

Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo

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Embryonic stem (ES) cells can be derived and propagated from multiple strains of mouse and rat through application of small-molecule inhibitors of the fibroblast growth factor (FGF)/Erk pathway and of glycogen synthase kinase 3. These conditions shield pluripotent cells from differentiation-inducing stimuli. We investigate the effect of these inhibitors on the development of pluripotent epiblast in intact pre-implantation embryos. We find that blockade of Erk signalling from the 8-cell stage does not impede blastocyst formation but suppresses development of the hypoblast. The size of the inner cell mass (ICM) compartment is not reduced, however. Throughout the ICM, the epiblast-specific marker Nanog is expressed, and in XX embryos epigenetic silencing of the paternal X chromosome is erased. Epiblast identity and pluripotency were confirmed by contribution to chimaeras with germline transmission. These observations indicate that segregation of hypoblast from the bipotent ICM is dependent on FGF/Erk signalling and that in the absence of this signal, the entire ICM can acquire pluripotency. Furthermore, the epiblast does not require paracrine support from the hypoblast. Thus, naïve epiblast and ES cells are in a similar ground state, with an autonomous capacity for survival and replication, and high vulnerability to Erk signalling. We probed directly the relationship between naïve epiblast and ES cells. Dissociated ICM cells from freshly harvested late blastocysts gave rise to up to 12 ES cell clones per embryo when plated in the presence of inhibitors. We propose that ES cells are not a tissue culture creation, but are essentially identical to pre-implantation epiblast cells.

KEY WORDS: Blastocyst, Epiblast, Hypoblast, Pluripotency, Erk, Mouse

INTRODUCTION

During early murine development, successive differentiation events lead to the segregation of firstly the trophectoderm, which forms an outer epithelial layer, and secondly the hypoblast on the blastocoelic surface of the inner cell mass (ICM). The remaining internal cells form the epiblast from which the foetus will arise. Subsequent to implantation in the uterus, the epiblast forms an epithelial structure that undergoes rapid cell division. Embryonic stem (ES) cells can be derived from the preimplantation epiblast (Batlle-Morera et al., 2008; Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981), but have not been derived from the post-implantation epiblast (Brons et al., 2007; Tesar et al., 2007). The relationship between pluripotent cells that exist transiently in the mammalian embryo and immortal ES cells in culture has not been fully defined (Gardner and Brook, 1997; Smith, 2001). On the one hand, ES cells have only been authenticated for a few mouse strains. This has been used to argue that genetic and/or epigenetic factors determine their derivation, and that they may be considered an artefact of cell culture (Buehr and Smith, 2003). On the other hand, the ability of ES cells to re-enter embryonic development and contribute extensively to chimaeras indicates that any adaptations to culture are fully and rapidly reversible (Bradley et al., 1984).

The dilemma surrounding definition of the status of ES cells has remained unresolved largely because the process of ES cell derivation from embryos has historically been inefficient and reliant on poorly defined and variable culture conditions and protocols. However, an understanding of the requirements for propagating ES

cells has evolved progressively since the initial reports in which a feeder layer of mitotically inactivated fibroblasts was used in combination with culture medium containing foetal calf serum (Evans and Kaufman, 1981; Martin, 1981). The feeder layer was then substituted with the cytokine leukaemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988) and, subsequently, serum was replaced with bone morphogenetic protein 4 (Ying et al., 2003). Most recently, defined conditions free from feeder cells, serum and cytokines were established based on combinations of small-molecule inhibitors of the fibroblast growth factor (FGF)/mitogen-activated protein kinase (Mek)/extracellular signal-related kinase (Erk1/2; Mapk3/1 – Mouse Genome Informatics) pathway and of glycogen synthase kinase 3 (Gsk3) (Ying et al., 2008). These defined conditions, known as 3i or 2i, have been applied to the derivation of ES cells and have enabled establishment of authentic ES cells from hitherto recalcitrant strains, such as non-obese diabetic (NOD) mice (Nichols et al., 2009). Furthermore, they have been applied to rat embryos, resulting in the production of the first germline-competent rat ES cells (Buehr et al., 2008; Li et al., 2008). The use of 3i/2i for efficient derivation of ES cells provoked the hypothesis that immortalising the pluripotent cells from developing embryos does not depend upon adaptation and selection in culture, but rather that the epiblast might be in a ground state that can be maintained by blocking inductive differentiation pathways. Thus, we speculated that cells with properties identical to ES cells exist, at least transiently, in the epiblast of the preimplantation embryo.

The roles of the extra-embryonic lineages, trophectoderm and hypoblast, in the development of a functional epiblast have not been described. Specification and maintenance of the trophectoderm in murine embryos are dependent upon activation of a sequence of transcription factors, including Tead4, Cdx2 and eomesodermin (Eomes) (Nishioka et al., 2009; Strumpf et al., 2005; Voss et al., 2000; Yagi et al., 2007). FGF/Erk signalling has been implicated in the differentiation of trophoblast (Lu et al., 2008) and in the proliferation of diploid trophoblast (Arman et al., 1998; Nichols et

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al., 1998; Tanaka et al., 1998). There have been reports implicating Erk signalling in the formation and propagation of the hypoblast. Embryos lacking Grb2, a key adaptor of the receptor tyrosine kinase signalling pathway, are defective in the formation of the hypoblast (Cheng et al., 1998). In the early embryo, Fgf4 is a potent activator of Erk signalling, and has been suggested to play a role in the maintenance of hypoblast (Silva, J. M. et al., 2008). Furthermore, addition of Fgf4 to isolated ICMs in culture promotes outgrowth of parietal endoderm, a derivative of the hypoblast (Rappolee et al., 1994). Reducing Erk activity has been shown to enhance the efficiency of ES cell derivation by promoting retention of Oct4 (Pou5f1)-positive epiblast during the outgrowth phase (Buehr and Smith, 2003). Also, the differentiation of established ES cells and their requirement for LIF can be diminished by addition of synthetic inhibitors of Erk signalling (Burdon et al., 1999).

Here, we examine the relationship and identity between pluripotent naïve epiblast and cultured ES cells. We investigate the effect of 3i/2i and the component inhibitors on the development of the embryonic and extra-embryonic lineages in preimplantation mouse embryos.

MATERIALS AND METHODS

Mice and embryos

The strains of mice used in this study were 129/Sv, C57BL/6/Ola, CBA/Ca, and F1 hybrids between C57BL/6/Ola and CBA/Ca. They were maintained by in-house breeding on a lighting regime of 14 hours light and 10 hours darkness with food and water supplied ad libitum. Prior to caging with stud males, females were selected for oestrous by visual inspection of the vagina (Champlin et al., 1973). Detection of a copulation plug the following morning was used to confirm successful mating; the resulting embryos were then considered to be 0.5 days post-coitum (dpc). For all experiments, the embryos from at least two females were pooled and randomly assigned to experimental groups.

Embryo culture

Zygotes in cumulus masses were dissected from oviduct ampullae at 0.5 dpc and placed in hyaluronidase (300 µg/ml, Sigma, Gillingham, UK) in M2 medium (Quinn et al., 1982) (made in house), to disperse the cumulus cells before rinsing in M2 medium. Embryos at the 8-cell stage were flushed from oviducts at 2.5 dpc; blastocysts were flushed from uteri at 3.5 or 3.75 dpc using M2. Embryos from the 1-cell stage to blastocyst were cultured in KSOM (MR-020P-5D, Millipore, Watford, UK). From the expanded blastocyst stage, embryos were cultured in N2B27 medium (Nichols and Ying, 2006; Ying and Smith, 2003). Where appropriate, inhibitors were added at the following concentrations and in the combinations specified in the text: PD184352, 0.8 µM; PD0325901, 1 µM; Chir99021, 3 µM (all synthesized in the Division of Signal Transduction Therapy, University of Dundee, Dundee, UK); SU5402 (Calbiochem, San Diego, CA, USA), 2 µM; PD173074 (Sigma), 100 nM.

Immunostaining

Embryos were fixed in 4% paraformaldehyde in PBS for 15 minutes, then rinsed in PBS containing 3 mg/ml polyvinylpyrrolidone (PBS/PVP; P0930, Sigma), permeabilised in PBS/PVP containing 0.25% Triton X-100 (23,472-9, Sigma) for 30 minutes and blocked in blocking buffer, which comprised PBS containing 0.1% BSA, 0.01% Tween 20 (P1379, Sigma) and 2% donkey serum. Primary antibodies were Oct4 (C-10 sc-5279, Santa Cruz Biotech, Santa Cruz, CA, USA), Nanog (ab21603, Abcam, Cambridge, UK), Gata4 (C-20 sc-1237, Santa Cruz), Eed (gift from Arie Otte, Slater Instituut, Amsterdam, The Netherlands; used at 1:10 dilution), Cdx2 (MU392A-UC, BioGenex, San Ramon, CA, USA) and Eomes (ab23345, Abcam). Antibodies were diluted 1:200 in blocking buffer and embryos were incubated in the appropriate antibody solution at 4°C overnight. They were rinsed three times in blocking buffer for ~15 minutes each, and incubated in secondary antibody solution for 1 hour. Secondary antibodies labelled with Alexa fluorophores (Invitrogen, Paisley, UK) were diluted 1:500 in blocking buffer. Embryos were then rinsed three times in blocking buffer, incubated briefly in increasing

concentrations of Vectashield (H-1200, Vector Labs, Peterborough, UK) before mounting on glass slides in small drops of concentrated Vectashield (with or without DAPI), and subsequently sealed with nail varnish.

Confocal analysis

Confocal images were collected using a Leica TCS SP5 confocal microscope. Reconstructions of three-dimensional images from confocal sections and cell counts were performed using Leica software and Adobe Photoshop.

Statistical analysis

Probability (*P*) values were established using Student's *t*-test for comparison between two samples.

Clonal analysis of ES cells from ICMs

Embryos at the 8-cell stage were flushed from oviducts of 129/Sv mice at 2.5 dpc, cultured in KSOM+2i for 2 days, then transferred to N2B27+2i±LIF for one more day. Alternatively, peri-implantation embryos were flushed directly from uteri of F1 mice that had been mated with F1 males 4.5 days previously. Embryos were subjected to immunosurgery to remove the trophectoderm using the protocol described previously (Nichols et al., 1998; Solter and Knowles, 1975). Isolated epiblasts were then disaggregated using trypsin. Residual clumps and obviously dying cells were discarded and the resulting single cells or couplets were deposited individually into each well of a gelatinised 96-well plate containing N2B27+2i±LIF. After 8 days, colonies were fixed and scored by morphology or immunostaining. In some cases, colonies were passaged further and injected into blastocysts for chimaera formation.

Chimaera analysis

Ten to 12 dissociated cells from immunosurgically isolated ICMs or ES cell cultures were injected into C57BL/6/Ola blastocysts. Injected embryos were transferred to the uteri of C57BL/6/Ola × CBA/Ca F1 females, previously mated with vasectomised males, at 2.5 dpc. Chimaeras were identified by coat colour mosaicism and tested for germline transmission by mating with an appropriate wild-type mouse.

RESULTS

Inhibition of Erk activity in early embryos eliminates hypoblast and expands epiblast

We previously demonstrated that ES cells can be maintained in an undifferentiated state in the presence of the Mek inhibitor PD184352 and the FGF receptor inhibitor SU5402, but that survival and expansion are enhanced by addition of an inhibitor of Gsk3, Chir99021 (Ying et al., 2008). We therefore cultured mouse embryos from the 8-cell stage in medium supplemented with PD184352 and SU5402, with and without Chir99021. In all conditions, expanded blastocysts formed and many of these hatched from the zona pellucida, indicating that the inhibitors do not interfere with trophoblast formation and differentiation. We then examined the ICMs by confocal immunofluorescence analysis. We utilised specific antibodies raised against Oct4 to identify cells of the epiblast and early hypoblast (Palmieri et al., 1994), and against Nanog, which is specific for the epiblast (Chambers et al., 2003; Mitsui et al., 2003), and Gata4, a hypoblast marker (Morrisey et al., 1996). In control cultured embryos, Gata4-positive cells were relatively abundant and Nanog-positive cells were scarce (Fig. 1A,B). By contrast, embryos cultured in PD184352 and SU5402 showed a dramatic reduction in Gata4-positive cells (Fig. 1A,B; $P < 0.0001$). They also exhibited a slight increase in the number of cells in the Nanog-positive compartment, and this was greatly enhanced by the addition of Chir99021 (3i, Fig. 1A,B; $P = 0.003$ and $P < 0.0001$ for PD184352+SU5402 versus control and 3i versus control, respectively). Embryos cultured in Chir99021 alone did not differ from untreated controls (Fig. 1A,B).

We substituted both PD184352 and SU5402 with the more potent Mek inhibitor PD0325901, or with PD173074, which has a higher affinity than PD184352 for the FGF receptor and is more selective

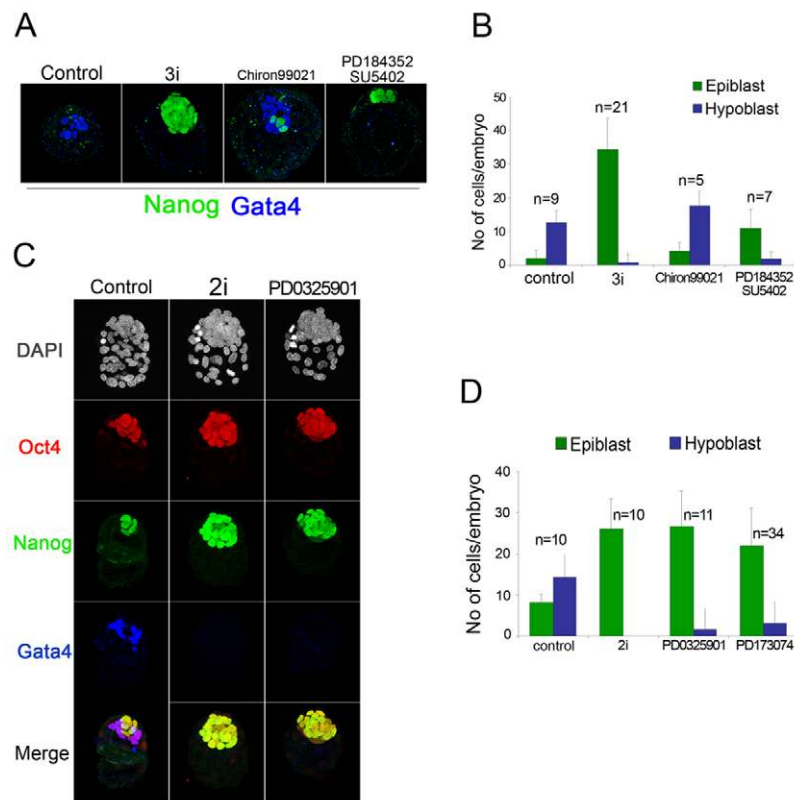


Fig. 1. Effect of FGF/Erk and Gsk3 inhibition on inner cell mass development. (A) Confocal images of mouse embryos grown from the 8-cell stage (E2.5) for 3 days in control medium, in 3i (medium supplemented with Chir99021, PD184352 and SU5402), medium supplemented with Chir99021 alone, or medium supplemented with PD184352 and SU5402. Embryos were immunostained using antibodies raised against Nanog (green) and Gata4 (blue). (B) Bar chart showing cell numbers of epiblast (Nanog positive, green) and hypoblast (Gata4 positive, blue) of embryos cultured in the conditions shown in A. Bars indicate the mean \pm s.d. (C) Confocal images of embryos grown from the 8-cell stage for 2 days in control medium, in 2i (medium supplemented with PD0325901 and Chir99021), or medium supplemented with PD0325901. Embryos were immunostained using antibodies raised against Oct4 (red), Nanog (green) and Gata4 (blue) and nuclei were counterstained with DAPI. (D) Bar chart showing cell numbers of epiblast (green) and hypoblast (blue) of embryos cultured in the conditions shown in C. Bars indicate the mean \pm s.d.

than SU5402 (Bain et al., 2007). In the presence or absence of Chir99021, there were few Gata4-positive cells compared with controls, similar to the embryos cultured in 3i (Fig. 1C,D; $P < 0.0001$). Interestingly, the Nanog-positive compartment was expanded in both cases without addition of Chir99021 (Fig. 1C,D; $P < 0.0001$ comparing either condition with controls, and $P = 0.88$ and $P = 0.21$ for PD0325901 and PD173074, respectively, compared with 2i). This suggests that off-target effects of SU5402 might inhibit ICM expansion in a manner that can be rescued by Gsk3 inhibition. The combination of PD0325901 with Chir99021, termed 2i, is very effective for propagating ES and induced pluripotent cells (Buehr et al., 2008; Silva, J. et al., 2008; Ying et al., 2008). For short-term culture of 2 days, 8-cell stage embryos cultured in PD0325901 or PD173074 alone were virtually indistinguishable from those cultured in 2i (Fig. 1C,D).

Effect of 3i/2i applied at the blastocyst stage

The absence of hypoblast in inhibitor-treated embryos could be due to selective ablation of this lineage. However, the maintenance of total ICM number in embryos cultured for 2 days in 2i compared with controls (Fig. 1C,D) suggests the alternative explanation that cells assigned to the ICM might have all been directed to the Nanog-positive lineage in preference to the Gata4-positive domain. In an attempt to distinguish between these possibilities, embryos were collected at the expanded blastocyst stage at ~ 3.75 dpc (E3.75), when the hypoblast is thought to be already determined (Chazaud et al., 2006), and cultured with or without inhibitors for 2 days. Numerous Gata4-positive cells were present at the end of this period in 3i, 2i and control culture conditions (Fig. 2A). This indicates that the inhibitors are not acutely toxic to hypoblast cells. Therefore, the absence of Gata4-positive cells in embryos treated from the 8-cell stage might be a result of preferential direction of the ICM cells into epiblast, rather than selective destruction of hypoblast.

During normal development, as embryos approach implantation, Nanog is transcriptionally downregulated in the epiblast until it becomes undetectable (Chambers et al., 2003; Hart et al., 2004). Freshly isolated peri-implantation stage embryos retain a few Nanog-positive cells, detectable by immunostaining (Fig. 2A). Embryos cultured from the blastocyst stage in control serum-free medium lose expression of Nanog completely, although Oct4 expression persists in a subset of ICM cells that is distinct from the Gata4-positive domain (Fig. 2A). By contrast, embryos cultured in serum-free medium with inhibitors (3i or 2i) maintained high levels of Nanog protein in the epiblast (Fig. 2A). Strikingly, expression of Oct4 was not restricted to the Nanog-positive domain, but was also maintained in the hypoblast in blastocysts (Fig. 2A). This profile of Nanog and Oct4 double-positive epiblast and Oct4-positive hypoblast is maintained in embryos in diapause (Batlle-Morera et al., 2008), the stage of arrested development from which ES cells are most readily derived (Batlle-Morera et al., 2008; Brook and Gardner, 1997; Kawase et al., 1994). Since Oct4 expression is progressively lost from the hypoblast during embryo maturation in vivo and in control culture conditions (Fig. 2A), the persistence of Oct4 in hypoblast during diapause and in 3i/2i blastocyst culture might reflect a block in developmental progression that maintains the embryo at the optimal stage for ES cell derivation.

Inhibition of hypoblast formation in early embryos is not reversible

Once the hypoblast has been microsurgically removed from epiblasts of peri-implantation mouse embryos it is not regenerated (Gardner, 1985). Moreover, hypoblast is not produced from mouse epiblasts mechanically isolated from diapause blastocysts (Batlle-Morera et al., 2008; Brook and Gardner, 1997). To investigate whether hypoblast can be regenerated from epiblasts developed in inhibitors, we incubated embryos from the 8-cell stage in 2i for 2

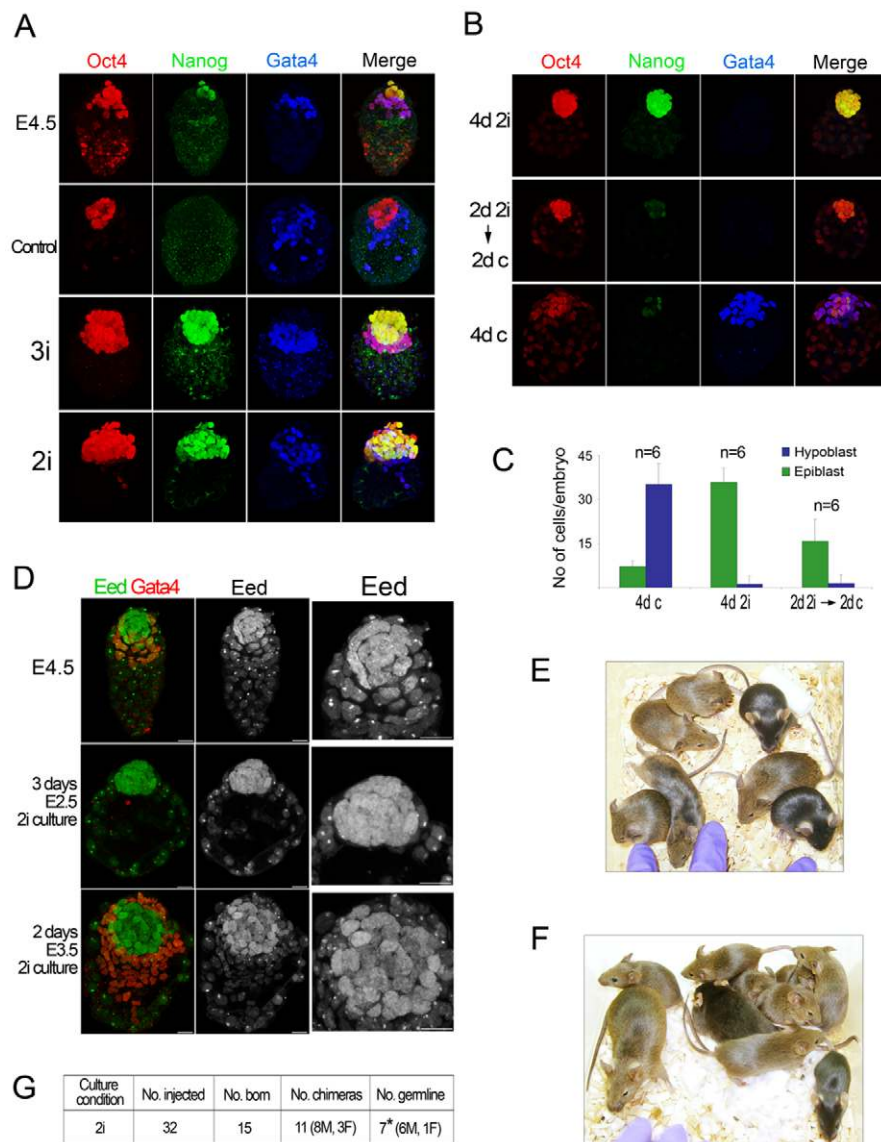


Fig. 2. Formation of functional epiblast in embryos cultured in ground state conditions.

(A) Confocal images of mouse embryos freshly isolated at 4.5 dpc (E4.5), embryos cultured for 2 days from E3.75 in control medium, in 3i or in 2i. Embryos were immunostained using antibodies against Oct4 (red), Nanog (green) and Gata4 (blue).

(B) Confocal images of embryos grown from the 8-cell stage (E2.5) for 4 days in 2i, for 2 days in 2i then a further 2 days in control medium, or in control medium for 4 days. Embryos were immunostained as in A. (C) Bar chart showing cell numbers of epiblast (Oct4 positive, Gata4 negative, green) and hypoblast (Gata4 positive, blue) of embryos cultured in the conditions shown in B. Bars indicate the mean \pm s.d. (D) Confocal images of embryos freshly isolated at E4.5, embryos cultured from the 8-cell stage (E2.5) for 3 days in 2i, or from E3.5 for 2 days in 2i. Embryos were immunostained using antibodies against Eed (green) and Gata4 (red). Scale bars: 20 μ m.

(E) Mice generated from injection of isolated epiblast cells from embryos grown for 3 days in 2i. Donor cells were from 129/Sv embryos (agouti coat colour); host blastocysts were of the C57BL/6/O1a strain (black coat colour).

(F) One of the female chimaeras and her C57BL/6/O1a mate with their offspring comprising seven agouti pups, demonstrating transmission through the germline of the donor cell genotype, and one black offspring produced from the germ cells of the host embryo. (G) Summary of blastocyst injection experiments to test the developmental capacity of epiblast cells from embryos cultured in ground state culture conditions. Asterisk indicates that nine mice were tested for germline transmission.

days, then transferred them to control medium for a further 2 days prior to fixing and immunostaining. Only one of seven embryos cultured in this way exhibited any Gata4-positive cells. One of six embryos cultured in 2i for the entire 4 days also exhibited a few Gata4-positive cells. By contrast, all six embryos cultured for 4 days in control medium possessed large numbers of Gata4-positive cells (Fig. 2B,C; $P < 0.0001$). The irreversible loss of hypoblast formation imposed by the inhibitors is consistent with commitment to the epiblast, indicating that ICM cells in treated embryos do not arrest at an early ICM stage.

ICM cells develop into functional epiblast in 3i/2i

We investigated the epigenetic status of ICM cells generated in 2i by examining the X chromosomes in female embryos. The Polycomb group protein Eed marks the inactive paternal X chromosome in early embryos (Mak et al., 2004; Okamoto et al., 2004). In female blastocysts at E3.5, all cells carry an inactive X chromosome. Reactivation of the silent X chromosome occurs exclusively in the epiblast between E3.5 and E4.5 (Silva et al., 2009). Accordingly, a prominent nuclear focus of immunostaining corresponding to enrichment of Eed on the inactive X could be detected in the

hypoblast and trophectoderm cells of female embryos at E4.5, but was absent in the epiblast (Fig. 2D). In female embryos cultured in 2i, the ICM cells lacked Eed foci, implying that X reactivation had occurred. The erasure of X chromosome silencing substantiates the argument that cells transit from ICM to epiblast state in the presence of 2i.

To confirm the development of functional epiblast in embryos cultured in 2i from the 8-cell stage, we disaggregated the ICMs and injected them into blastocysts. From 32 blastocysts injected, 11 coat colour chimaeras were obtained. Seven of these subsequently exhibited germline transmission of the injected cells (Fig. 2E-G). These results establish that the inhibitor treatment does not compromise epiblast identity or potency. This suggests that the inhibitors hold naïve epiblast cells in the ground state, from which they can readily resume normal development.

Culture from the zygote stage in 2i reduces the number of trophectoderm cells

To determine whether culture in 2i affects development of the trophectoderm, we cultured embryos with or without inhibitors from the zygote stage for 5 days before fixation and immunostaining.

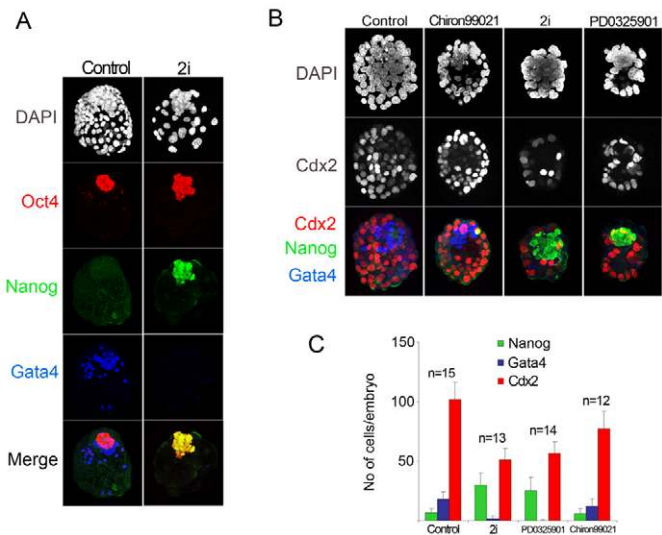


Fig. 3. Effect of 2i on trophoderm development. (A) Confocal images of mouse embryos cultured from the zygote stage for 6 days in control medium or 2i. Embryos were stained for Oct4 (red), Nanog (green) and Gata4 (blue) and nuclei counterstained with DAPI. (B) Confocal images of embryos cultured from zygotes for 6 days in control medium, Chir99021, 2i or PD0325901. Embryos were immunostained using antibodies against Cdx2 (red), Nanog (green) and Gata4 (blue) and nuclei counterstained with DAPI. (C) Bar chart showing cell numbers of epiblast (Nanog positive, green), hypoblast (Gata4 positive, blue) and trophoderm (Cdx2 positive, red) of embryos cultured in the conditions shown in B. Bars indicate the mean \pm s.d.

Treated embryos formed expanded blastocysts that hatched from the zona pellucida, properties that are dependent on functional trophoderm (Fig. 3A). Immunostaining for Cdx2 and Eomes showed that these trophoblast markers were expressed in the outside cells of embryos cultured in inhibitors (Fig. 3B; data not shown). These results indicate that, unlike hypoblast, formation of trophoderm is not dependent upon Erk signalling. However, embryos cultured in 2i or in PD0325901 alone developed significantly fewer trophoderm cells than the controls (Fig. 3C; $P < 0.0001$), consistent with reports that trophoblast proliferation is stimulated by Fgf4 (Nichols et al., 1998; Tanaka et al., 1998). As with culture of embryos from the 8-cell stage, Nanog-positive epiblast cells increased in number at the expense of Gata4-positive hypoblast in ICMs formed in the presence of 2i or PD0325901 (Fig. 3; $P < 0.0001$).

Addition of LIF does not enhance the response of embryos to 2i

Although derivation and culture of ES cells in 3i/2i medium does not require the addition of any cytokines, clonogenic efficiency is improved by supplementation with LIF (Ying et al., 2008). However, we found that addition of LIF to embryos cultured in 2i did not enhance expansion of the ICM (Fig. 4A,B). This might be because endogenous LIF is sufficient. In the embryo, the components of LIF signalling are present by the blastocyst stage, with LIF expressed specifically by the trophoderm, and the receptor components predominating in the ICM (Nichols et al., 1996).

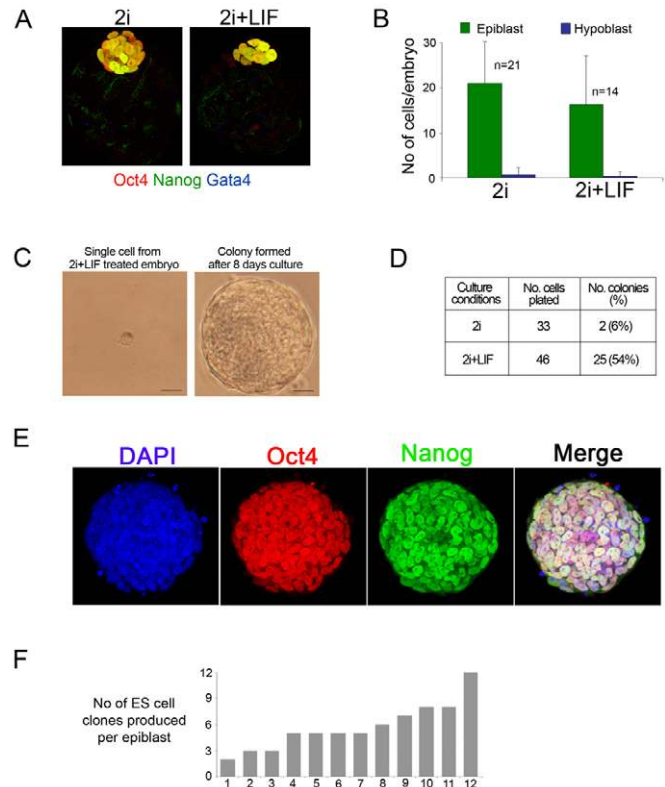


Fig. 4. Effect of LIF on epiblast cells in embryos cultured in 2i and explanted. (A) Confocal images of mouse embryos cultured from the 8-cell stage for 2 days in 2i or 2i+LIF. Embryos were immunostained using antibodies against Oct4 (red), Nanog (green) and Gata4 (blue). (B) Bar chart showing cell numbers of epiblast (green) and hypoblast (blue) of embryos cultured in the conditions shown in A. Bars indicate the mean \pm s.d. (C) (Left) Bright-field image of a single epiblast cell immediately after isolation from an embryo cultured for 3 days in 2i+LIF, plated into one well of a 96-well plate. (Right) Bright-field image of a colony produced from the same cell after growth in 2i+LIF for 8 days. Scale bars: 20 μ m. (D) Summary of colonies produced from single epiblast cells plated into 2i or 2i+LIF. (E) Confocal images of a colony grown from a single epiblast cell plated into 2i+LIF for 8 days, as shown in C. Colonies were immunostained using antibodies against Oct4 (red) and Nanog (green) and nuclei were counterstained with DAPI. (F) Bar chart showing the number of ES cell clones produced per embryo from single ICM cells isolated from freshly flushed F1 embryos at E4.5.

Clonal propagation of ES cells from dissociated epiblast

ES cells have been derived from single epiblast cells, but at low efficiency (Brook and Gardner, 1997). This has provoked discussion as to whether epiblast cells are equipotent or whether only a minority have the capacity to become ES cells (Gardner and Brook, 1997). We observed that the derivation of ES cells from embryos cultured in 3i/2i from the 8-cell stage was very efficient (Ying et al., 2008). We therefore used this system to address the ability of the individual epiblast cells to form ES cells. We used immunosurgery to isolate ICMs from strain 129 embryos cultured for 3 days in 2i from the 8-cell stage. We dissociated the ICMs and deposited single cells into individual wells of 96-well plates in 2i or 2i plus LIF. Of individual cells plated in 2i alone, two out of 33 (6%) gave rise to undifferentiated ES cell colonies. By contrast, 25 out of 46 cells

(54%) plated in 2i plus LIF yielded ES cell colonies (Fig. 4C,D). Addition of LIF to 3i has previously been shown to enhance clonogenicity of ES cells, presumably by activation of the Stat3 pathway (Ying et al., 2008). The colonies produced in 2i alone were morphologically indistinguishable from those derived in 2i plus LIF. Several colonies were stained for Oct4 and Nanog and were found to exhibit uniformly high levels of both pluripotency markers (Fig. 4E). The remaining colonies were expanded as ES cell lines. Two of these were injected into blastocysts and gave rise to chimaeras that subsequently transmitted the ES cell genome through the germline (data not shown).

Finally, to exclude the possibility that prior culture of embryos in 2i induces some epigenetic adaptation, we isolated and dissociated the ICMs from freshly harvested E4.5 blastocysts and plated single cells or couplets into individual wells of 96-well plates containing 2i plus LIF. All ICMs produced colonies. The mean number of undifferentiated colonies per embryo scored after 8 days in culture was 5.75 ($n=12$), ranging from two to 12 colonies (Fig. 4F). These data indicate that naïve epiblast cells in the murine embryo can transit directly to self-renewing ES cell status. Furthermore, approximately half of the cells plated from E4.5 ICMs will be committed hypoblast, and given that the cloning efficiency of established ES cells is less than 50% (Ying et al., 2008), the frequency of ES cell colony formation suggests that all epiblast cells are likely to have this property.

DISCUSSION

The precise relationship between cultured mouse ES cells and pluripotent cells in the embryo has been contentious (Buehr and Smith, 2003; Gardner and Brook, 1997; Smith, 2001; Zwaka and Thomson, 2005). Authentic ES cells are characterised by the unusual capacity to proliferate in the presence of selective inhibition of the Mek/Erk1/2 signalling pathway (Buehr et al., 2008; Burdon et al., 1999; Chen et al., 2006; Kunath et al., 2007; Li et al., 2008; Ying et al., 2008). The present findings demonstrate that in intact embryos, the naïve epiblast can also develop and expand in conditions in which FGF/Erk signalling is inhibited. We further show that epiblast cells can be directly and clonally expanded at high efficiency into ES cell lines in these culture conditions. These results establish a close similarity between naïve epiblast present in the mature blastocyst and ES cells *ex vivo*.

ICMs of embryos developed to the blastocyst stage in the presence of FGF/Erk signalling inhibition exhibit uniformly high levels of Nanog or Oct4 (Fig. 1; Fig. 2A,B). They entirely lack overlying hypoblast, yet have similar or even increased total ICM cell numbers compared with embryos cultured without inhibitors or freshly isolated peri-implantation embryos. In embryos cultured in the Gsk3 inhibitor alone, hypoblast formation is not overtly impaired (Fig. 1A,B). This result appears at variance with a recent report in which ES cells were efficiently derived from embryos of recalcitrant strains of mice using the Gsk3 inhibitor BIO, and ICM outgrowths specifically exhibited reduced hypoblast proliferation (Umehara et al., 2007). However, BIO is less selective for Gsk3 than Chir99021 and is likely to inhibit various other kinases including Cdks, FGF receptor and, possibly, Erk (Zhen et al., 2007). Furthermore, in the Umehara study, the outgrowths were maintained on murine embryonic fibroblast feeders in medium supplemented with serum.

The mechanism underlying the absence of hypoblast in embryos cultured in 2i is likely to be a fate change rather than specific ablation. Embryos exposed to the inhibitors at the expanded blastocyst stage of development, when the hypoblast is already specified, develop numerous Gata4-positive cells (Fig. 2A). Furthermore, the diversion

of ICM cells to an epiblast fate by inhibition of FGF/Erk signalling seems irreversible, as further culture of embryos following removal of inhibitors does not restore the hypoblast (Fig. 2B,C). This suggests that the ICM cells in 2i undergo normal developmental progression and lose the capacity to form hypoblast coincident with acquiring pluripotent epiblast identity. Transition to epiblast is further indicated by erasure of epigenetic silencing of the inactive paternal X chromosome in female embryos cultured in 2i (Fig. 2D). The presence of two active X chromosomes is a hallmark of the pluripotent ground state of ES cells that is not shared by epiblast stem cells (EpiSCs), extra-embryonic trophoblast stem (TS) or extra-embryonic endoderm (XEN) cell lines (Guo et al., 2009). Female human ES cell lines vary in the activation status of their paternal X chromosome; they are apparently subject to dynamic epigenetic reprogramming *ex vivo* that is not necessarily reflective of their pluripotent status (Silva, S. S. et al., 2008). Neither human ES cells nor EpiSCs exhibit X chromosome reactivation when cultured in 3i/2i, and ultimately fail to survive (Nichols and Smith, 2009). We show in this study that epiblast cells from embryos cultured in 2i are able to contribute normally to embryonic development and form viable chimaeras and functional germ cells when injected into host blastocysts (Fig. 2E-G). These results demonstrate that functional epiblast can develop when FGF/Erk and Gsk3 signalling are inhibited, and that neither the development nor potency of murine epiblast requires interaction with hypoblast.

In contrast to the requirement for development of hypoblast, specification of the trophoblast lineage does not appear to require activation of the Erk pathway. Embryos cultured in the presence of 2i from the single-cell stage cavitated and hatched from the zona pelucida, demonstrating the formation of functional trophoblast. Moreover, the cells of this outer epithelium expressed both *Cdx2* and *Eomes*, two definitive markers of trophoblast (Fig. 3B; data not shown). Nonetheless, expansion of the trophoblast is affected by inhibition of Erk activity; embryos cultured in 2i or in PD0352901 alone exhibited a decrease in trophoblast cell numbers compared with controls (Fig. 3). Expression of FGF receptors has been demonstrated in trophoblast and its derivative extra-embryonic ectoderm (Arman et al., 1998; Holdener et al., 1994; Rappolee et al., 1994), and a requirement for FGF signalling has previously been implicated in proliferation of the diploid population of cells that reside in the polar trophoblast (Nichols et al., 1998; Tanaka et al., 1998). Thus, Erk signalling appears to be dispensable for trophoblast specification and differentiation in the early embryo, but is likely to be required for maintenance of a proliferating diploid population.

We have proposed that naïve pluripotency might be a basal mammalian cell state that is intrinsically self-maintaining if shielded effectively from inductive differentiation stimuli (Ying et al., 2008). The present findings indicate that this state is shared between pre-implantation epiblast and ES cells. The intrinsic self-replication exhibited by mouse ES cells is thus not an adaptation to culture, but directly represents autonomous expansion of early epiblast. In the unperturbed embryo, self-renewal is short-lived owing to the inductive action of *Fgf4* and other extrinsic Erk stimuli, but this feed-forward stimulus can be arrested in diapause, during which the epiblast can remain in a naïve state for weeks. ES cell lines can most readily be derived from diapause blastocysts (Kawase et al., 1994). Upon explant culture, the epiblast will rapidly lose pluripotency and differentiate under the influence of Erk signalling (Buehr et al., 2003; Buehr and Smith, 2003). Disrupting this signal with 3i/2i prevents this progression and releases ES cells. This has been exploited to enable efficient derivation of ES cells from embryos of recalcitrant CBA and MF1 mouse strains (Ying et al., 2008), NOD

mice (Nichols et al., 2009) and rats (Buehr et al., 2008; Li et al., 2008). We conclude that in rodents, naïve epiblast and ES cells are the same ground state entity, differing only by their environment. It will be important to determine whether this principle extends to other mammals, in particular primates, from which authentic ES cells have yet to be described.

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