Maternal-effect *Floped* gene is essential for the derivation of embryonic stem cells in mice

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Abstract: *Floped* (official name *Ooep*) is specifically and abundantly expressed in mouse oocytes and embryonic stem cells (ESCs). Depletion of *Floped* from oocytes leads to early embryonic arrest at the 2-cell stage. Although crucial in cleavage stage development, its roles in early embryos as well as in ESCs remain completely unknown. Here, we compared the efficiency of mouse ESC derivation from inner cell mass (ICM) with and without *Floped* to study its possible roles in mESCs. Derivation rates of mESC from wild-type, heterozygous, and homozygous blastocysts were 33.3%, 21.43%, and 3.85%, respectively, indicating that *Floped-/-* blastocysts had significantly decreased derivation rates. Respective outgrowth appearing rate five days after blastocyst attachment were 83.3%, 85.7%, and 15.4%. Morphologically, the outgrowth of ICM from *Floped-/-* blastocysts appeared severely death three to five days after blastocyst attachment, and the respective derived stem cells showed long-term instability with long-standing epithelial-like colonies. This result suggests a possible role of *Floped* in the course of ICM-ESCs transition.

Keywords: *Floped*; ICM; ESCs

Embryonic stem cells (ESCs) and inner cell mass (ICM) of blastocysts, from which ESCs are derived, possess both similar and dissimilar characteristics (Downing & Battey et al, 2004). The ICM of blastocysts has the potential to develop into a whole embryonic property but loses self-renewal ability. Embryonic stem cells can undergo limitless self-renewal division and maintain the capacity to differentiate into all body cells types (Ying et al, 2008). *In vitro* derivation of ESCs from ICM is a process in which cells switch off the developmental program while retaining pluripotency and gaining self-renewal ability. Although some studies have investigated gene expression changes during derivation, the molecular basis underlying ICM-ESC transition remains unclear.

Floped (factor located in oocytes permitting embryonic development, official name *Ooep*) was originally identified as an oocyte specific gene in mice (Li et al, 2008). The mRNAs of *Floped* are restrictively detected in growing oocytes, but the protein can persist until the blastocyst stage. Genetic ablation of *Floped* from mouse oocytes does not impair the growth, meiotic maturation, or fertilization of the oocytes. Nevertheless, embryos without maternal deposition of the *Floped* protein cannot develop beyond the 2-cell stage when the major zygotic genome activation event occurs (Li et al, 2008). *Floped* is also abundantly expressed in undifferentiated mouse ESCs. Using targeted mutation strategies, a recent study investigated the possible roles of *Floped* in mESCs and found that *Floped* was not necessary for maintaining ESC identity under undifferentiated conditions. However, the absence of *Floped* in mESCs accelerated the differentiation pace when ESCs were induced to undergo differentiation (Miura et al, 2010). Moreover, tracing the derivation of ESCs from ICM by single cell gene expression analysis uncovered a significant increase in mRNA expression of *Floped* during transition, suggesting possible involvement in this reprogramming process (Tang et al,

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2010). Based on these observations, we aimed to investigate the potential roles of *Floped* in the establishment of mESCs from ICM. By utilizing the *Floped* knockout mouse line, our work showed that ICM of *Floped-/-* blastocysts displayed lower potential to transit into ESCs when compared to their wild-type counterparts.

MATERIALS AND METHODS

Ethics statement

All experimental procedures and care of animals were performed according to protocols approved by the Ethics Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences (permit number: SYDW-2006-006).

Feeder cells preparation

Mouse embryonic fibroblasts (MEF) from E14 embryos of CD1 mice were prepared and cultured as previously described (Nagy, 2003). Briefly, MEF were plated in culture dishes precoated with a 0.1% (w/v) solution of gelatin (AMRESCO. 9764-500g) and cultured in DMEM (Gibco; 430-1600) supplemented with 10% newborn calf serum (NCS; Gibco. 16010-159). To prepare the feeder cells in the ESC culture, MEF growing near confluence were treated with mitomycin C (5 μg/mL, Sigma M4287) for 3 hours followed by rinsing five times with Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (PBS). These cells were then trypsinized with 0.05% Trypsin-EDTA (Gibco; 25200-056) and seeded at a density of 5×10^4 cells/cm^2 after washing.

Animals and blastocysts collection

The *Floped-/-* mice were kindly provided by Dr. Lei LI from the Institute of Zoology, Chinese Academy of Sciences. The *Floped+/-* females were mated with *Floped+/-* males at four weeks of age and the mating plug was examined every morning. Females who achieved successful mating were immediately separated from males and blastocysts were collected at E3.5. Morphologically healthy blastocysts were used to derive ESCs. The zonae pellucidae of the blastocysts were removed by incubation with acid Tyrode solution (pH 3; Sigma T1788-100 mL).

Derivation and propagation of ES cells

We used serum-free media supplemented with knockout serum replacement to facilitate the derivation of C57BL/6J mouse ES cell lines (Cheng et al, 2004; Tanimoto et al, 2008) and derive ESCs from blastocysts.

The ES medium was composed of DMEM/F12 supplemented with 20% Knockout Serum Replacement (Gibco; 10828-028), penicillin (100 U/mL)/streptomycin (100 μg/mL) (Gibco; 15140-122), 2 mmol/L of L-glutamine (Sigma; G8540-25 g), 1×MEM Non-Essential Amino Acids Solution (Gibco; 11140-035), 100 μmol/L of beta-mercaptoethanol (Sigma; M7522), and recombinant mouse leukemia inhibitory factor (1 000 U/mL; Chemicon no. ESG1107).

Blastocysts without zona pellucidae were plated on mitomycin C-treated MEFs. Blastocysts were allowed to attach to MEFs and grew without any interference for 48 hours. After five days, the outgrowths were selectively collected and transferred into droplets of 0.05% trypsin-EDTA solution for 1−3 min with a mouth-controlled micropipette (Bryja et al, 2006). Dissociated individual cells and small cell clumps were pipetted directly into a 4-well plate containing new MEFs and ES medium. After continued purification, the putative ES cells were frozen at a concentration of 2×10^6 cells per vial. Cultures were maintained in a humidified incubator at 37 °C, under 5% CO_2 and 95% air.

PCR genotype of the blastocysts

Each blastocyst used for ESC derivation was genotyped by PCR amplification according to previous research (Li et al, 2008). Genomic DNA was extracted from individual blastocysts or its derivatives using a Tianamp Micro DNA Kit according to manufacturer's instructions (TIANGEN BIOTECH; DP316). Primers P1 (5'-CCCTGCTGACAGTGGACTC-3'), P2 (5'-CCAGCCAGTTTTA GCCCTTT-3'), and P3 (5'-TGCGCAACTGTTGGGAAG-3') were used for PCR genotyping.

The PCR conditions consisted of 1 cycle at 95 °C for 3 min; 32 cycles at 94 °C for 30 s, 55 °C for 30 s, 68 °C for 60 s, and a full extension cycle at 72 °C for 7 min. The PCR with three primers produced a 512 bp band (primers P1, P2) in wild-type and an 890 bp band (primers P1, P3) in null and heterozygous mice.

To follow up the ESC lines establishment of three genotypic kinds of *Floped* blastocysts, we collected all derivatives of the blastocysts for respective genome DNA extraction. If one blastocyst did not hatch within 48 hours after seeding, we collected this blastocyst for DNA extraction. As such, derivatives from respective hatched blastocysts were collected regardless of the ES-like cells, peripheral primitive endoderm-like cells, or trophectoderm-derived cells.

Alkaline phosphatase staining

During the log phase, ES cells were fixed by 4% ice-cold paraformaldehyde (PFA) for 1 minute, after which rinsed wells and added enough staining solution (BCIP/NBT Alkaline Phosphatase Color Development Kit; Beyotime C3206) for 15 minutes at room temperature, then count the number of atropurpureus stem cell colonies (AP) versus the number of differentiated colonies (colorless).

Immunocytostaining

Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature, permeabilized with 0.1% TritonX-100 in PBS for 10 minutes, incubated with PBS containing 1% BSA for 60 minutes to block non-specific reactions, and incubated with anti-Nanog, Oct4, and SSEA-1 antibody (Santa Cruz) overnight at 4 °C. Dilution ratio of the first antibodies was 1:200. After washing with PBS, the cells were incubated with Alexa-Fluor-488-conjugated secondary antibody (Life Technology). Negative controls for fluorophore-conjugated secondary antibody were carried out without primary antibody, and nonspecific binding of secondary antibodies was detected. Fluorescent images were captured using a Leica TCS SP5 confocal microscope system (Leica Microsystems).

Statistical analysis

Data analysis was performed using SPSS 13.0 statistical software. Fisher's exact test was performed. $P<0.05$ was considered statistically significant.

RESULTS

***Floped* for outgrowth formation**

As summarized in Table 1, a total of 15 outgrowths were obtained after five days of culture from 18 wild-type blastocysts (outgrowth rate: 83.33%, 15/18, three females). A similar outgrowth rate was obtained from blastocysts with heterozygous genotype under the same experimental conditions (85.71%, 36/42, three females); in contrast, only 15.38% (4/26, three females) of *Floped-/-* blastocysts demonstrated outgrowth formation.

Table 1 Outgrowth and embryonic stem cell line derivation efficiency from different genotypic blastocysts

Floped	Number of blastocysts used	Number of (%) outgrowths at day 5	Number of ES lines (% blastocyst)	Percentage of ES lines (from outgrowth)
+/+	18	15 (83.33) [a]	6 (33.3) [c]	40 [e]
+/–	42	36 (85.71) [a]	9 (21.43) [c, d]	25 [e]
–/–	26	4 (15.38) [b]	1 (3.85) [d]	25 [e]

Values with different superscripts are significantly different in one column ($P < 0.05$) by Fisher's exact test. Values with same superscripts indicate numbers in the same column are not significantly different ($P > 0.05$).

We found that *Floped-/-* blastocysts could normally attach to feeder cells and grow in the first two days. However, the sizes of the *Floped-/-* outgrowths were smaller than their wild-type or heterozygous counterparts (Figure 1C1, C2). Outgrowths from wide-type and heterozygous blastocysts maintained typical stem-cell like morphology five days after attachment to MEFs, while the majority of *Floped-/-* outgrowths (22/26, 84.6%) started to display signs of death, with ICM derived outgrowth disappearing and only trophectoderm-derived cells left (Table 1; Figure 1C3).

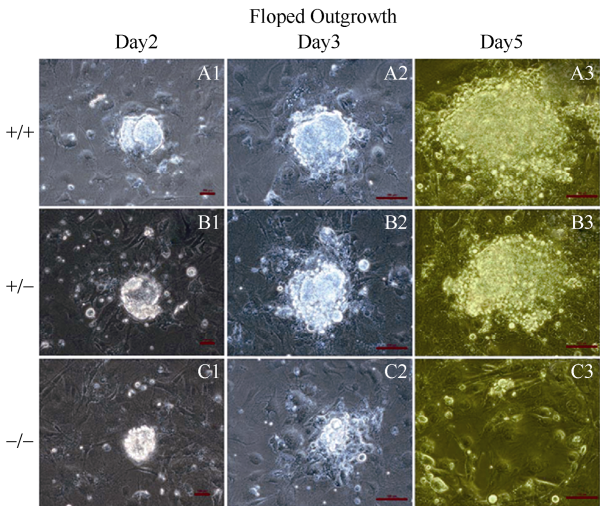

Figure 1 Representative outgrowth morphology from day 2 to day 5 after blastocysts attachment (scale bar=100 μm)

Depletion of *Floped* and derivation of ES cell lines from blastocysts

To establish ESCs from outgrowth, each outgrowth after five days of culturing was manually collected and dispensed into a single cell or small clusters through trypsinization. These cells were cultured and purified continuously for 20−30 days to obtain stable ES cell lines. From this, we obtained six wild-type ESC lines from 15 outgrowths (6/15, 40%), nine ESC lines from *Floped+/-* outgrowths (9/36, 25%), and one ESC line from *Floped-/-* outgrowths (1/4, 25%). From blastocysts, the derivation rates of wild-type, *Floped+/-*, and *Floped-/-* ESC lines were 33.3%, 21.43%, and 3.85%, respectively (Table 1).

It took much longer to purify the *Floped-/-* ESC line than it did to purify the wild-type or heterozygous counterparts. During passage and purification, stable ES cell lines from wide-type and/or heterozygous blastocysts were easily obtained. We purified these cells without the appearance of obvious epidermal-like colonies. In contrast, purification of the *Floped* null ES cell line was very difficult. As shown in Figure 2, epithelial-like colonies appeared at the first passage from outgrowth and continued for two months, even though we collected typical ES-like colonies manually for further culture at every passage. After two months, these epithelial-like colonies disappeared gradually and the stable *Floped* null ES cell line was established.

***Floped* null ES cell characteristics**

Morphology of the ES cell lines is shown in Figure

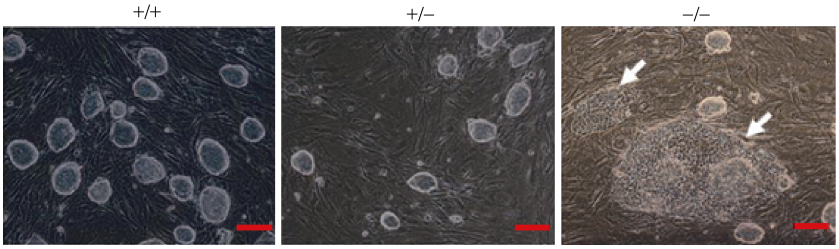

Figure 2 Purification of stem cells after five days of outgrowth

White arrows indicate epithelial-like colonies, scale bar=100 μm.

3A. *Floped* null ES cells, together with wide-type and heterozygous cells, showed typical mouse ES cell morphology, e.g. high ratio of nucleus to cytoplasm and prominent nucleoli, with colonies consisting of small juxtaposed spheroid cells and strong positive staining for alkaline phosphatase (Figure 3A). The ES cells showed high expression of pluripotency markers including Oct4, SSEA-1, and Nanog (Figure 3B-D, respectively). These results indicated that *Floped* was dispensable for ES cell self-renewal after successful derivation from ICM.

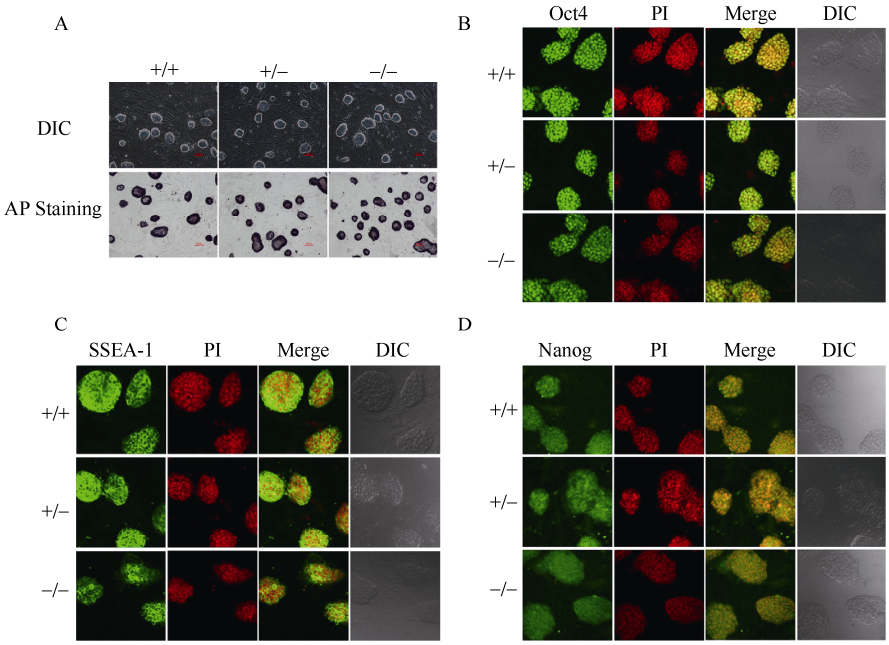

Figure 3 Pluripotency analysis of the ESCs

A) Morphology and alkaline phosphatase activity in ESCs after successful purification; B, C, D) Oct4, SSEA-1, and Nanog immunocytostaining analysis, respectively. scale bar=100 μm.

DISCUSSION

Establishing an ES cell line consists of dynamic molecular changes that accompany cell-fate changes, during which the ICM transform into cells with a capacity for infinite self-renewal while retaining pluripotency (Tang et al, 2010). Cultured ES cells have been assumed to be equivalent, or even identical to cells of the ICM of the blastocyst stage embryo from which they are derived (Reijo Pera et al, 2009). However, no evidence suggests that ICM cells can self-renew extensively. Comparison of global gene expression between individual ICM clusters and human embryonic stem cells indicate that these two cell types are significantly different in regards to gene expression, with less than one half of all genes expressed in both cell types (Reijo Pera et al, 2009). The ICM-ESCs transition is accompanied by complex genomic and epigenetic changes, and further study is required to fully understand this transition. We reported that the maternal effect *Floped* gene had a key function during ICM-ESCs transition, without which the transition rate of success was compromised, but had no influence on ES cell cultivation after successful derivation.

Gene expression data suggests a potential role of *Floped* in ICM-ESCs transition. With Oct4-DPE-GFP transgenic mice, Tang et al (2010) traced the

transcriptional, epigenetic, and miRNA transitions during the ICM-ESCs switching process. In their single cell RNA-seq database, the *Floped* expression counts were 1 352 in ICM, 598 in day 3 outgrowth Oct4 positive cells, 7525 in day 5 outgrowth Oct4 positive cells, and 11259 in ESCs; however, were 72 in day 5 outgrowth Oct4 negative cells. The expression counts of *Floped* decreased from ICM to day 3 outgrowth, but increased sharply from day 3 to day 5 outgrowth, and increased further from day 5 outgrowth to ESCs. These data indicated that *Floped* may have an important role during ESC line derivation from day 3 outgrowth to ESCs. In addition, the 72 *Floped* count on day 5 outgrowth Oct4 negative cells indicated that *Floped* was specifically expressed in pluripotent cells that progress toward ESCs, instead of differential cells. These are in agreement with the RNA-seq counts above.

We found that *Floped* null outgrowths died between days 3 and 5. We concluded, therefore, that *Floped* viably effected outgrowth, with a subsequent increased total cell number resulting from cell culture. This effect is beneficial for the later establishment of a stem-cell line.

Floped was highly expressed in self-renewing ES cells, and its expression was downregulated upon differentiation. The ES cells without *Floped* expression showed no obvious abnormalities in their undifferentiated state (Miura et al, 2010). Our results confirmed that *Floped* null ES cells had typical morphology, with stable passaging. In the process of stem cell purifying, however, many epithelial-like colonies appeared in the *Floped* null line, which suggested these cells were in an unstable state. After a period of continuous culture, the *Floped* null stem cells adapted to the in vitro environment and became stable. These data indicated that *Floped* had a key function during the establishment process of the ES cell lines, but no notable function in maintaining the ES cell self-renewal state after successful purification.

Further study is required to fully understand the molecular mechanisms of *Floped* during ESC derivation, which will increase our knowledge on the molecular changes of this process and accelerate the application of embryonic stem cells.

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