Chemical Enhancement in Embryo Development and Stem Cell Derivation from Single Blastomeres

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Abstract

Several chemicals targeting the mitogen-activated protein (MAP) kinase signaling pathway, which play an important role in regulating cell growth and differentiation, have shown enhancing effects on the development of the inner cell mass (ICM) and the derivation of ES cells. However, investigation of such chemicals on early embryonic development and the establishment of ES cell lines has not been elucidated. This study was aimed to determine if ACTH, MAP2K1 inhibitor [MAP2K1 (I)], and MAPK14 inhibitor [MAPK14 (I)] could enhance the development of the ICM in preimplantation mouse embryos and blastocyst outgrowths, and the establishment of ES cell lines from blastomeres of early embryos. We have demonstrated that both MAP2K1 (I) and MAPK14 (I) delay early embryo development and inhibit the development of embryos from early blastomeres. On the other hand, ACTH had a positive effect on embryos derived from early blastomeres. As a result, 17 ES cell lines were established. Among these ES cell lines, nine and five ES cell lines were established from single blastomeres of two-cell embryos with and without the supplement of ACTH, respectively. In addition to two-cell isolated blastomeres, three ES cell lines were established from blastomeres of four-cell embryos only with the supplement of ACTH. Our results suggest that ACTH can enhance the derivation of ES cells from single blastomere-derived embryos.

Introduction

ES CELLS ARE ONE OF THE MOST PROMISING stem cell sources for cell therapy and regenerative medicine. One of the major barriers of stem cell therapy is the identification of immune-compatible ES cells or adult stem cells for patients. ES cells have been successfully established from several species in the past decades including mice (Evan and Kaufman, 1981; Wakayama, et al., 2007), monkeys (Suemori, et al., 2001; Thomson et al., 1995), and humans (Baharvand, et al., 2006; Heins, et al., 2006). Although most of the currently available ES cell lines were derived from the ICM cells of a blastocyst stage embryo, blastomeres of eight-cell and morula stage embryos have also been used for the derivation of stem cell lines (Chung, et al., 2006, 2007; Delhaise, et al., 1996; Eistetter, 1989; Klimanskaya, et al., 2006; Strelchenko, et al., 2004; Tesar, 2005). Blastomeres collected by biopsy of mouse and human eight-cell embryos were capable of establishing ES cells

(Chung et al., 2006, 2007; Klimanskaya, et al., 2006), which suggests the likelihood of success in deriving personal ES cells. Although embryo transfer and full-term development of the biopsied blastocysts were not demonstrated, a similar blastomere biopsy procedure is commonly used in fertility clinics for preimplantation genetic diagnosis (PGD); thus, viable blastocysts and pregnancy are expected. In addition to ES cell coculture, MAP kinase inhibitors (MAPK inhibitor) such as MAP2K1 (I) have also been used as a supplement for the derivation of ES cells from a single blastomere (Chung et al., 2006). However, it is unclear whether ES cell coculture, the supplement of MAP2K1 (I), or both play an enhancing role on the establishment of ES cells from blastomeres of early embryos.

The MAPK family consists of four categories of kinases: MAPK2/3, MAPK7, MAPK8, and MAPK14. Each isoform is encoded by a different gene (Binetruy et al., 2007). Among the MAPK family, the MAPK2/3, MAPK8, and MAPK14

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pathways were the most studied in stem cell research because of their roles in regulating proliferation, differentiation, and apoptosis (Binetruy et al., 2007). Several MAPK inhibitors have also been investigated for their roles in early embryo and stem cell development (Chung et al., 2006; Maekawa et al., 2005). Among these MAPK inhibitors, MAP2K1 (I) has been used for the derivation of mouse ES cells from early blastomeres cocultured with mouse ES cells (Chung et al., 2006). Although ES cell lines have been successfully established, the role of MAP2K1 (I) and the need for coculture with ES cells have not yet been determined. Additionally, the inhibiting effect of MAPK14 (I) on the development of TE cells in mouse morula has been reported (Maekawa et al., 2005). This suggests the potential of enhancing ICM development by suppressing TE. Furthermore, Wakayama and colleagues (2007) have reported the establishment of mouse ES cell lines from a single blastomere of two-, four- and eight-cell stage embryos with the supplement of ACTH. Thus, the ICM enhancement effect of MAPK14 (I), and the impact on ES cell derivation by MAP2K1 (I) and ACTH merit further investigation.

We recently reported the establishment of mouse ES cell lines from a single blastomere of two-cell embryos without the coculture of ES cells or additional supplement besides hLIF (Lorthongpanich et al., 2008). Our current study was evolved based on the recent advancements in the derivation of ES cell lines from early blastomeres with the supplement of MAPK inhibitors and ACTH. Here we aim to evaluate and determine the effects of MAP2K1 (I), MAPK14 (I), and ACTH on early mouse embryo development and the derivation of ES cells from blastomeres of two- and four-cell embryos.

Materials and Methods

Animals

Female CD-1 mice (4–6 weeks old) were superstimulation with 10 IU of PMSG, (Sigma, St. Louis, MO). This was followed by 10 IU of hCG (Sigma) 48 h later for superovulation, and then natural mating with CD-1 male mice. Two-cell embryos were collected 43–45 h after hCG injection from the oviducts and then cultured in 20 μ L drop of KSOM media (Specialty Media, Lavallette, NJ) under mineral oil with 5% CO₂ in air at 37°C. All procedures were approved by the IACUC and Biosafety Committee of Emory University.

Nomenclature

The blastomeres were recovered by isolating blastomeres of two- and four-cell (2C and 4C) stage mouse embryos. The 4C stage embryos used in the experiment were obtained from *in vitro* culture of the 2C stage embryos. The blastomeres were named to indicate the embryonic stage from which they were derived. The embryos derived from blastomere isolation of 2C and 4C stage embryos were named as 2CIB and 4CIB embryos, respectively. KSOM was used as an initial for potassium simplex optimized medium (Erbach et al., 1994). The ACTH, MAP2K1 (I), and MAPK14 (I) were used as initials for adrenocorticotrophic hormone (ACTH, fragments 1.24; American Peptide Company, Sunnyvale, CA), MAP2K1 (I) (Cell Signaling Technology, Beverly, MA) and MAPK14 (I) (SB203580, Calbiochem, LaJolla, CA), respectively. The mES media was composed of DMEM (Invitrogen, Carlsbad,

CA), supplemented with 10% FBS (Hyclone, Logan, UT), 200 mM L-Glutamine (Invitrogen), 0.1 mM β -Mercaptoethanol (Sigma), 1 $\times$ Minimum essential amino acid (Invitrogen), 1 $\times$ Penicillin/Streptomycin (Invitrogen) and 1000 IU/ml hLIF (Chemicon, Temecula, CA).

Isolation of blastomeres

The zona pellucida of two- and four-cell stage mouse embryos was removed by 0.5% Protease (Sigma). The blastomeres were then separated by incubating the zona-free embryo in PBS without calcium and magnesium, followed by gentle pipeting.

Feeder cell preparation

Mouse fetal fibroblasts (MFF) were prepared from day 13.5 mouse fetuses. The MFFs were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone), 200 mM L-glutamine, and 1 $\times$ Penicillin/Streptomycin (Invitrogen). The MFFs were inactivated with 5 μ g/mL mitomycin C (Sigma) for 2 h followed by a thorough wash before plating.

Single blastomere culture

The isolated blastomeres of 2C and 4C embryos (2CIB and 4CIB) were cultured individually alongside their sister blastomeres in a 72-well plate (Nunc, Naperville, IL), which was pre-coated with 0.1% Gelatin (Sigma). The MFFs were plated onto the precoated 72-well plate at 1000 cells/well in 10 μ L of culture media 24 h prior to blastomere and embryo culture.

Individual 2CIBs, 4CIBs, and embryos were co-cultured with MFFs in four different treatment groups. (1) control: individual blastomeres and embryos were cultured in KSOM until attachment onto the feeder. Medium was then changed to mES medium and cultured until the isolation of ICM for ES cell derivation. (2) ACTH group: individual blastomeres and embryos were cultured in KSOM + 0.1 mg/mL ACTH (fragments 1.24; American Peptide Company, Sunnyvale, CA; modified from Wakayama et al. (2007) until attachment onto the feeder. Medium was then changed to mES media + 20% Knock-out Serum Replacement (KSR) + 0.1 mg/mL ACTH, and cells were cultured until isolation of the ICM for ES cell derivation. (3) MAP2K1 (I) group: individual blastomeres and embryos were cultured in KSOM + 50 μ M MAP2K1 (I) (Cell Signaling Technology) for 3 days and then cultured in KSOM until attachment onto the feeder. Medium was then changed to mES medium and cells were cultured until isolation of the ICM for ES cell derivation, modified from Chung et al., (2006). (4) MAPK14 (I) group: individual blastomeres and embryos were cultured in KSOM + 20 μ M MAPK14 (I) (SB203580; Calbiochem) for 15 h, modified from Maekawa et al. (2005), and then medium was replaced with fresh KSOM until attachment onto the feeder. Medium was then changed to mES medium and cells were cultured until isolation of the ICM for ES cell derivation. The optimal dosage of MAPK14 (I) was determined based on the development of early mouse embryos to blastocyst stage (Supplementary Tables 1 and 2; see supplementary material at www.libertonline.com). The 2CIB and 4CIB embryos were cultured at 37°C with 5% CO₂ in air until analysis or further treatment.

FIG. 1. The development of intact and isolated blastomere-derived embryos cultured in various supplements. (A) The development of 2C stage intact embryos after cultured in ACTH, MAP2K1 (I), and MAPK14 (I). (B) The development of 2CIB-derived embryos cultured in ACTH, MAP2K1 (I), and MAPK14 (I). Embryo development was indicated by the day after blastomere isolation followed by *in vitro* culture. (C) The development of 4CIB-derived embryos cultured in ACTH, MAP2K1 (I), and MAPK14 (I). Embryo development was indicated by the day after blastomere isolation followed by *in vitro* culture.

TABLE 1. DIFFERENTIAL EXPRESSION OF Cdx2 AND Sox2 IN BLASTOCYSTS CULTURED WITH VARIOUS CHEMICALS

Treatment	No. embryo	Cdx2 $\pm$ SEM	Sox2 $\pm$ SEM	Ratio $\pm$ SEM (Cdx2/Sox2)
Control	15	46.3 $\pm$ 2.5	12.4 $\pm$ 0.9 ^a	3.7 $\pm$ 0.3 ^a
ACTH	15	40.0 $\pm$ 3.2	6.0 $\pm$ 0.6 ^b	6.7 $\pm$ 2.1 ^b
MAP2K1 (I)	15	43.9 $\pm$ 2.9	6.7 $\pm$ 0.8 ^b	6.5 $\pm$ 0.6 ^b
MAPK14 (I)	15	38.2 $\pm$ 4.1	6.1 $\pm$ 0.9 ^b	6.3 $\pm$ 0.9 ^b

^{a,b}Superscript in the same column indicates a significant difference at $p < 0.05$.

Measurement of ICM size

The width and height of the ICM in embryo outgrowths was measured using a microscopic ruler (Supplementary Fig. 1; see Supplementary material at www.liebertonline.com and the estimated area of the ICM was calculated using the following equation.

$$\text{The ICM size } (\mu\text{m}^2) = \text{width } (\mu\text{m}) \times \text{height } (\mu\text{m})$$

Blastocyst cell count

To distinguish cells of the ICM and TE, embryos were immunostained with antibodies specific against the ICM (Sox2) and TE (Cdx2) cells. DNA was counter stained with Hoechst 33342. Secondary antibodies for Sox2 and Cdx2 were conjugated with Rhodamine and Alexa-488, and were encoded in red and green, respectively. The Sox2 and Cdx2 positive cells were visualized and counted under a fluorescent microscope at different focal planes across the embryos.

Stem cell establishment from single blastomeres

Ten days after blastomere separation, the embryo outgrowths with a prominent ICM were manually selected and subcultured onto freshly prepared MFF. Visible ES colonies were then selected for subculture based on cell morphology and maintained by standard methodology (Nagy and Gertsenstein, 2003). Besides morphology, ES cell lines were characterized by the expression of stem cell markers using specific antibodies and a commercially available kit for detecting alkaline phosphatase. Oct-4 (Pou5f1; 1:250; Santa Cruz Biotechnology), Sox2 (1:100; Stem Cell Technologies), Nanog (1:50; Santa Cruz Biotechnology), SSEA-1 (1:50; Chemicon) and Alkaline phosphatase activity (AP; Vector Lab, Burlingame, CA).

In vitro differentiation

ES cells were cultured in suspension for 7 days for the formation of embryoid bodies (EBs). EBs were then allowed to

attach onto a gelatin-coated plate and cultured in N1 medium for 7 days, N2 medium for 14 days, and N3 medium for 7 days to allow differentiation into neuronal cell types. The N1 medium was composed of DMEM/F12 (Invitrogen) supplemented with minimum essential amino acid (Invitrogen), 200 mM of L-glutamine (Invitrogen), and N2 supplement (Invitrogen). The N2 medium was composed of N1 medium supplemented with 20 ng/mL bFGF. The N3 medium was composed of DMEM/F12 supplemented with 1% FBS (Hyclone) and B27 supplement (Invitrogen). EBs were stained with alpha-fetoprotein (AFP) and vimentin. Neuroprogenitor cells were stained with nestin, whereas successful differentiation of neuronal cell types was confirmed by the expression of neuron specific β -III tubulin (TuJ1), tyrosine hydroxylase (TH), and choline acetyltransferase (ChAT) (Kuo et al., 2003).

TUNEL assay

A DeadEnd™ Fluorometric TUNEL system kit (Promega, Madison, WI) was used to identify the apoptotic cells. In brief, blastocyst stage embryos from each group were fixed, permeabilized, washed, and equilibrated in equilibration solution for 5–10 min at room temperature. Embryos were then incubated in rTDT buffer for 1h at 37°C in the dark. After a thorough wash with 2x SSC and PBS to remove the unincorporated fluorescein-12-dUTP, embryos were counterstained with 5 $\mu\text{g}/\text{mL}$ Hoechst 33342 (Sigma). TUNEL positive cells (green) and cell nuclei (blue) were visualized and counted under a fluorescent microscope at different focal planes. Dead cell index (DCI) was calculated by dividing the number of TUNEL positive cells by the total cell number (Neuber et al., 2002).

Statistical analysis

Data analyses for differences in the embryonic development were carried out by ANOVA in Statistical Analysis Systems (SAS, version 9.0, SAS Inc., Cary, NC).

TABLE 2. APOPTOTIC RATE IN BLASTOCYSTS SUPPLEMENTED WITH DIFFERENT CHEMICALS

Treatment	No. of embryo	Total cells (Average $\pm$ SEM)	TUNEL-positive cells (Average $\pm$ SEM)	DCI $\pm$ SEM
Control	20	56.1 $\pm$ 1.9 ^a	3.4 $\pm$ 0.8	0.06 $\pm$ 0.01 ^a
ACTH	20	43.7 $\pm$ 3.0 ^{b,c}	2.8 $\pm$ 0.7	0.07 $\pm$ 0.02 ^a
MAP2K1 (I)	20	50.5 $\pm$ 2.7 ^{a,b}	2.6 $\pm$ 0.4	0.05 $\pm$ 0.01 ^a
MAPK14 (I)	20	42.0 $\pm$ 3.5 ^c	4.5 $\pm$ 1.4	0.11 $\pm$ 0.04 ^b

^{a,b,c}Superscript in the same column indicates a significant difference at $p < 0.05$.

DCI, Death Cell Index = TUNEL positive cells/total cell number.

Results

Embryo development in various chemicals

The effect of ACTH, MAP2K1 (I) and MAPK14 (I) was determined by the culture of two-cell mouse embryos with or without the supplements. Control, ACTH, and MAP2K1 (I) groups have comparable development rates, which were much higher than that of the MAPK14 (I) group ($p < 0.05$) (Fig. 1A). In order to determine if the ratio of ICM and TE cells was altered in the resulted blastocysts, double immunostaining using antibodies specifically recognizing the TE (Cdx2) and ICM or stem cells (Sox2) was performed. The ratio of TE versus ICM was consistent in all three treatment

groups and was significantly higher than that of the control ($p < 0.05$; Table 1; Supplementary Fig. 2; see Supplementary material at www.liebertonline.com). Additionally, the number of Sox2-positive cells in all treatment groups was approximately 50% less than that of the control blastocysts ($p < 0.05$; Table 1).

Apoptotic effect of various chemicals on early mouse embryos

Because a reduction in the number of Sox2-positive cells and an increase in the ratio of Cdx2 and Sox2-positive cells in all treatment groups were observed (Table 1), one may be concerned with potential adverse effects of these supple-

FIG. 2. TUNEL assay on mouse blastocysts cultured in ACTH, MAP2K1 (I), and MAPK14 (I). Apoptosis in mouse blastocysts was determined by TUNEL assay. Each row indicates a different treatment. (a) Control, (e) ACTH, (i) MAP2K1 (I) and (m) MAPK14 (I) are transmission light images of blastocysts cultured in the different supplements. The embryonic cell nuclei were stained with Hoechst 33342 (b,f,j,n) and TUNEL-positive cells were labeled with green fluorescence (c,g,k,o). The colocalization of the TUNEL-positive cells and nuclei was demonstrated by overlaying images captured by confocal microscopy (d,h,l,p). Bar = 50 μ m.

TABLE 3. LOCALIZATION OF TUNEL-POSITIVE CELLS IN BLASTOCYSTS DERIVED FROM DIFFERENT TREATMENTS

Treatment	No. of embryos	Total cells (Average $\pm$ SEM)	TUNEL-positive cells (Average $\pm$ SEM)	
			TE (%)	ICM (%)
Control	3	52.0 $\pm$ 2.9	2.7 $\pm$ 0.3 (5.2)	0.7 $\pm$ 0.7 (1.3) ^a
ACTH	3	51.0 $\pm$ 3.9	2.3 $\pm$ 0.3 (4.6)	0.7 $\pm$ 0.3 (1.4) ^a
MAP2K1 (I)	3	60.6 $\pm$ 4.3	4.0 $\pm$ 1.0 (6.6)	1.3 $\pm$ 0.2 (2.1) ^a
MAPK14 (I)	3	46.7 $\pm$ 7.8	5.0 $\pm$ 3.7 (10.7)	5.3 $\pm$ 2.7 (11.3) ^b

^{a,b}Superscript in the same column indicates a significant difference at $p < 0.05$.

ments on early embryonic development. Here we investigated if these supplements increased apoptosis in the resulted blastocysts. To determine the magnitude of apoptosis, we first determined the total number of apoptotic cells and then calculated the ratio of apoptotic TE and ICM cells. The total cell count of MAP2K1 (I)-derived blastocysts did not differ from that of the control and ACTH groups, whereas those supplemented with MAPK14 (I) had a reduced number compared to the control and MAP2K1 (I) but did not differ from the ACTH group (Table 2). The DCI indicated that MAPK14 (I)-derived blastocysts have the highest dead cell rate among all treatment groups (Fig. 2; Table 2). Confocal images further confirmed that MAPK14 (I)-derived blastocysts had the highest number of apoptotic cells in both the ICM and TE (Table 3; $p < 0.05$). These results strongly suggest an adverse effect of MAPK14 (I) on cellular integrity that may result in an increase of cell death.

Development of single blastomeres supplemented with various chemicals

This study aimed to develop an optimal culture condition for single blastomeres of 2C and 4C embryos supplemented with ACTH, MAP2K1 (I) and MAPK14 (I). Embryos and outgrowths derived from single blastomeres were then used for the establishment of ES cells. Among these supplements, ACTH had no adverse effect on the development of early blastomeres, and was comparable to that of the control (Fig. 1B and C). On the other hand, both MAP2K1 (I) and MAPK14 (I) inhibited the development of 2CIB and 4CIB derived embryos (Fig. 1B and C), MAPK14 (I) having the strongest inhibitory effect.

Effect of supplements on ICM size and the establishment of ES cells from single blastomere derived embryos

We have demonstrated that each supplement has a different degree of impact on the development of blastomeres, embryos, and outgrowths. We were also interested in determining the impact of supplements on ICM formation and the establishment of ES cells. In 2CIB, only 9.7% (7 of 72) of the blastocyst outgrowths from the MAPK14 (I) group formed an ICM, which was much lower than that of the control (27.8%; 20 of 72), ACTH (38.9%; 28 of 72), and MAP2K1 (I) (36.1%; 26 of 72) (Table 4). Among these supplements, MAP2K1 (I)-derived blastocyst outgrowths had the largest ICM and the MAPK14 (I) treatment group had the smallest ICM. The average size of the ICM in ACTH ($10.8 \times 10^4 \mu\text{m}^2 \pm 2.5 \times 10^4$) and MAP2K1 (I) ($19.3 \times 10^4 \mu\text{m}^2 \pm 2.0 \times 10^4$) treatment groups were not different from that of control ($9.6 \times 10^4 \mu\text{m}^2 \pm 4.2 \times 10^4$; $p > 0.05$), and significantly differed from that of the MAPK14 (I) group ($5.4 \times 10^4 \mu\text{m}^2 \pm 1.2 \times 10^4$; $p < 0.05$). However, no ESC line was able to be established from the ICMs of either MAPK inhibitor group, and only those ICMs of the control and ACTH derived blastocyst outgrowths were capable of establishing ES cell lines.

In 4CIB cultured with and without the supplements, the ICM formation rate did not differ between treatments compared to the control (Table 4). Similar to the 2CIB, the MAP2K1 (I) group had the biggest ($8.7 \times 10^4 \mu\text{m}^2 \pm 1.4 \times 10^4$) ICM and MAPK14 (I) group had the smallest ($3.5 \times 10^4 \mu\text{m}^2 \pm 1.0 \times 10^4$) ICM. No ES cell line was established from ICMs derived from either of these treatment groups, which was consistent with the 2CIB study. However, ES cell lines

TABLE 4. THE EFFECT OF DIFFERENT CHEMICALS ON THE DEVELOPMENT OF SINGLE BLASTOMERE-DERIVED OUTGROWTHS AND THE DERIVATION OF ES CELL LINES

Embryo stage	Treatment	No. embryo	No. blastomere	No. subcultured ICM (%)	Average ICM size $\pm$ SD (μm^2)	ES cell line (%)
2CBD	Control	36	72	20 (27.8) ^a	$9.6 \times 10^4 \pm 19.0 \times 10^4$; ^{a,b}	5 (6.9) ^a
	ACTH	36	72	28 (39.9) ^a	$10.8 \times 10^4 \pm 13.7 \times 10^4$; ^{a,b}	9 (12.5) ^a
	MAP2K1 (I)	36	72	26 (36.1) ^a	$19.3 \times 10^4 \pm 9.9 \times 10^4$; ^a	0 (0.0) ^b
	MAPK14 (I)	36	72	7 (9.7) ^b	$5.4 \times 10^4 \pm 3.2 \times 10^4$; ^b	0 (0.0) ^b
4CBD	Control	18	72	8 (11.1)	$6.8 \times 10^4 \pm 6.3 \times 10^4$	0 (0.0) ^b
	ACTH	18	72	5 (6.9)	$6.6 \times 10^4 \pm 5.3 \times 10^4$	3 (4.2) ^a
	MAP2K1 (I)	18	72	7 (9.7)	$8.7 \times 10^4 \pm 3.2 \times 10^4$	0 (0.0) ^b
	MAPK14 (I)	18	72	7 (9.7)	$3.5 \times 10^4 \pm 2.3 \times 10^4$	0 (0.0) ^b

^{a,b}Superscript in the same column indicates a significant difference at $p < 0.05$.

could be established from 4CIB-derived embryos supplemented with ACTH (Table 4).

In blastomeres derived from 2CIB embryos, five ES cell lines were established from the control group (6.9%; 5 of 72) and nine ES cell lines were established from the ACTH group (12.5%; 9 of 72). Three (4.2%; 3 of 72) ES cell lines were established from 4CIB supplemented with ACTH (Table 4). However, no ES cell line was established from any of the sister blastocyst outgrowths derived from either 2CIB or 4CIB.

Characterization of single blastomere derived ES cell lines

We have established a total of 17 ES cell lines from 2CIB and 4CIB-derived embryos. To confirm their stem cell properties, immunostaining was performed using stem cell markers commonly used for mouse ES cells. All ES cell lines were characterized after six passages. Stem cell markers such as alkaline phosphatase, Oct-4, Sox2, Nanog, and SSEA-1 are expressed in mouse ES cells (Supplemental Fig-

FIG. 3. *In vitro* differentiation of the ES cells derived from single blastomere-derived mouse embryos. *In vitro* differentiation of ES cells derived from single blastomere-derived mouse embryos. Each row represents the same sample. The first row is EB stained with endoderm marker (AFP: α -fetoprotein) and ectoderm marker (vimentin), the second row is ES cell-derived progenitor cells (PGC; Nestin) in N1 medium and the third to fifth rows are ES cell-derived neuronal cell types (tyrosine hydroxylase, TH; β -III tubulin, TuJ1; choline acetyltransferase, ChAT) in N3 medium. The first column from the left is the transmission light image, the second column is DNA stained with Hoechst, the third column is immunostaining using specific antibodies, and the fourth column is the overlaid images of columns 2 and 3 except for EB, which was stained with vimentin. Bar = 100 μ m.

ure 3; see online Supplementary material at www.liber-tonline.com). Additionally, *in vitro* differentiation capability was evaluated by differentiation toward a neuronal lineage and then confirmed by immunostaining using stage and cell-type specific antibodies. All ES cell lines were capable of forming EBs and were positive for endoderm (AFP) and ectoderm (vimentin) markers (Fig. 3). EBs were then allowed to attach onto a gelatin coated dish and cultured in N1, N2, and N3 mediums (Kuo et al., 2003), subsequently. Neuroprogenitor cells (NPCs) were induced, selected, and enhanced in N1 and N2 mediums, and were then confirmed by the expression of Nestin. Mature neurons were induced in N3 medium and were confirmed by the expression of TuJ1, TH, and ChAT (Fig. 3). Here we demonstrated the successful establishment of mouse ES cell lines from single blastomere-derived embryos and their pluripotent differentiation capability was also confirmed. These results also suggest the positive effect of ACTH on the derivation of mouse ES cells from a single blastomere.

Discussion

The objective of this study was to determine if ACTH, MAP2K1 (I) and MAPK14 (I) affect early embryo development and if they can enhance the derivation of ES cells from early embryonic blastomeres. The effect on blastomere development and the establishment of ES cell lines was determined in parallel with the control group. ACTH and MAP2K1 (I) had no adverse effect on the development of control embryos (Fig. 1A). However, the development of 2CIB-derived embryos was inhibited by both MAP2K1 (I) and MAPK14 (I) (Fig. 1B), while 4CIB derived embryos were only inhibited by MAPK14 (I) (Fig. 1C). Although the development rate of 2CIB derived embryos was reduced in MAP2K1 (I) culture (Fig. 1B), the development of blastocyst outgrowths was comparable to that of the ACTH and control groups (Table 4). We observed a reduced negative effect of MAPK inhibitors on 4CIB derived embryos when compared to the culture of 2CIB derived embryos (Fig. 1C). This suggested the impact of MAPK inhibitors on 2CIB might be related to the maternal embryonic transition (MET) and the activation of the embryonic genome at the two-cell stage (Schultz, 1986).

ACTH is a peptide hormone produced from the pituitary gland, and plays an important role in early development. ACTH could enhance ES cell proliferation and the inhibitory effect of ACTH on the G-protein coupled receptor pathway might be the underlying mechanism (Ogawa et al., 2004). A recent study by Wakayama and colleagues (2007) has further confirmed the role of ACTH in supporting the propagation and derivation of pluripotent ES cells. Here we demonstrated the derivation of ES cells from 2CIB and 4CIB derived embryos with and without the supplement of ACTH (Table 4). Despite a comparable embryo development rate (Fig. 1B), the ES cell establishment rate was higher in the 2CIB-ACTH group (12.5%; 9 of 72) than that of the 2CIB-control group (6.9%; 5 of 72) (Table 4). However, ES cell lines could only be established from 4CIB if ACTH was presented. These results suggest a positive effect of ACTH in supporting ES cell derivation from blastomeres of early mouse em-

bryos (Table 4), which was consistent with the prior report (Wakayama et al., 2007).

The supplement of ACTH in single blastomere culture has resulted in a comparable development rate to that of the control, which was superior to that of MAP2K1 (I) and MAPK14 (I). The positive effect of ACTH on ES cell derivation was further demonstrated in our study. A total of nine and three ES cell lines were established from 2CIB and 4CIB derived embryos (Table 4). Interestingly, ES cell lines could only be established from 4CIB derived embryos with the supplement of ACTH (Table 4).

Although no ES cell line was established from MAP2K1 (I) treated embryos, MAP2K1 (I) has a positive impact on ICM development in both 2CIB and 4CIB derived blastocyst outgrowths (Table 4). MAP2K1 (I) targets the upstream events of the MAPK2/3 pathway and selectively inhibits the phosphorylation of the MAPK2/3, thus inhibiting the activity of the MAPK kinase cascade (Alessi et al., 1995; Dudley, et al., 1995). Instead of inducing apoptosis in TE cells, MAP2K1 (I) enhanced ICM growth, which in return suppressed the development of TE cells (Table 2). However, the competency of the ICM cells remains questionable because no ES cell line was established from such an ICM in this study. One question is whether MAP2K1 (I) alone is sufficient to enhance ICM development and maintain stem cell properties. Establishment of ES cell lines with the supplement of MAP2K1 (I) was only reported with the coculture of mouse ES cells (Chung et al., 2006); thus, the synergistic role of "stem-cell autocrine factor" derived from the ES cells and the role of MAP2K1 (I) in stemness merit further investigation.

MAPK14 (I) inhibits p38 activity by binding at the ATP site (Davies et al., 2000; Young et al., 1997). Frantz and colleagues (1999) reported that SB203580 (MAPK14-I) bind to both unphosphorylated and phosphorylated MAPK14. Due to the robust inhibitory effect of MAPK14 (I) in the MAPK cascade, a more severe outcome was observed compared to that of the MAP2K1 (I). Single blastomere-derived embryos cultured in MAPK14 (I) have the lowest number of subcultured ICMs compared to the other treatment groups (Table 4). Although MAPK14 (I) was also known for having a positive effect on pluripotency of mouse ES cells and the derivation of ES cell lines from *Alk3*^{-/-} embryos (Duval et al., 2004; Qi et al., 2004), a higher percentage of apoptotic cells in the ICMs of the blastocysts was revealed in the MAPK14 (I) group (11.3%) (Table 3). Furthermore, we also found that MAPK14 (I) increases the apoptotic rate in blastocysts (Table 2) and suppressed preimplantation embryo development (Supplementary Table 1). The opposite effect of the MAPK14 (I) on early embryos and ES cells is unclear. One possible explanation is the nonspecific inhibitory effect of MAPK14 (I) on important kinases during early embryonic development. These findings suggest a distinct role of MAPK14 in preimplantation embryos and ES cells.

Our study demonstrated the inhibitory effect of MAPK inhibitors on TE development in early embryos (Table 1). This is consistent with a study measuring the level of secreted hCG after the inhibition of the MAPK2/3 or MAPK14 pathway in the human placenta, which resulted in the delay of TE differentiation (Daoud et al., 2006). Our study was based on the hypothesis that the manipulation of cell proliferation

and differentiation through the MAPK2/3 or MAPK14 pathway might increase the likelihood of success in establishing ES cells. However, our results clearly demonstrated that inhibiting the MAPK2/3 and the MAPK14 pathways was not sufficient to enhance stem cell properties of the ICM cells or to improve the derivation rate of ES cells from a single blastomere.

In conclusion, we performed a comparative study on a peptide hormone and two kinase inhibitors and determined their impact on early embryo development and ES cell derivation from single blastomeres. We have established 17 pluripotent ES cell lines from blastomeres of 2CIB and 4CIB derived embryos. Of these, five (6.9%) and nine (12.5%) ES cell lines were established from 2CIB-control and 2CIB-ACTH, respectively. Three (4.2%) ES cell lines were established from blastomeres of 4CIB-derived embryos supplemented with ACTH. The supplement of MAPK inhibitors, MAP2K1 (I) and MAPK14 (I), were not able to support and enhance the derivation of ES cells from a single blastomere. We have demonstrated that ES cell lines can be efficiently established from single blastomere-derived blastocysts with the supplement of ACTH.

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Author Disclosure Statement

The authors declare that no financial conflicts exist.

References

- Alessi, D.R., Cuenda, A., Cohen, P., et al. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J. Biol. Chem.* 270, 27489–27494.
- Baharvand, H., Ashtiani, S.K., Taei, A., et al. (2006). Generation of new human embryonic stem cell lines with diploid and triploid karyotypes. *Dev. Growth Differ.* 48, 117–128.
- Binetruy, B., Heasley, L., Bost, F., et al. (2007). Regulation of embryonic stem cell lineage commitment by mitogen activated protein kinases. *Stem Cells* 25, 1090–1095.
- Chung, Y., Klimanskaya, I., Becker, S., et al. (2006). Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. *Nature* 439, 216–219.
- Chung, Y., Klimanskaya, I., Becker, S., et al. (2007). Human embryonic stem cell lines generated without embryo destruction. *Cell Stem Cell* 2, 113–117.
- Daoud, G., Amyot, M., Rassart, E., et al. (2006). MAPK2/3 and MAPK14 regulate trophoblasts differentiation in human term placenta. *J. Physiol.* 566, 409–423.
- Davies, S.P., Reddy, H., Caivano, M., et al. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* 351, 95–105.
- Delhaise, F., Bralio, V., Schuurbijs, N., et al. (1996). Establishment of an embryonic stem cell line from 8-cell stage mouse embryos. *Eur. J. Morphol.* 34, 237–243.
- Dudley, D., Pang, L., Decker, S., et al. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* 92, 7686–7689.
- Duval, D., Malaise, M., Reinhardt, B., et al. (2004). A P38 inhibitor allows to dissociate differentiation and apoptotic processes triggered upon LIF withdrawal in mouse embryonic stem cells. *Cell Death Differ.* 11, 331–341.
- Eistetter, H.R. (1989). Pluripotent embryonic stem cells can be established from disaggregated mouse morulae. *Dev. Growth Differ.* 31, 275–282.
- Erbach, G.T., Lawitts, J.A., Papaioannou, V.E., et al. (1994). Differential growth of the mouse preimplantation embryo in chemically defined media. *Biol. Reprod.* 50, 1027–1033.
- Evan, M., and Kaufman, M. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154–156.
- Frantz, B., Klatt, T., Pang, M., et al. (1998). The activation state of p38 mitogen-activated protein kinase determines the efficiency of ATP competition for pyridinylimidazole inhibitor binding. *Biochemistry* 37, 13846–13853.
- Heins, N., Lindahl, A., Karlsson, U., et al. (2006). Clonal derivation and characterization of human embryonic stem cell lines. *J. Biotechnol.* 122, 511–520.
- Klimanskaya, I., Chung, Y., Becker, S., et al. (2006). Human embryonic stem cell lines derived from single blastomeres. *Nature* 444, 481–485.
- Kuo, H., Pau, K., Yeoman, R., et al. (2003). Differentiation of monkey embryonic stem cells into neural lineages. *Biol. Reprod.* 68, 1727–1735.
- Lorthongpanich, C., Yang, S.H., Piotrowska-Nitsche, K., et al. (2008). Development of single mouse blastomeres into blastocysts, outgrowths and the establishment of embryonic stem cells. *Reproduction* 135, 805–813.
- Maekawa, M., Yamamoto, T., Tanoue, T., et al. (2005). Requirement of the MAP kinase signaling pathways for mouse pre-implantation development. *Development* 132, 1773–1783.
- Nagy, A. and Gertsenstein, M. (2003). Manipulating the mouse embryo. In *A Laboratory Manual*. K. Vintersten and R. Behringer, eds. (Cold Spring Harbor, NY, Cold Spring Harbor Press) pp. 1–764.
- Neuber, E., Luetjens, C., Chan, A.W.S., et al. (2002). Analysis of DNA fragmentation of in vitro cultured bovine blastocysts using TUNEL. *Theriogenology* 57, 2193–2202.
- Ogawa, K., Matsui, H., Ohtsuka, S., et al. (2004). A novel mechanism for regulating clonal propagation of mouse ES cells. *Genes Cells* 9, 471–477.
- Qi, X., Li, T., Hao, J., et al. (2004). BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. *Proc. Natl. Acad. Sci. USA* 101, 6027–6032.
- Schultz, G.A. (1986). Molecular biology of the early mouse embryo. *Biol. Bull.* 171, 291–309.
- Strelchenko, N., Verlinsky, O., Kukharensky, V., et al. (2004). Morula-derived human embryonic stem cells. *Reprod. Biomed. Online* 9, 623–629.

- Suemori, H., Tada, T., Torii, R., et al. (2001). Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. *Dev. Dyn.* 222, 273–279.
- Tesar, P. (2005). Derivation of germ-line-competent embryonic stem cell lines from preblastocyst mouse embryos. *Proc. Natl. Acad. Sci. USA* 102, 8239–8244.
- Thomson, J., Kalishman, J., Golos, T., et al. (1995). Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. USA* 92, 7844–7848.
- Wakayama, S., Hikichi, T., Suetsugu, R., et al. (2007). Efficient establishment of mouse embryonic stem cell lines from single blastomeres and polar bodies. *Stem Cells* 25, 986–993.
- Young, P., McLaughlin, M.M., Kumar, S., et al. (1997). Pyridinyl imidazole inhibitors of p38 mitogenactivated protein kinase bind in the ATP site. *J. Biol. Chem.* 272, 12116–12121.

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