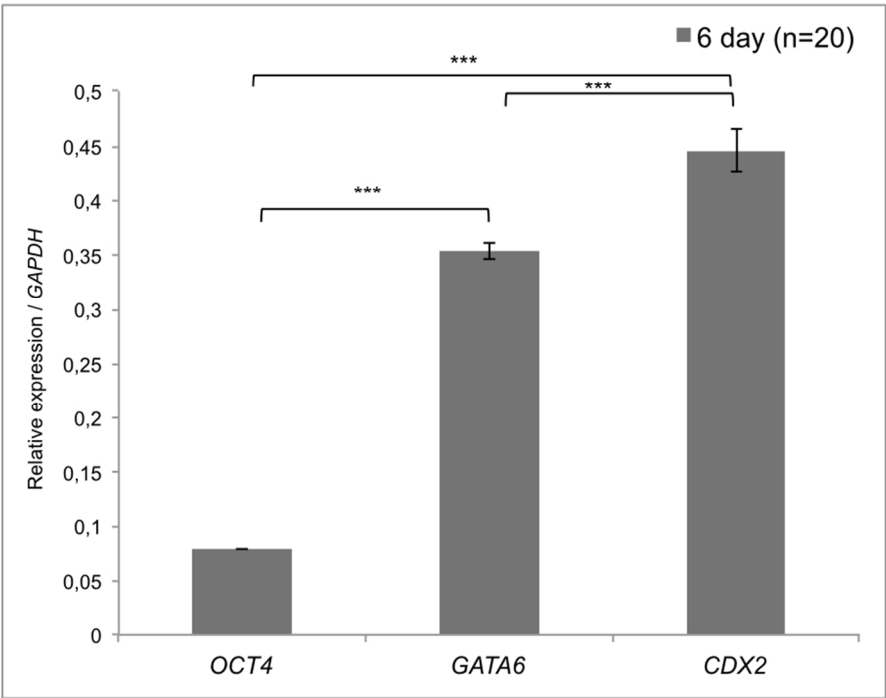


Figure 1E
Quantitative expression of OCT4, GATA6 and CDX2 in 6-day rabbit embryos. OCT4 transcript was down-regulated at 6-day embryos. Expression levels were calculated relative to GAPDH transcript level and normalized to the 7-days mRNA level. Error bars indicate SD (***) $p \leq 0.001$.
361x270mm (72 x 72 DPI)

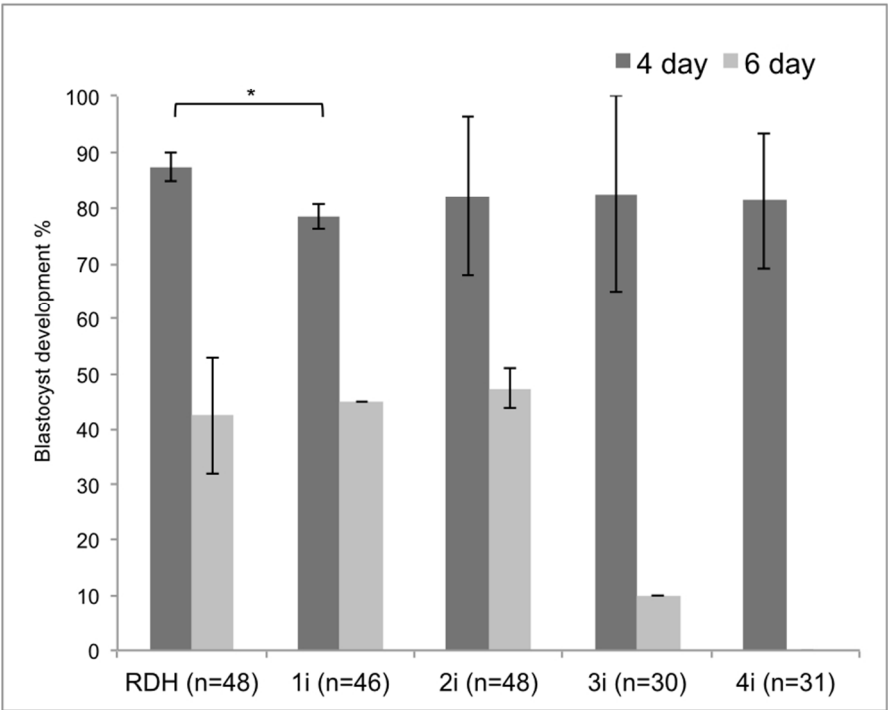


Figure 2A
The percentage of good quality embryos (devoid of zona pellucida) cultured in vitro from the 8-cell stage and compared to the control RDH-cultured embryos (dark-grey lines). The percentage of the same embryos, after continued culture, that successfully developed to the expanded blastocyst stage on the day 6 post coitum (light-grey lines). 3i and 4i treatments resulted in death of the embryos. Error bars indicate SD (*p ≤ 0.05).
361x270mm (72 x 72 DPI)

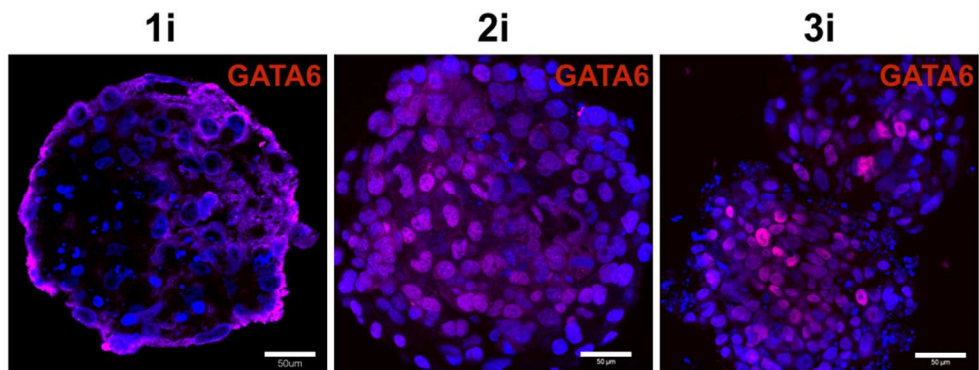


Figure 2B
Immunostaining of 6 day blastocysts cultured under different condition (Scale bars: 50µm).
361x137mm (72 x 72 DPI)

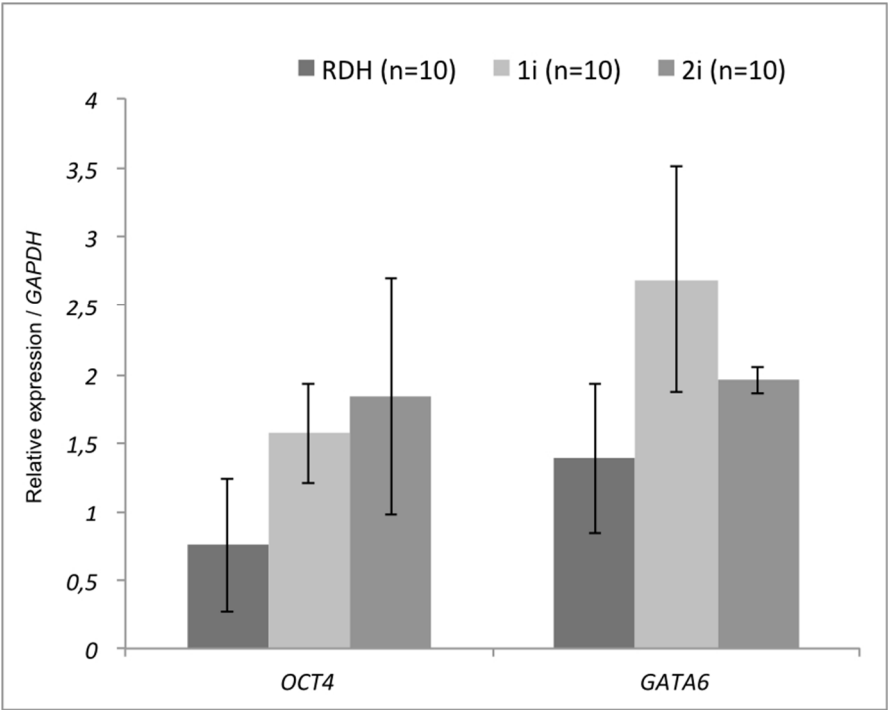


Figure 2C
Quantitative real-time PCR analysis showing the expression of OCT4 and GATA6 in 6-day embryos cultured from the 8-cell stage in 1i, 2i and the control RDH medium. The highest expression of OCT4 transcript was detected in 2i condition, while the expression of GATA6 transcript was slightly higher in 1i condition.
361x270mm (72 x 72 DPI)

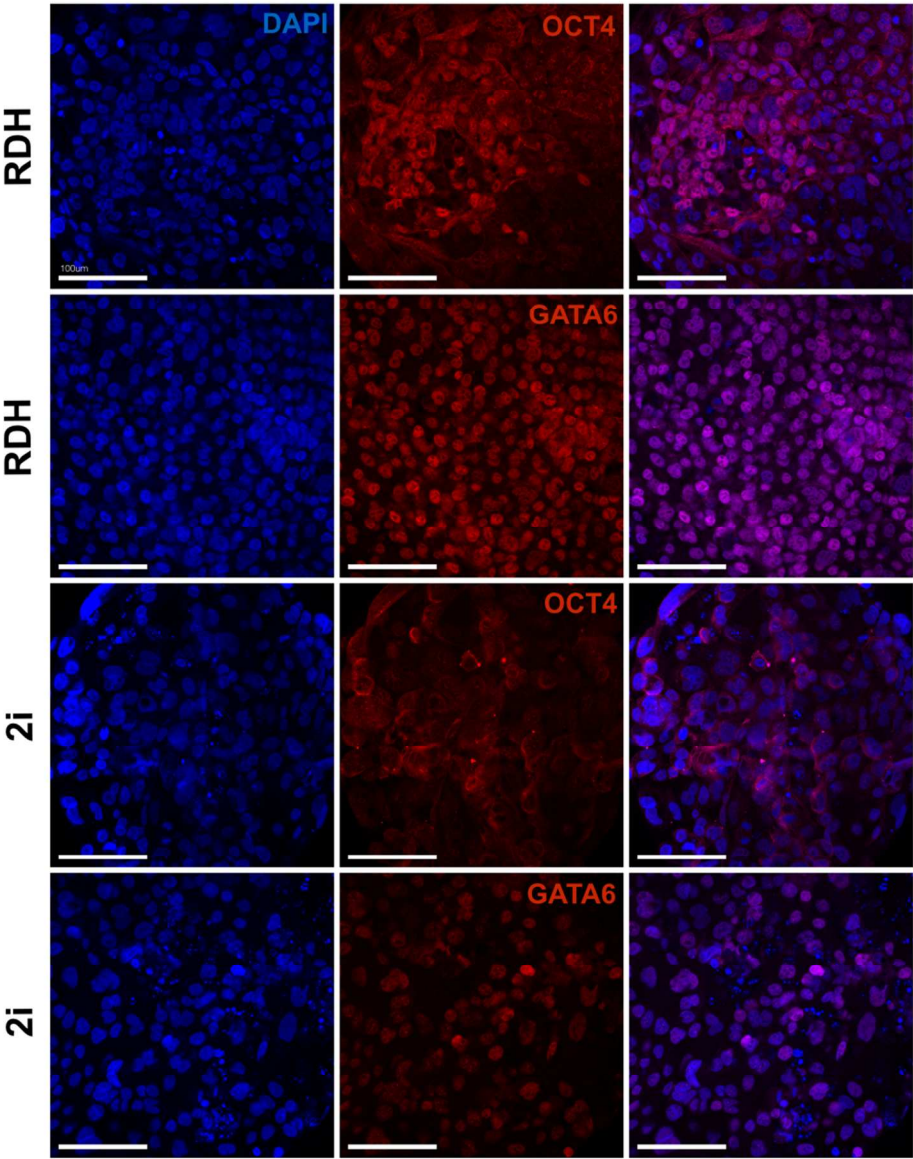


Figure 2D
Immunohistochemical analysis of OCT4 and GATA6 expression in 6-day embryos cultured from the 8-cell stage in control RDH medium (first two rows and 2i last two rows)(Scale bars: 100µm).
361x458mm (72 x 72 DPI)

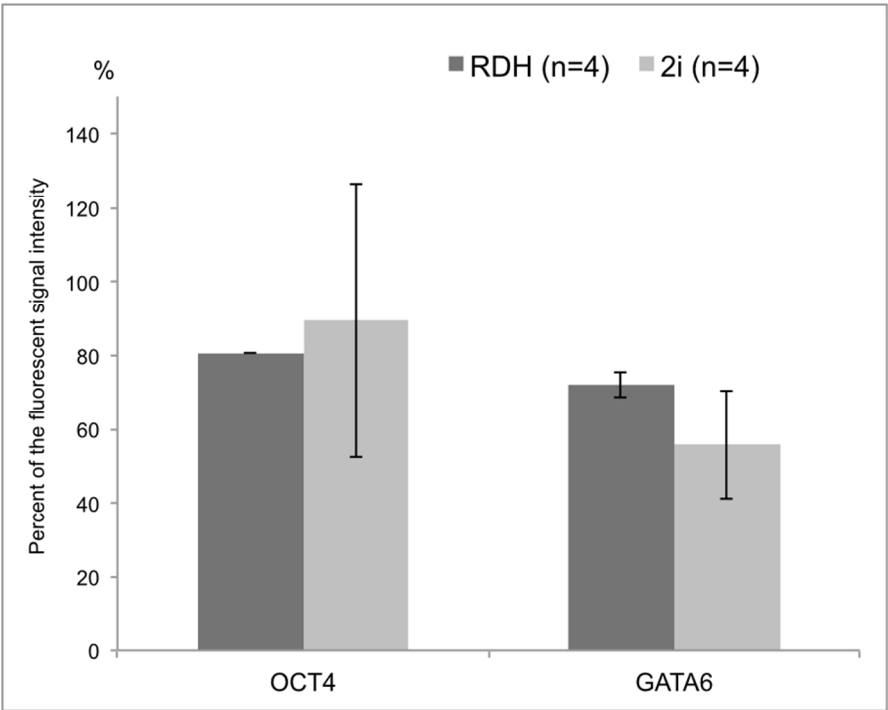


Figure 2E
Quantitation of the fluorescent signal intensities pertaining to fluorescently labelled antibodies specific to OCT4 and GATA6 calculated as a percentage of fluorescent signals together with DAPI signal out of total DAPI signal. The percentage represents a mean value calculated from the individual confocal image slices of 6-day embryos.
361x270mm (72 x 72 DPI)

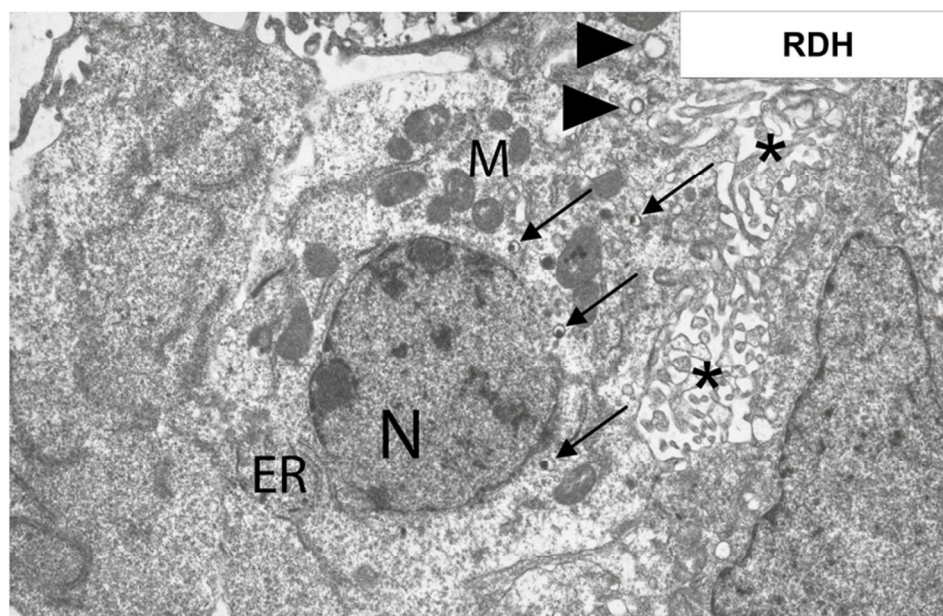


Figure 2F

Transmission electron micrographs of the ICM of the 6-day embryos cultured in the control RDH condition. The electromicrograph shows three cells of the inner cell mass from the control group of embryos in RDH.

One of them was localized in the centre of the image with oval nucleus (N) and nucleoli localized in the centre of cytoplasm. Intercellular spaces are partially extended (*). Individual structures: Mitochondria (M) (dark and oval), granular endoplasmic reticulum (ER), small residual bodies (→) and small vacuoles (►) around the ESC. Magnification: 7200X.

317x211mm (72 x 72 DPI)

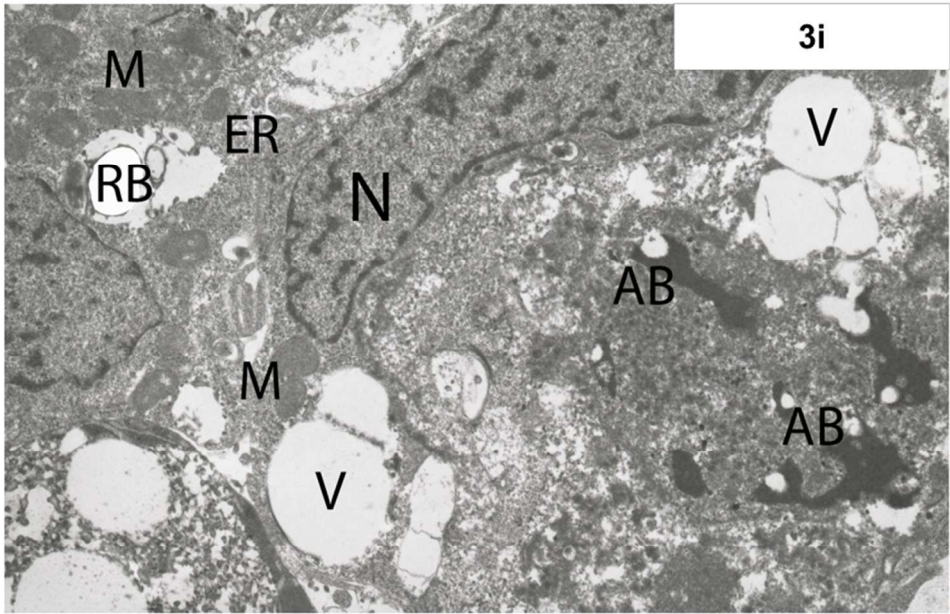


Figure 2G
Transmission electron micrographs of the ICM of the 6-day embryos cultured in 3i condition. The electromicrograph of two cells of the inner cell mass of rabbit embryos treated with 3i shows numerous large oval vacuoles (V), residual bodies (RB) and apoptotic bodies (AB) located in the vicinity of an elongated nucleus (N) of heterochromatic type. The presence of mitochondria (M) and granular endoplasmic reticulum (ER) is very rare. Individual structures: Mitochondria (M) (dark and oval), granular endoplasmic reticulum (ER), small residual bodies (→) and small vacuoles (▶) around the ESC. Magnification: 7200X.
317x211mm (72 x 72 DPI)

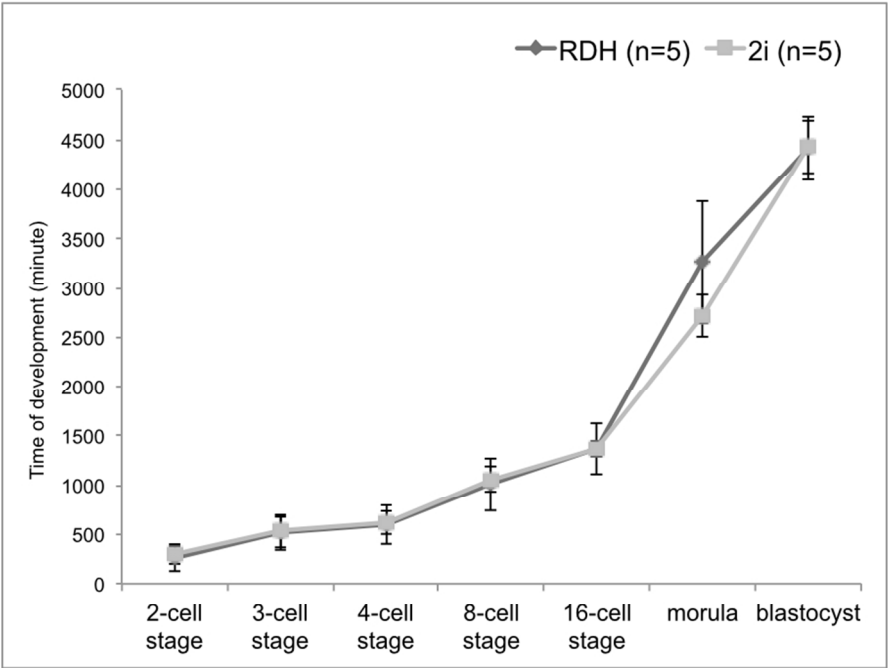


Figure 3A
Tracking of the embryonic development in the control culture condition (RDH) against 2i-supplemented culture medium using time lapse video technology, measuring the time between the different embryonic developmental stages, from 2-cell stage up to early blastocyst stage over the span of 75 hours.
361x270mm (72 x 72 DPI)

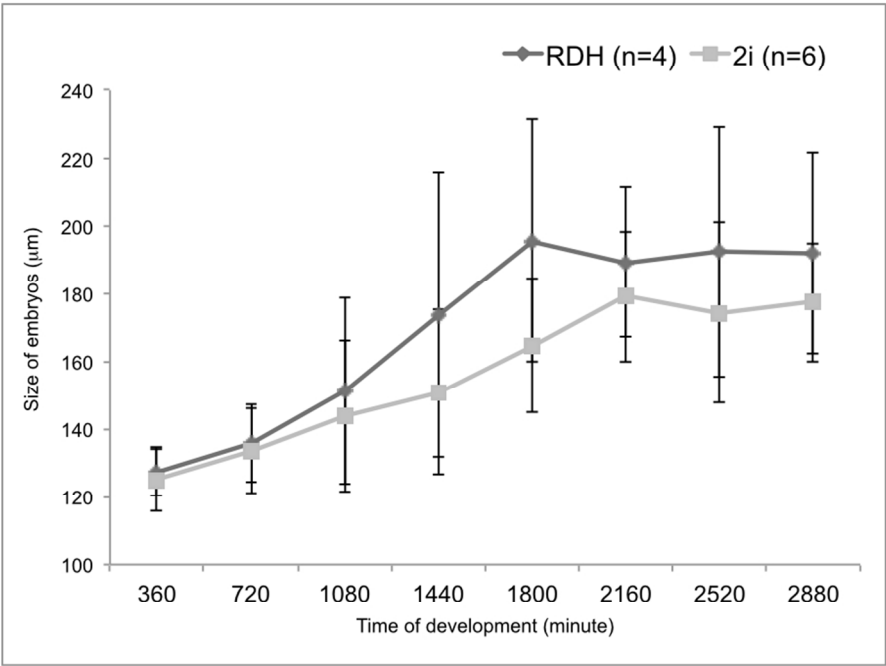


Figure 3B

Tracking of the embryonic development in the control culture condition (RDH) against 2i-supplemented culture medium using time lapse video technology, measuring the time between the different embryonic developmental stages, from morula up to the blastocyst stage over the span of 48 hours. Embryos cultured in 2i condition displayed slightly faster development and smaller size compared to the control embryos in RDH.

361x270mm (72 x 72 DPI)

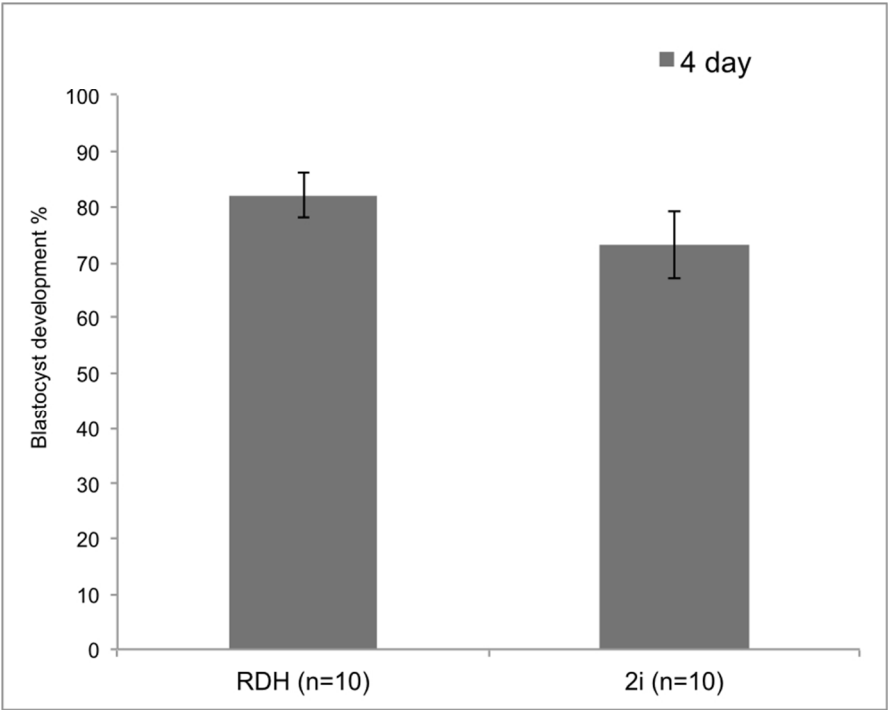


Figure 4A
Rabbit embryos were cultured from 8-cell stage to early blastocyst stage in 2i and control medium. No significant difference in the developmental rate was observed.
361x270mm (72 x 72 DPI)

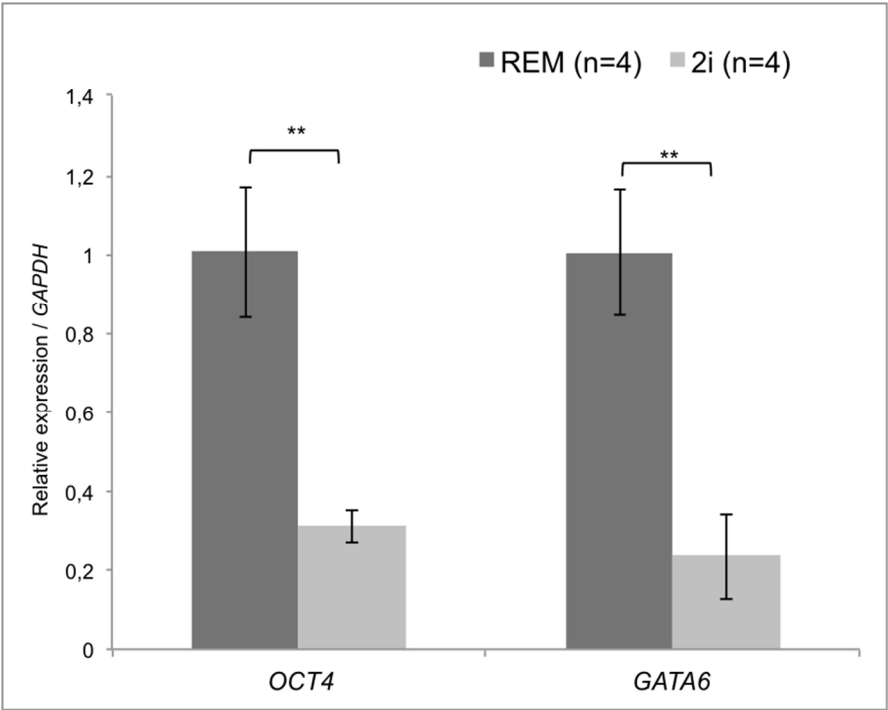


Figure 4B
Quantitative real-time PCR analysis of OCT4 and GATA6 expression in attached ICM outgrowths displayed significant depletion of both OCT4 and GATA6 transcription in 2i supplemented medium compared to control rabbit ES cells culture medium (REM). Expression levels were calculated relative to GAPDH level and normalized to the 7-days mRNA level. (Error bars indicate SD **p ≤ 0.01).
361x270mm (72 x 72 DPI)

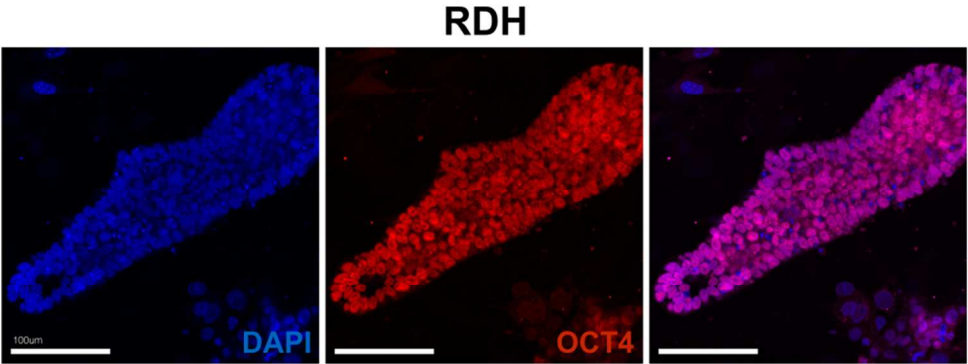


Figure 4C1C2C3
Expression of OCT4 in attached ICM outgrowths cultured in control RDH medium. We found colonies with high OCT4 expression. The cells in dome-shaped colonies displayed high nucleus-to-cytoplasm ratio. (Scale bars: 100µm).
361x137mm (72 x 72 DPI)

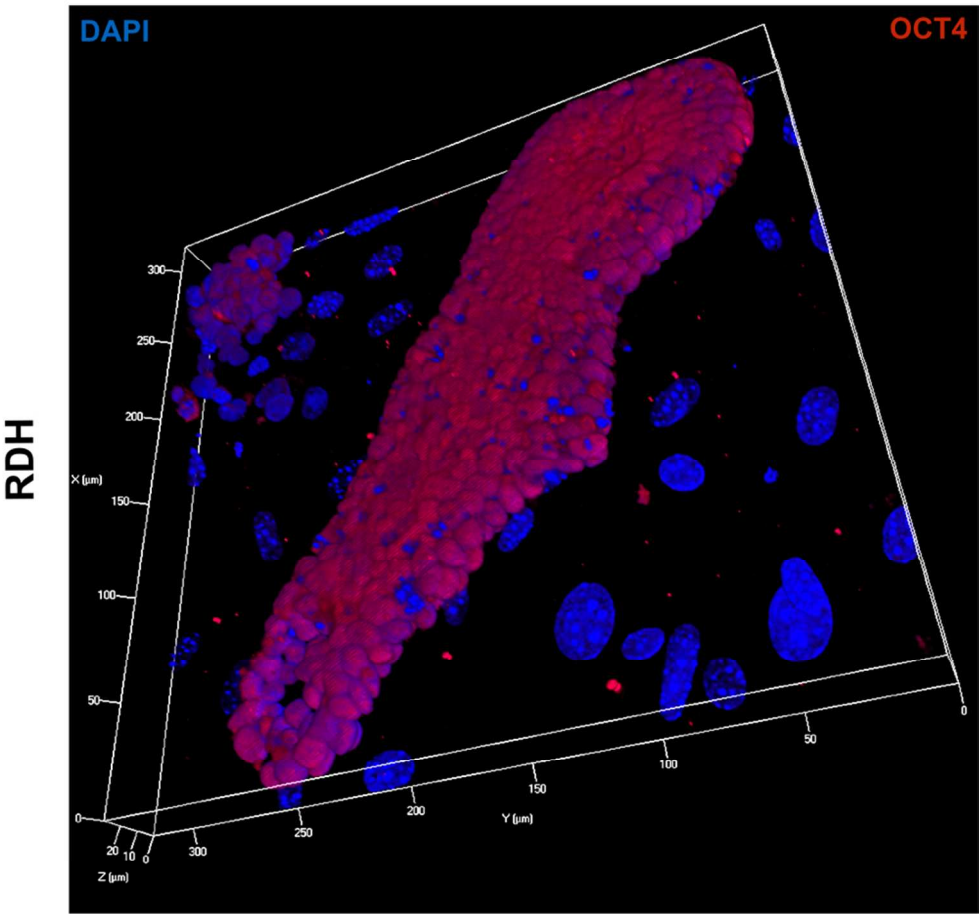


Figure 4C4
The corresponding 3D reconstructions assembled from the individual confocal image slices (C1C2C3): Colonies with a high OCT4 expression that are also dome-shaped with cells displaying high nucleus-to-cytoplasm ratio.
361x361mm (72 x 72 DPI)

RDH

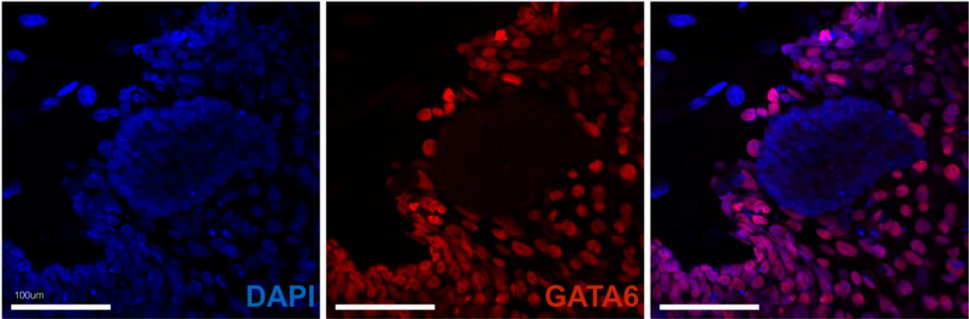


Figure 4D1D2D3

This figure shows the colonies of compact dome-shaped cells in RDH medium, that were GATA6-negative, surrounded by GATA6-positive cells. (Scale bars: 100µm)

361x137mm (72 x 72 DPI)

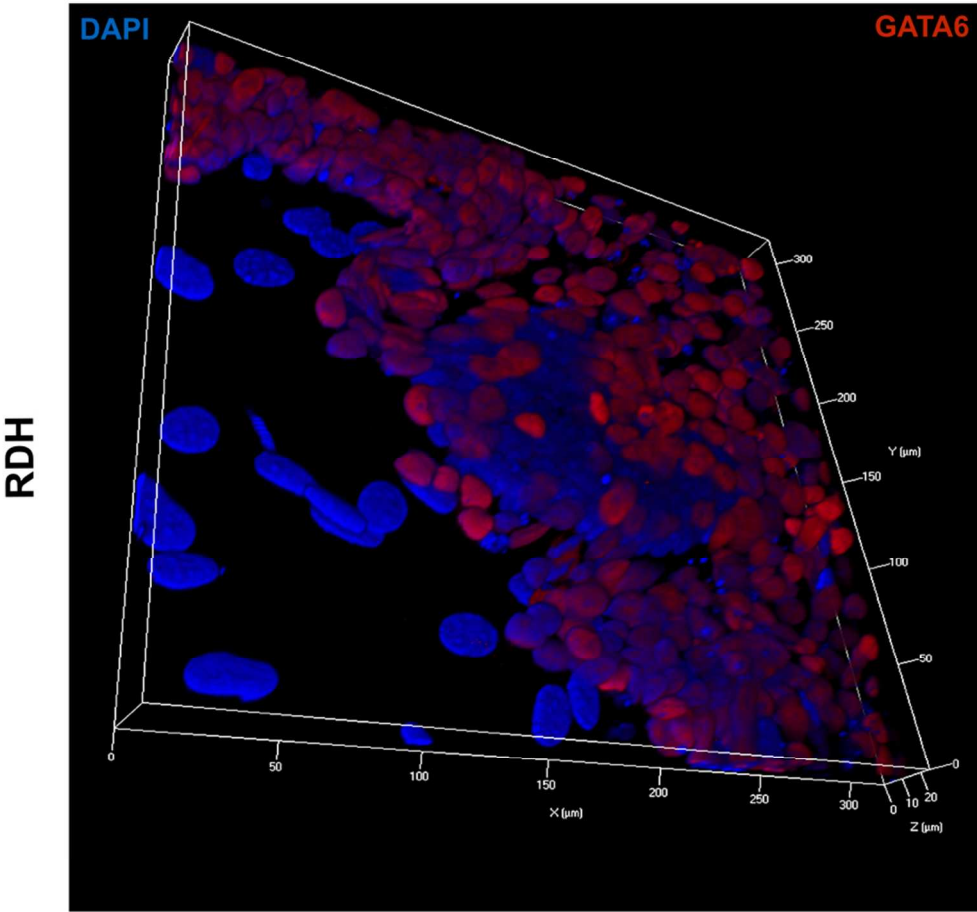


Figure 4D4
The corresponding 3D reconstructions assembled from the individual confocal image slices (D1D2D3): colonies of compact dome-shaped cells in RDH medium, that were GATA6-negative, surrounded by GATA6-positive cells.
361x361mm (72 x 72 DPI)

RDH

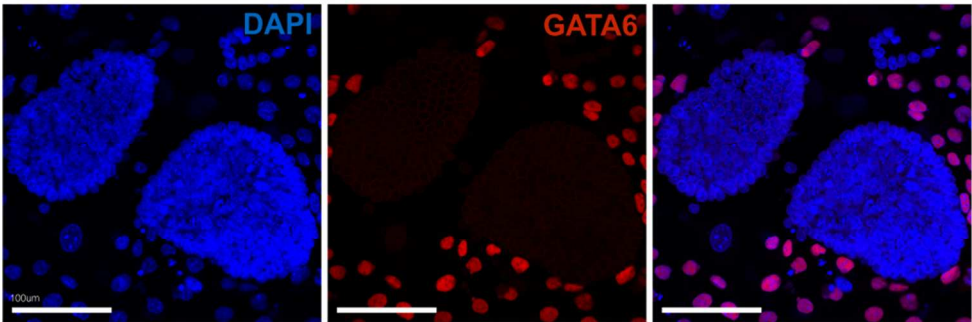


Figure 4E1E2E3
GATA6 expression in attached ICM outgrowths cultured in RDH medium (Scale bars: 100µm)
361x137mm (72 x 72 DPI)

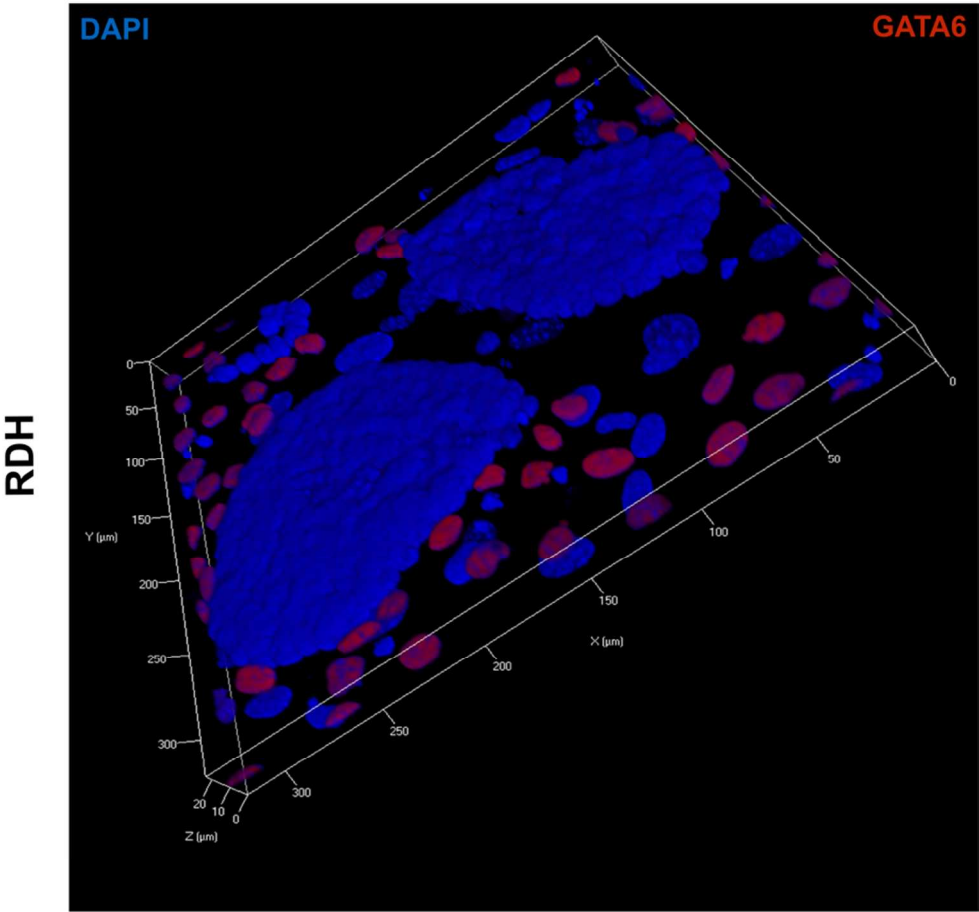


Figure 4E4
The corresponding 3D reconstructions assembled from the individual confocal image slices (E1E2E3): the colonies display GATA6 negative colonies in RDH medium.
361x361mm (72 x 72 DPI)

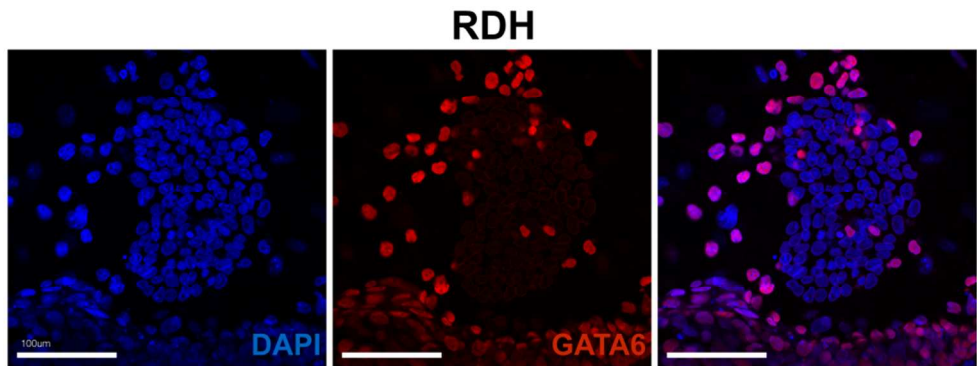


Figure 4F1F2F3
The picture show colonies of compact dome-shaped cells that were GATA6-negative, surrounded by GATA6-positive cells in RDH medium. (Scale bars: 100µm).
361x137mm (72 x 72 DPI)

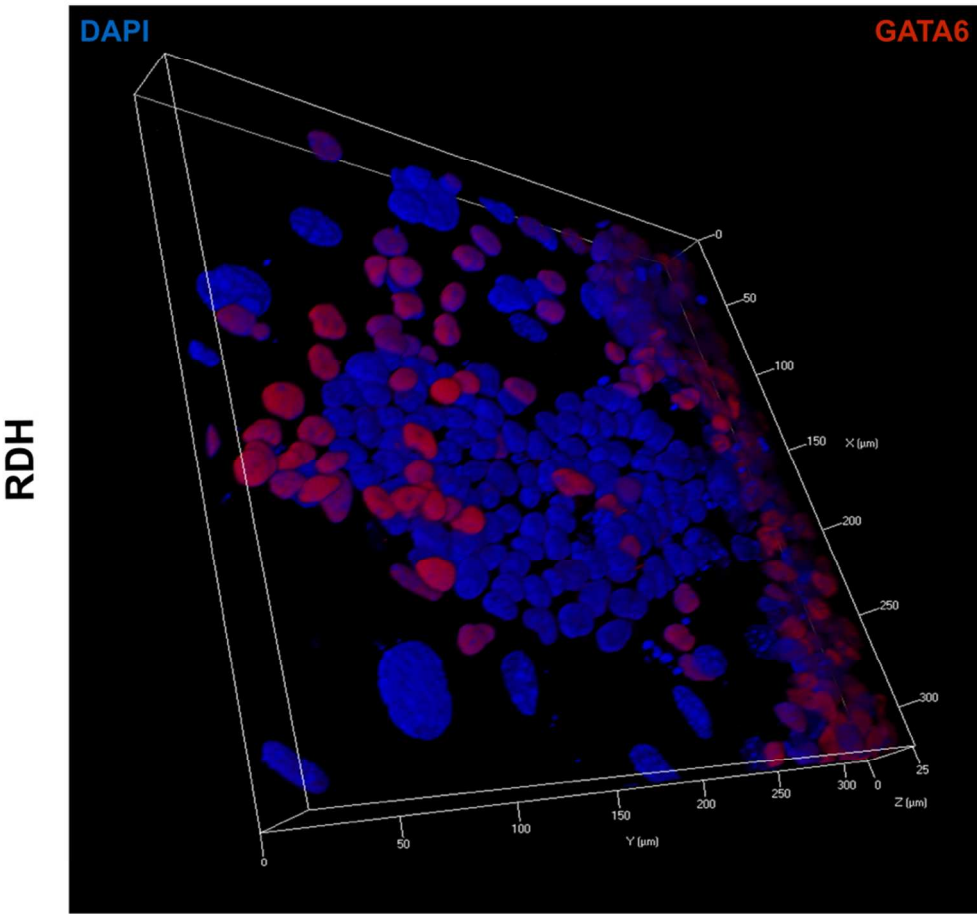


Figure 4F4
The corresponding 3D reconstructions assembled from the individual confocal image slices (F1F2F3): the colonies display GATA6 negative colonies in RDH medium.
361x361mm (72 x 72 DPI)

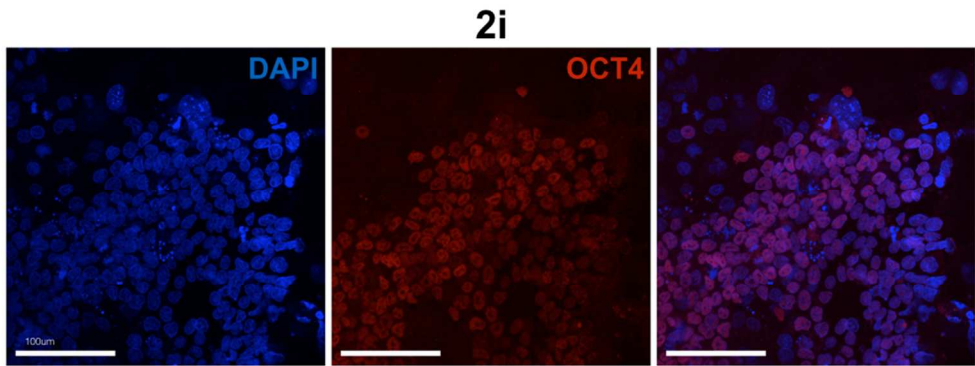


Figure 4G1G2G3
Expression of OCT4 in attached ICM outgrowths cultured in 2i-supplemented medium. (Scale bars: 100μm).
361x137mm (72 x 72 DPI)

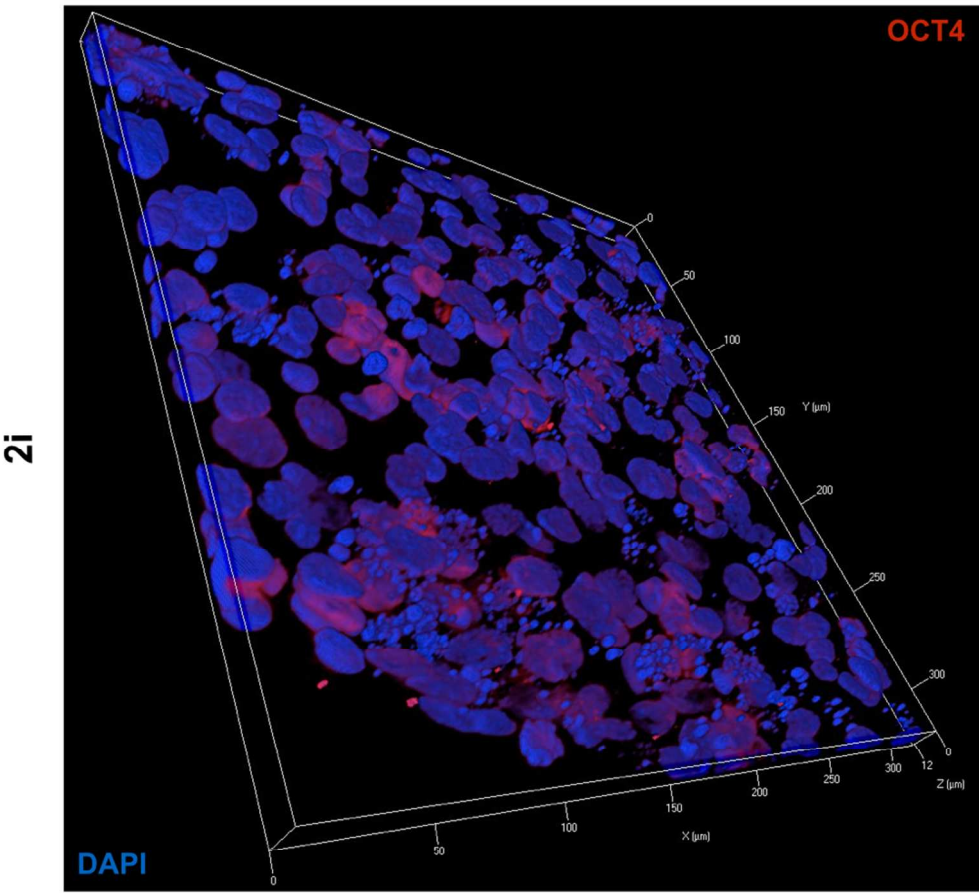


Figure 4G4
The corresponding 3D reconstructions assembled from the individual confocal image slices (G1G2G3): OCT4 expression in attached ICM outgrowths cultured in 2i-supplemented medium.
361x361mm (72 x 72 DPI)

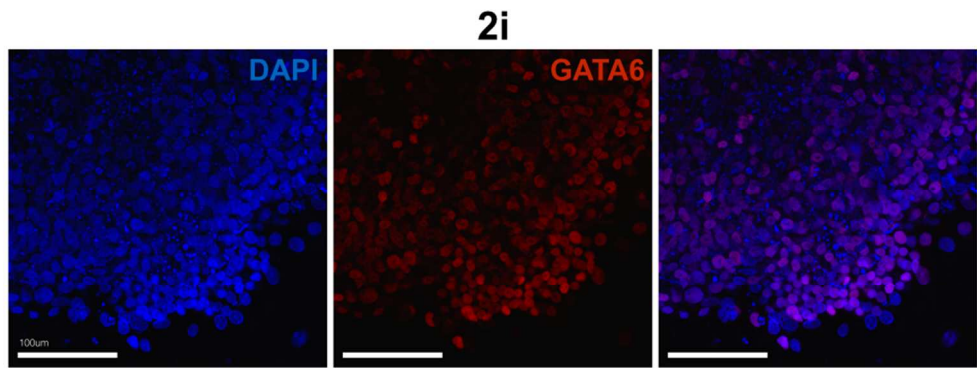


Figure 4H1H2H3
Expression of GATA6 in attached ICM outgrowths cultured in 2i-supplemented medium. The outhgrowth colonies contained GATA6expressing cells. (Scale bars: 100µm).
361x137mm (72 x 72 DPI)

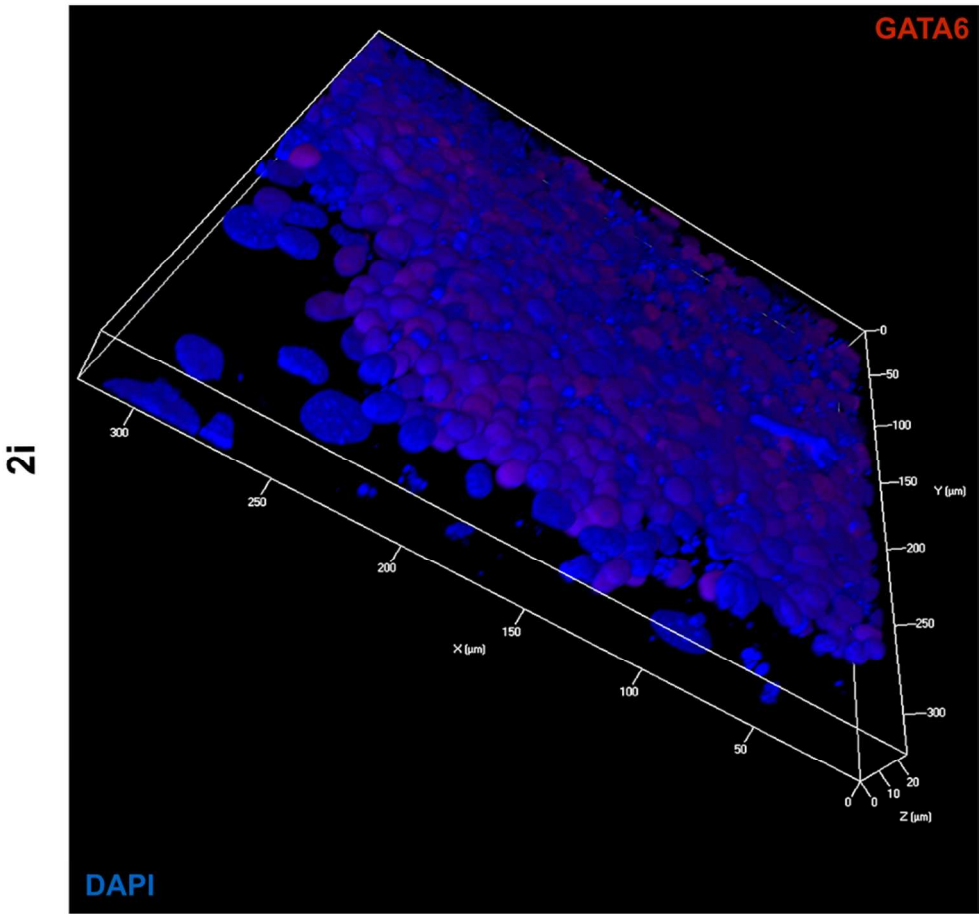


Figure 4H4
The corresponding 3D reconstructions assembled from the individual confocal image slices
(H1H2H3) GATA6 expression in ICM outgrowth cultured in 2i medium.
361x361mm (72 x 72 DPI)

Table 1

	RDH (n=10)	2i (n=10)	3i (n=11)	4i (n=10)
Mitochondria (M)	7,50 ^a	6,67	5,15 ^b	1,93 ^b
Endoplasmic Reticulum (ER)	9,67 ^a	6,87	4,73 ^b	4,53 ^b
Golgi Complex (GC)	3,40 ^a	2,87	2,78	1,73 ^b
Vacuoles (V)	4,87 ^a	6,2	6,18	7,20 ^b
Residual Bodies (RB)	5,13 ^a	9,73 ^b	14,12 ^c	16,73 ^c
Apoptotic Bodies (AB)	0,40 ^a	0,4	2,20 ^b	0,6

Table 1. Relative volume (%) of organelles in the cells of ICM of 6-day rabbit embryos cultured in inhibitor supplemented media. The individual treatments were the following: Dual inhibition (2i) preserved the integrity of mitochondria, endoplasmic reticulum and Golgi complex, but only the volume of residual bodies significantly increased. The integrity of organelles was impaired in 3i and 4i, which proved the viability data. avs.b significant difference at $p < 0.05$, avs.c 361x270mm (72 x 72 DPI)