Development of single mouse blastomeres into blastocysts, outgrowths and the establishment of embryonic stem cells

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Abstract

The recently developed technique of establishing embryonic stem (ES) cell lines from single blastomeres (BTMs) of early mouse and human embryos has created significant interest in this source of ES cells. However, sister BTMs of an early embryo might not have equal competence for the development of different lineages or the derivation of ES cells. Therefore, single BTMs from two- and four-cell embryos of outbred mice were individually placed in sequential cultures to enhance the formation of the inner cell mass (ICM) and the establishment of embryonic outgrowth. The outgrowths were then used for the derivation of ES cell lines. Based on the expression of ICM (*Sox2*) and trophoblast (*Cdx2*) markers, it was determined that ICM marker was lacking in blastocysts derived from 12% of BTMs from two-cell stage and 20% from four-cell stage. Four ES cell lines (5.6%; 4/72) were established after culture of single BTMs from two-cell embryos, and their pluripotency was demonstrated by their differentiation into neuronal cell types. Our results demonstrate that sister BTMs of an early embryo are not equally competent for ICM marker expression. However, we demonstrated the feasibility of establishing ES cells from a single BTM of outbred mice.

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Introduction

The competence of early embryonic blastomeres (BTMs) is one of the fundamental questions in developmental biology that has not been fully elucidated (Tarkowski 1959, Tarkowski & Wroblewska 1967, Rossant 1976, Tsunoda & McLaren 1983, Papaioannou *et al.* 1989, Chan *et al.* 2000, Piotrowska *et al.* 2001, Piotrowska-Nitsche & Zernicka-Goetz 2005, Piotrowska-Nitsche *et al.* 2005). The successful implantation of a mouse four-cell BTM-derived embryo has been reported, but no full-term development resulted (Rossant 1976). Later studies on two-cell mouse embryos have demonstrated that only one BTM of a two-cell mouse embryo was capable of normal development resulting in a live birth (Tarkowski 1959, Papaioannou *et al.* 1989). On the other hand, Tsunoda & McLaren (1983) have reported four sets of monozygotic twins at day 18–19 of gestation. Recent studies on embryo splitting have further demonstrated the pluripotency of embryonic BTMs. Unfortunately, not all BTMs were able to develop to term (Heyman *et al.* 1998, Chan *et al.* 2000, Mitalipov *et al.* 2002). On the contrary, two-, four-, and eight-cell sheep embryos show no difference in producing

monozygotic twins (Willadsen 1979). Although monozygotic twins have been produced by separating two-cell embryos in some species (Willadsen 1979, Willadsen & Polge 1981, Willadsen *et al.* 1981, Ozil 1983, Allen & Pashen 1984, Tsunoda *et al.* 1985, Matsumoto *et al.* 1989), there was only one report showing the pluripotency of four-cell BTMs that produced quadruplets (four identical calves) (Johnson *et al.* 1995).

The switching of embryonic BTMs at the two-, four-, and eight-cell stage (chimaerism) further demonstrates the competency of BTMs without affecting later development. These studies suggest that sister BTMs are capable of compensating for lost or damaged BTMs in an embryo (Fehilly *et al.* 1984, Tsunoda *et al.* 1987, Avis & Anderson 1988). For years, individual BTMs in early embryos, prior to polarization at the morula stage, were thought to be pluripotent, if not totipotent. The question now is whether the development of a single BTM can be seen as a linear clonal process whereby a particular stem cell gives rise to the inner cell mass (ICM) or trophoblast (TE). The results of lineage-tracing experiments on labeled two- and four-cell BTMs support the idea of a developmental bias. It has also been found that although each two-cell BTM gives

rise to both the ICM and the TE lineages, one cell tends to contribute more to the embryonic part of the blastocyst and the other contributes to the abembryonic part of the blastocyst (Gardner 2001, Fujimori *et al.* 2003, Piotrowska-Nitsche & Zernicka-Goetz 2005). Additionally, the four-cell stage BTM that inherits vegetal membrane tends to contribute to mural trophoctoderm (Piotrowska-Nitsche *et al.* 2005). This bias in the allocation of a two- and four-cell BTM progeny suggests that the developmental competence of the sister BTMs, and their lineage fates, perhaps are established at an even earlier embryonic stage.

Embryonic stem (ES) cells have been successfully established from early embryos rather than using the ICM of a blastocyst (Eistetter 1989, Strelchenko *et al.* 2004, Tesar 2005). It is noted that only a small number of BTMs from 8-cell (Delhaise *et al.* 1996) and 16-cell (Eistetter 1989) mouse embryos were viable for deriving ES cells. Wilton & Trounson (1989) attempted to establish ES cells from the biopsy BTM of a four-cell mouse embryo, but the attempt was unsuccessful in that only trophoblast-like cells resulted. Recently, Wakayama *et al.* (2007) demonstrated that ES cell lines could be derived from the BTMs of early mouse embryos, with the two-cell BTM having the highest success rate (Wakayama *et al.* 2007). It was also noted that the success rate was reduced by ~50% at each additional cell division. This study suggests that only one or two BTMs of an embryo retain stem cell properties and are capable of deriving ES cells up to the eight-cell stage.

The latest reports support the idea of establishing personal ES cell lines from the single biopsy BTMs of eight-cell mouse embryos and the establishment of an extraembryonic stem cell line from a single BTM recovered from eight- to ten-cell human embryos (Chung *et al.* 2006, Klimanskaya *et al.* 2006). Because the establishment of ES cells from a single biopsy BTM has a low success rate (Chung *et al.* 2006, Klimanskaya *et al.* 2006) and as only a few of the BTMs retain their stem cell properties and are capable of deriving ES cells, the question becomes: can an ES cell line be established from sister BTMs that have a distinct lineage fate? It is important to determine if the random retrieval of early embryonic BTMs for the derivation of ES cells would jeopardize the integrity of an embryo and its later development. We aim to investigate if the sister BTMs of the two- and four-cell mouse embryos have equal developmental capacity and lineage markers expression. Finally, we also aim to determine if ES cells could be established from the single BTMs of outbred mouse embryos.

Results

Development of two- and four-cell mouse embryos and isolated BTMs

We first compared the developmental rate of the zona-free two-cell whole (2CW) and four-cell whole (4CW) mouse embryos, as well as the individual culture of the single

BTMs derived from two-cell isolated BTM (2CIB) and four-cell isolated BTM (4CIB) mouse embryos, in both the KSOM and mES media. Approximately 65.3% (47/72) of the 2CW embryos developed to blastocyst on day 3 and 87.5% (63/72) attached to the feeder cells by day 6 when cultured in the KSOM medium (2CW-K; Table 1). When compared with KSOM, only 9.7% (7/72) of the 2CW embryos cultured in the mES medium developed to blastocyst stage (Table 1). These results suggest that KSOM is better suited to support the development of zona-free two-cell mouse embryos when compared with that of the mES medium (Table 1; $P < 0.05$).

When 2CIB KSOM (2CIB-K) embryos were cultured individually, over 90% (67/72) of the blastocysts attached to the feeder cells and formed an outgrowth (Table 1). On the other hand, only 6.9% (5/72) of the 2CIB-ES BTM cultures reached the blastocyst stage and attached to the feeder cells on day 6. This was much lower than that of the 2CIB-K (Table 1; $P < 0.05$).

Although both of the 2CIB BTM-derived embryos formed blastocysts and outgrowths when cultured in KSOM, ~60% (22/36) of outgrowths from both sister BTMs have prominent ICM, and only 25% (9/36) of the outgrowth has prominent ICM from one of the sister BTMs (Fig. 1B and I; Table 1).

In addition to two-cell embryos, the development of 4CW and 4CIB mouse embryos in sequential culture was also evaluated (Table 1). In the 4CIB culture, the developmental rate was not affected when cultured in either media. However, the attachment rate was greatly enhanced in the 4CIB-K-derived blastocysts (Table 1). Even though most of the 4CIB-derived blastocysts were able to attach and form an outgrowth, only 44.4% (8/18), 22.2% (4/18), and 5.5% (1/18) of the 4CIB-derived embryos had one, two, or three out of the four sister outgrowths form visible ICM clumps respectively (Fig. 1E–L; Table 1).

Besides the effect of the culture medium on the developmental rate, the size of the embryos was also affected. Blastocysts derived from the 2/4CW-K embryos were bigger with more prominent and distinctive ICMs and TE cells than those cultured in the mES medium (data not shown). In the isolated BTM study, the sizes of the 2CIB and 4CIB BTM-derived blastocysts are about one-half and one-quarter of a normal embryo respectively (data not shown).

Spatial expression of cell fate determining genes in the isolated BTMs-derived embryos and outgrowths

We were interested in investigating the expression pattern of stem cell and TE markers in isolated BTMs-derived embryos and outgrowths, thus predicting their lineage fate. We chose *Sox2* to represent the pluripotent genes in the following experiments because it provided a more specific expression pattern and it was tightly restricted in ICM cells, whereas the expression of *Oct4* was primarily in the ICM with a low level expression in the TE cells

Table 1 The effects of culture medium on development and outgrowth formation of whole embryos and isolated blastomeres.

Embryo stage	Media	2CW	2CIB	4CW	4CIB
% Blastocyst formation (D3)	mES	7/72* (9.7)	2/72* (2.8)	46/72 (63.9)	19/72 (26.4)
	KSOM	47/72 [†] (65.3)	28/72 [†] (38.9)	54/72 (75.0)	27/72 (37.5)
% Outgrowth (D6)	mES	7/72* (9.7)	5/72* (6.9)	47/72* (65.3)	18/72* (25.0)
	KSOM	63/72 [†] (87.5)	67/72 [†] (90)	68/72 [†] (94.4)	56/72 [†] (77.8)
% Outgrowth with ICM (D6)	mES	7/7 (100.0)	1 BTM=0/36 (0)	47/47 (100)	1 BTM=3/18 (16.7)
			2 BTM=0/36 (0)		2 BTM=1/18 (5.5)
					3 BTM=0/18 (0)
					4 BTM=0/18 (0)
					1 BTM=8/18 (44.4)
	KSOM	63/63 (100.0)	1 BTM=9/36 (25.0)	68/68 (100)	2 BTM=4/18 (22.2)
			2 BTM=22/36 (61.1)		3 BTM=1/18 (5.5)
					4 BTM=1/18 (5.5)

BTM, blastomere; D3 and D6 were day 3 and day 6 after culture respectively. *[†]Superscripts in the same column of the same embryonic stage indicate significant difference at $P < 0.05$. % Outgrowth with ICM was calculated based on the number of embryos with ICM divided by the total number of embryonic outgrowths and statistical analysis was not performed in this group. 1, 2, 3, and 4 BTM refers to the number of ICM appearance in 1, 2, 3, and 4 blastomere-derived outgrowth. % Outgrowth (D6) and % outgrowth with ICM (D6) came from individual experiments that were based on the number of isolated blastomeres and the number of cultured embryos respectively.

(Supplementary Figure 1, which can be viewed online at www.reproduction-online.org/supplemental).

The 2CIB- and 4CIB-derived embryos at 4 days after culture were used for determining the ratio of TE cells to ICM cells. Immunostaining was performed by colabeling with *Sox2*- and *Cdx2*-specific antibodies, and the positive cells were counted under a fluorescence microscope (Fig. 2A and B). The normal embryos were used as a control. A total of 58 BTMs derived from embryos

were used: 2CIB (9×2 BTMs = 18), 4CIB (5×4 BTMs = 20), and control (20 embryos) respectively. The total number of cells in the 2CIB- (28.1 ± 12.5) and 4CIB- (13.9 ± 4.5) derived blastocysts was reduced by ~ 55 and $\sim 78\%$ of a control embryo (62.8 ± 11.1 ; Fig. 3). A similar ratio was also observed in the *Cdx2*-positive cells (TE) and the *Sox2*-positive cells (ICM) while the overall ratio of the *Cdx2* (TE) and *Sox2* (ICM) remained the same in the 2CIB, 4CIB, and normal embryos (Fig. 3). Although the ratio of

Figure 1 Blastocysts and outgrowths derived from 2CIB and 4CIB. (A–D) 2CIB-derived blastocysts with visible ICM clump (A) and non-visible ICM clump (trophoblastic vesicle; C). Visible ICM clump could be observed in one embryonic outgrowth after attachment to the feeder cells (B) whereas the other outgrowth developed to TE (D); arrows indicate ICM; Bar, 30 μ m. (E–H) 4CIB-derived blastocysts, in which only one blastomere (E) has visible ICM, and non-visible ICM was observed in (F–H) sister blastomere-derived blastocysts; arrow indicates ICM; Bar, 30 μ m. (I–L) 4CIB-derived embryo outgrowths. There is only one outgrowth that could form a visible ICM clump (I; arrow) while the others could attach to the feeder but do not have ICM clump (J–L); Bar, 50 μ m.

Figure 2 The expression of stem cell (*Sox2*) and trophectoderm (TE; *Cdx2*) markers in blastocysts and outgrowths derived from 2CIB- and 4CIB-derived embryos and outgrowths. (A) Blastocysts derived from two sister blastomeres of a 2CIB embryo. Transmission light image (a); Hoechst DNA staining (b, blue); *Cdx2* (c, green); *Sox2* (d, red); and overlay images of *Cdx2* and *Sox2* (e); arrow indicates *Sox2*-positive cells; Bar, 100 μ m. (B) Blastocysts derived from four sister blastomeres of a 4CIB embryo (a, f, k and p); Hoechst DNA staining (b, g, l and q; blue); *Cdx2* (c, h, m and r; green); *Sox2* (d, i, n and s; red) and overlay images of *Cdx2* and *Sox2* (e, j, o and t); arrows indicate *Sox2*-positive cells; Bar, 30 μ m. (C) Embryonic outgrowths derived from two sister blastomeres of a 2CIB embryo. The first outgrowth (a–e) was derived from 2CIB blastocyst with visible ICM, whereas the second outgrowth (f–j) was derived from blastocyst without visible ICM. Transmission light image (a and f); Hoechst DNA staining (b and g; blue); *Cdx2* (c and h; green); *Sox2* (d and i; red), and overlay image of *Cdx2* and *Sox2* (e and j); arrow indicates ICM; Bar, 100 μ m. (D) Embryonic outgrowths derived from four sister blastomeres of a 4CIB embryo. The first outgrowth (a–e), second outgrowth (f–j), third outgrowth (k–o), and fourth outgrowth (p–t) were cultured individually and had only one outgrowth with visible ICM clump and *Sox2*-positive cells with intense signal. There are cell negative for both *Cdx2* and *Sox2* suggesting spontaneous differentiation beyond the TE lineage. Transmission light image (a, f, k and p); Hoechst DNA staining (b, g, l and q; blue); *Cdx2* (c, h, m and r; green); *Sox2* (d, i, n and s; red), and overlay image of *Cdx2* and *Sox2* (e, j, o and t); arrow indicates ICM clump; Bar, 100 μ m.

the total cell numbers between the groups and the ratio of *Sox2* to *Cdx2* remain unchanged, only 88.1% (16/18) of the 2CIBs and 80% (16/20) of the 4CIBs had a visible ICM and were positive for *Sox2*, whereas all blastocysts from the normal embryos had a visible ICM.

In addition to immunocytochemistry on the 2CIB- and 4CIB-derived blastocysts, we determined the expression patterns of a stem cell marker (*Sox2*) and a TE marker (*Cdx2*) in the embryo outgrowths (Fig. 2C and D). We aimed to determine if each BTM had a distinct fate and if they were equally competent to form the ICM or develop a stem cell lineage based on the expression pattern of the stem cell marker. Our results in the 2CIB- and 4CIB-derived embryonic outgrowths were consistent with the blastocysts (Fig. 3). The blastocysts derived from the 2CIBs were capable of attaching and developing an embryonic outgrowth regardless of the presence of the ICM. However, only those with a visible ICM in a blastocyst formed an ICM clump in an outgrowth (Fig. 1). In addition to the *Sox2*- and *Cdx2*-positive cells, there were cell types with a distinctive morphology that were not recognized by either antibody. This suggests a spontaneous differentiation toward more advanced cell types than the TE lineage occurring in the outgrowth (Fig. 2D).

ES cell derivation from a single BTM of outbred mouse embryos

We have demonstrated that a single BTM can be continually cultured and developed *in vitro*. However, the embryos and outgrowths derived from single BTMs of two- and four-cell embryos did not show equal competence for the formation of the ICM, and a biased expression of stem cell determinant genes was also observed. Here, we aimed to determine if ES cells could be derived from single BTMs of outbred stock (CD-1) mouse embryos

Figure 3 Cell number of blastocysts derived from isolated blastomeres. ^{a,b,c}Superscripts in the same column indicates significant difference at $P < 0.05$.

rather than inbred mice, such as 129/SV, which are commonly used for the derivation of ES cell lines.

The 2CIBs and 4CIBs were individually cultured in the KSOM medium until the resulting blastocysts attached to the feeder cells. At that point, the KSOM was replaced by the mES medium for subsequent culture. Visible ICM clumps were mechanically isolated, trypsinized, and continued in culture. The established ES cell lines were then used for further characterization and analyses. As a result, we established four ES cell lines from the 2CIBs (5.6%, 4/72; Table 2), while no ES cell lines could be established from the 4CIBs (0%, 0/72; Table 2). There was no ES cell line established from the sister outgrowth that was derived from the 2CIB-derived embryo.

These results suggested that ES cell lines could be derived from a single BTM of the 2CIB-derived embryos. The stem cell properties of the resulting ES cell lines were confirmed by the expression of stem cell markers, which included *Oct4*, *Sox2*, *Nanog*, *SSEA-1* (*Fut4*), *TRA-1-60*, *TRA-1-81*, and *AP* (Supplementary Figure 2, which can be viewed online at www.reproduction-online.org/supplemental). In order to determine the pluripotency of the ES cell lines derived from 2CIBs, *in vitro* differentiation toward neuronal lineage was performed, followed by immunostaining using endoderm- (α -fetoprotein) and ectoderm- (vimentin) specific antibodies on EBs, as well as antibodies that specifically recognize neuroprogenitor cells (nestin) and neuronal cell types (β -III tubulin, TuJ1; tyrosine hydroxylase, TH; choline acetyltransferase, ChAT; Fig. 4). Here, we demonstrated the successful establishment and differentiation of ES cells from single BTM-derived embryos, outgrowths, and ICM clumps. This validated our single BTM culture system and confirmed that pluripotent ES cell lines can be derived from the 2CIB of outbred CD-1 mice.

Discussion

We first developed a sequential culture scheme for BTM development. A much higher blastocyst rate ($P < 0.05$) resulted when 2CIBs were cultured individually with mouse fetal fibroblasts (MFF) in the KSOM medium than those cultured in the mES medium (Table 1). This suggests that KSOM is sufficient to support the development of a single BTM to the blastocyst stage at a comparable rate with that of whole embryo culture (Table 1). While most of the 2CIB-derived embryos developed to blastocysts and attached to feeder cells in the KSOM medium, nearly all of the blastocysts established embryo outgrowth (Table 1).

Continuous culture of the embryonic outgrowths in the mES medium has resulted in the establishment of four mouse ES cell lines. All mouse ES cell lines express common mouse ES markers (Supplementary Figure 2). Our results clearly show that KSOM is superior to the mES medium in supporting single BTM development to the blastocyst stage, although a previous study suggested that the mES medium is sufficient for the culturing of single BTMs and the isolation of ES cells (Wakayama *et al.* 2007). The discrepancy between the two studies could be explained by the mouse strains that were used for preparing embryos. Several reports have demonstrated the importance of the genetic background of mice on early embryonic development (Bagis *et al.* 2001) and the establishment of ES cells (Kawase *et al.* 1994). Most of the ES cell lines, including the single BTM-derived ES cells described by Wakayama *et al.*, were derived from the 129/SV strain (Sukoyan *et al.* 2002, Tesar 2005, Wakayama *et al.* 2007), which has been shown to be the best mouse strain for establishing an ES cell line.

In this study, we used an outbred CD-1 stock instead of the commonly used 129/SV strain. Although there have been several attempts to establish mouse ES cell lines from CD-1 stock (Suzuki *et al.* 1999, Brook *et al.* 2003), reports on the derivation of CD-1 ES cell lines are very limited. Wakayama *et al.* (2005) have attempted to use somatic cells from male and female CD-1 mice as donor cells for nuclear transfer (NT) and for the establishment of ES cells from the reconstructed blastocyst (Wakayama *et al.* 2005). A much lower morula/blastocyst rate resulted when compared with B6D2 mice. Although no live offspring resulted from the cloned embryos, they were successful in establishing CD-1 ES cells from cloned embryos at a relatively low rate. These studies suggest that ES cells could be established from outbred mice with much less efficiency when compared with that of an inbred strain, which could explain our low establishment rate when deriving ES cells from a single BTM of CD-1 mouse embryos. However, the effect of genetic background on single BTM-derived ES cells merits in depth investigation.

We have also demonstrated that not all sister BTMs from 2CIB and 4CIB embryos form a visible ICM clump in the embryo outgrowth (Fig. 1). The immunocytochemistry study revealed that not all of the 2CIB- and 4CIB-derived embryos expressed *Sox2* (Fig. 2A and B). This is not surprising because there were only a few reports of live offspring derived from a full set of bisect/split embryos that is even more unusual in an advanced embryo. Although the production of monozygotic twins in various species by

Table 2 Efficiency of embryonic stem (ES) cell derivation from 2CIB- and 4CIB-derived embryos.

Embryo stage	# Embryo	# Blastomere	# ICM subcultured (%)	# ES cell line at passage 1 (%)	# ES cell line at passage 6 (%)
2CIB	36	72	30 (41.7)	8 (11.1)	4 (5.6)
4CIB	18	72	8 (11.1)	0 (0)	0 (0)

The percentage was calculated based on the total number of blastomeres.

Figure 4 *In vitro* differentiation of ES cells derived from 2CIB-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 rows 3–5 are ES cell-derived neuronal cell types (β -III tubulin, TuJ1; tyrosine hydroxylase, TH; choline acetyltransferase, ChAT) in N3 medium. The first column from the left is the transmission light image, the second column is DNA (blue) stained with Hoechst, the third column is immunostaining using specific antibodies (green and red) and the fourth column is the overlay images of the columns 2 and 3 except for EB, which was stained with vimentin. Bar, 100 μ m.

the separation of a two-cell embryo has been reported by several groups (Willadsen 1979, Willadsen & Polge 1981, Willadsen *et al.* 1981, Ozil 1983, Allen & Pashen 1984, Tsunoda *et al.* 1985, Papaioannou *et al.* 1989), there was only one report on the production of a complete set of quadruplet calves (Johnson *et al.* 1995). In most cases, live offspring have not resulted from a full set of bisected embryos (Tarkowski 1959, Tsunoda & McLaren 1983, Chan *et al.* 2000). Instead of embryo splitting, Kwon & Kono (1996) demonstrated that a full set of offspring could be produced from a four-cell mouse embryo by NT (Kwon & Kono 1996). This study suggested that four-cell BTMs are competent for reprogramming and capable of regaining pluripotency through nuclear transplantation. This study also supported the notion that sister BTMs may be capable of compensating the lost or damaged BTMs of an embryo. Nonetheless, a very limited number of successful cases of producing a full set of identical offspring by either embryo splitting or nuclear transplantation were reported (Johnson *et al.* 1995, Kwon & Kono 1996). Our results support the idea that not all of the BTM-derived embryos and outgrowths expressed ICM marker. The development of a personal ES cell line for cell therapy has been considered as a potential cure for various diseases including neurodegenerative diseases and diabetes. The idea of establishing personal ES cells was aggressively pursued after the birth of Dolly, a sheep created by nuclear transplantation (Wilmut *et al.* 1997). Rideout *et al.* (2002) then demonstrated the possibility of producing cloned mouse embryos using somatic cells and through genetic modification created personal mouse ES cells that were

transplanted into the genetically defected somatic cell donors. This resulted in significant improvement of the donors' clinical symptoms (Rideout *et al.* 2002). In theory, somatic cell cloning (or therapeutic cloning) and the creation of personal ES cells from a cloned embryo is an ideal approach for the production of unlimited matched cell types for cell therapy; however, the advancement in clinical applications has been limited by the low efficiency of nuclear transplantation and the ethical concerns associated with therapeutic cloning.

Recent success in establishing mouse and human ES cell lines from a single BTM of pre-implantation embryos has led to a new development in stem cell technology (Chung *et al.* 2006, Klimanskaya *et al.* 2006, Wakayama *et al.* 2007). This breakthrough demonstrates the possibility that personal ES cells could eliminate the ethical concerns surrounding therapeutic cloning. ES cells have been derived from single BTMs, first demonstrated in BTM biopsies of *in vitro* mouse embryos, which continued to develop to full term (Wakayama *et al.* 2007). The feasibility of deriving ES cells from human BTMs was also demonstrated using a similar isolation method (Klimanskaya *et al.* 2006). Embryos from a BTM biopsy have been used for deriving ES cells but have not been used for embryo transfer or resulted in any live offspring (Klimanskaya *et al.* 2006), even though the procedure is commonly used in fertility clinics for pre-implantation genetic diagnosis and is considered a safe and practical method for BTM retrieval. As a result, there is a strong interest in personal human ES cells established from a single BTM and their clinical applications (Chung *et al.* 2006, Klimanskaya

et al. 2006, Wakayama *et al.* 2007). However, a relatively low success rate in establishing ES cells from a single biopsy BTM and the unsuccessful derivation of ES cell lines from sister BTMs has raised the question of whether all sister BTMs have equal potential to derive ES cells, or if they have a distinct fate. In fact, reprogramming of BTMs by co-culture with living ES cells as described by Chung *et al.* (2006) and Klimanskaya *et al.* (2006) may have profound effect on BTM fate, which may not be comparable with those cultured with the support of inactivated fibroblast feeder cells. In mice, the ES cell establishment rate was inversely related to the number of divisions or cell numbers of an embryo. The efficiency in establishing ES cell lines from BTMs of two-cell, early four-cell, late four-cell, and eight-cell embryos were 50–69%, 28–40%, 22%, and 14–16% respectively (Wakayama *et al.* 2007). This result demonstrates an interesting pattern indicating that only one or two, but not all, BTMs from each embryonic stage are capable of deriving ES cells. This is consistent with our findings that the expression pattern of stem cell markers and the development of ICMs were not uniform among sister BTM-derived embryos and outgrowths (Fig. 2A–D). Our results share similar conclusions with studies on the generation of identical animals (Heyman *et al.* 1998, Chan *et al.* 2000, Mitalipov *et al.* 2002, Schramm & Paprocki 2004) and the generation of an ES cell from a single BTM (Mitalipov *et al.* 2002, Chung *et al.* 2006, Wakayama *et al.* 2007), in that sister BTMs are in general not equally competent at establishing ES cell lines or the generation of identical offspring. However, the capability of sister BTMs in complementing the lost or damaged BTM of an embryo could not be determined. The fundamental questions revolve around the lineage fate of the BTMs; whether lineage fate is already determined in an early embryo, whether this lineage fate can be reversed, and whether it is limited to a particular BTM(s) due to the fact that not all sister BTMs retain pluripotency. Our findings demonstrated that sister BTMs are not identical based on the expression pattern of stem cell markers and the unequal capability of forming ICMs.

Although sister BTMs may be capable of complementing a lost or damaged sister BTM, limitation is expected based on previous studies and our recent findings. Therefore, it is very important to determine if the random retrieval of a single BTM for the derivation of an ES cell would jeopardize an embryo's integrity and its later development, as BTMs with ICM lineage preference would be retrieved and could result in reduced developmental capability of the donor embryo. Therefore, before attempting the retrieval of single BTMs from human embryos for the establishment of ES cells as a clinical practice, an in depth investigation should be performed. Recent breakthrough developments in which ES cells were derived by the reprogramming of somatic cells under defined culture conditions have led to a new era of stem cell research that draws great interest in alternative strategies for the derivation of personal stem cells (Takahashi & Yamanaka 2006, 2007, Hanna *et al.*

2007, Meissner *et al.* 2007, Okita *et al.* 2007, Wernig *et al.* 2007, Yu *et al.* 2007). Although the potential impact of the ability to reprogram somatic cells into pluripotent stem cells is exciting for biomedicine, alternative strategies for the development of personal stem cells should continue.

In conclusion, we have demonstrated that single BTMs of outbred mouse embryos can be cultured *in vitro*, develop to blastocyst, establish embryonic outgrowth, and yield pluripotent ES cell lines. We also demonstrated that sister BTMs of two- and four-cell embryos are not identical. Hence, there is a potential risk in retrieving a single human BTM from an early embryo for the establishment of an ES cell, because it may jeopardize the integrity of the embryo for continued development.

Materials and Methods

Animals

Female outbred stock mice (CD-1; 4–6 weeks) were super-ovulated with 10 IU pregnant mare serum gonadotropin (Sigma; i.p.). This was followed by 10 IU human chorionic gonadotropin (hCG, Sigma; i.p.) 48 h later, and then by natural mating with CD-1 male mice. Two-cell (2C) embryos were collected 43–45 h after the hCG injection from the oviducts and then cultured in 20 µl drops of KSOM+AA media (specialty media) under mineral oil with 5% CO₂ in air at 37 °C. The 4C stage embryos used in the experiment were obtained from *in vitro* culture of the 2C stage embryos.

Nomenclature

Embryos derived from the isolated BTMs of two- and four-cell embryos were named 2CIB (or 1/2C) and 4CIB (or 1/4C) embryos respectively. The zona pellucida-free 2C and 4C embryos were named as 2C whole (2CW) and 4C whole (4CW) embryos.

BTM isolation

The zona pellucida of the two- and four-cell mouse embryos was removed by 0.5% protease (Sigma). The BTMs were then separated by incubating the zona-free embryo in PBS without calcium and magnesium followed by gentle pipetting. The BTMs of the same embryo were cultured individually alongside with their sister BTMs.

Feeder cell preparation

Mouse fetal fibroblasts (MFFs) were prepared from 13.5 dpc mouse fetuses. The MFFs were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 200 mM L-glutamine and 1× penicillin/streptomycin (Invitrogen). The MFFs were inactivated with 5 µg/ml mitomycin C (Sigma) for 2 h followed by a thorough wash before plating.

Single BTM culture

The 2CIB- and 4CIB-derived embryos were cultured individually in a 72-well plate pre-coated with 0.1% gelatin (Sigma).

The MFFs were plated at 1000 cell/well in 10 µl culture medium 24 h prior use. The BTMs and embryos were initially cultured in KSOM (K; specialty media) until attached to the feeder cells when mouse ES cell medium (mES) was replaced for the continued culture of outgrowth and ES cells. The mES medium contained DMEM (Invitrogen) supplemented with 10% FBS (Hyclone), 200 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 1 × minimum essential amino acid (Invitrogen), 1 × penicillin/streptomycin (Invitrogen) and 1000 IU/ml hLIF (Chemicon, Temecula, CA, USA). BTMs and embryos were cultured in air at 37 °C with 5% CO₂.

Immunocytochemistry on pre-implantation embryos and embryo outgrowth

The embryos were washed twice in PBS, fixed in 4% paraformaldehyde (PFA; Polyscience Inc., Warrington, PA, USA) for 30 min, and followed by incubation with blocking buffer that consists of 0.2% Triton X-100 (Sigma), 3 mM sodium azide (Sigma), 0.1% saponin (Sigma), 2% BSA (Sigma), and 5% house serum (HyClone, Logan, UT, USA) in PBS without calcium and magnesium for 1 h. The samples were then thoroughly washed with PBS without calcium and magnesium and incubated with primary antibodies. Primary antibodies include: anti-*Oct4* (1:250; Santa Cruz, Biotechnology, Santa Cruz, CA, USA), anti-*Sox2* (1:100; Stem Cell Technologies, Vancouver, BC, Canada), and anti-*Cdx2* (1:1000; BioGenex). The samples were then incubated with secondary antibodies conjugated with appropriate fluorescent tag, stained with 5 µg/ml Hoechst 33342 (Sigma), mounted on slide and covered with 10 µl Vectashield (Vector Laboratories, Burlingame, CA, USA), and examined by fluorescence or confocal microscopy. Instead of mounting, embryo outgrowths were covered with PBS followed by microscopic examination.

Blastocyst cell count

Immunocytochemistry was performed on blastocysts using specific antibodies against the ICM (*Sox2*) and TE (*Cdx2*) cells. *Sox2*- and *Cdx2*-positive cells were visualized and counted under a fluorescence microscope at different focal planes across the embryos.

Establishment of embryonic stem cells from a single BTM

The 2CIBs and 4CIBs were cultured in KSOM until attaching to the feeder cells when KSOM was replaced by the mES medium. Ten days after BTM separation, the embryo outgrowth with a prominent ICM clump was 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 *et al.* 2003). Besides morphology, ES cell lines were characterized by the expression of stem cell markers, which included *Oct4* (1:250; Santa Cruz Biotechnology), *Sox2* (1:100; StemCell Technologies), *Nanog* (1:50; Santa Cruz Biotechnology), *SSEA-1* (*Fut4*; 1:50; Chemicon), *TRA-1-60* (1:100; Chemicon), *TRA-1-81* (1:100; Chemicon), and alkaline phosphatase activity (Vector Laboratories).

In vitro differentiation of ES cells derived from single BTMs

The ES cells were cultured in suspension for 7 days for the formation of embryoid bodies (EBs). EBs were then allowed to attach to a gelatin-coated plate and cultured in N1, N2, and N3 medium for 7, 14, and 7 days respectively. The N1 medium was composed of DMEM/F12 (Invitrogen) supplemented with minimum essential amino acid (Invitrogen), 200 mM L-glutamine (Invitrogen), and N2 supplement (Invitrogen). The N2 medium was composed of the N1 medium supplemented with 20 ng/ml basic fibroblast growth factor (bFGF). The N3 medium was composed of DMEM/F12 supplemented with 1% FBS (Hyclone) and B27 supplement (Invitrogen). EBs were stained with α-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), TH, and ChAT.

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, USA).

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