

A modified culture method significantly improves the development of mouse somatic cell nuclear transfer embryos

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Abstract

Many strategies have been established to improve the efficiency of somatic cell nuclear transfer (SCNT), but relatively few focused on improving culture conditions. The effect of different culture media on preimplantation development of mouse nuclear transfer embryos was investigated. A modified sequential media method, named D media (M16/KSOM and CZB-EG/KSOM), was successfully established that significantly improves SCNT embryo development. Our result demonstrated that while lacking any adverse effect on *in vivo* fertilized embryos, the D media dramatically improves the blastocyst development of SCNT embryos compared with other commonly used media, including KSOM, M16, CZB, and α MEM. Specifically, the rate of blastocyst formation was 62.3% for D1 (M16/KSOM) versus 10–30% for the other media. An analysis of media components indicated that removing EDTA and glutamine from the media can be beneficial for early SCNT embryo development. Our results suggest that *in vitro* culture environment plays an important role in somatic cell reprogramming, and D media represent the most efficient culture method reported to date to support mouse SCNT early embryo development *in vitro*.

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Introduction

The incomplete or aberrant reprogramming of donor nuclei by oocytes is the main obstacle to efficient cloning (Jouneau & Renard 2003). Although a considerable number of reconstructed embryos can reach the blastocyst stage, their post-implantation developmental competency remains very limited. To improve somatic cell nuclear transfer (SCNT) efficiency, many parameters have been investigated including the timing of oocyte activation, the effects of DMSO (Wakayama & Yanagimachi 2001), inhibition of cytokinesis (Wakayama & Yanagimachi 2001) and the timing of enucleation, or injection of the nucleus (Wakayama *et al.* 2003). Different nucleus injection methods such as electrofusion (Ogura *et al.* 2000), piezoelectric microinjection (Wakayama *et al.* 1998), and 'one-step micromanipulation' (OSM; Zhou *et al.* 2003) have also been explored. Efficiency has also been enhanced by epigenetic modification prior to nuclear transfer (NT) by treating donor cells with pharmacological reagents. For example, S-adenosyl homocysteine treatment reduced the level of DNA methylation and increased the *in vitro* development rate of NT embryos (Jones *et al.* 2001, Enright *et al.* 2003, Jeon *et al.* 2008). Improvement of somatic cell reprogramming ability was reported when the donor cells or the reconstructed embryos were treated with

trichostatin A, an inhibitor of histone deacetylases, before or after NT (Kishigami *et al.* 2006, Rybouchkin *et al.* 2006). Unfortunately, the long-term effects of these reagents on embryo development and their safety in therapeutic cloning are not known.

Although much effort has been devoted to improving media composition and culture conditions for better development of *in vivo* fertilized mouse embryos or human IVF embryos, relatively few studies have focused on SCNT culture conditions, which are known to play a vital role in embryo development (Summers & Biggers 2003). For instance, mouse embryos were shown to be sensitive to osmolarity (Dawson *et al.* 1998), which appears to be one of the major causes of developmental arrest at the two-cell stage (Hadi *et al.* 2005). Genetic make-up and media composition are also important factors in the survival of blastocysts (Kamjoo *et al.* 2002). The role of amino acids for *in vitro* culturing of preimplantation embryos was also extensively discussed (Summers & Biggers 2003, Biggers & Summers 2008). Epigenetic regulation of important genes such as *H19* can be affected by the *in vitro* culture environment (Doherty *et al.* 2000, Khosla *et al.* 2001). Furthermore, cloned embryos are even more sensitive to culture requirements compared with fertilized embryos, as early as the one-cell stage (Chung *et al.* 2002). The rate of blastocyst formation in NT-reconstructed embryos,

Table 1 The effect of D culture media on preimplantation development of nuclear transfer (NT) and *in vivo* fertilized embryos.

Embryo derivation	Medium	Number of reconstructed embryos	Number of PN formation	Number of two-cell embryos	Number of (%) four-cell embryos	Number of (%) morula	Number of (%) blastocysts
NT	CZB	91	72	70	47 (66.3 ± 8.9) ^a	16 (22.4 ± 4.2) ^c	10 (14.5 ± 1.9) ^{g,i}
	αMEM	91	74	73	67 (91.8 ± 6.1) ^b	28 (38.2 ± 4.2) ^c	23 (31.1 ± 4.6) ^{g,j}
	M16	89	67	66	55 (82.6 ± 4.5) ^{a,b}	13 (18.8 ± 4.7) ^{c,e}	7 (10.1 ± 2.9) ^{g,i}
	KSOM	92	78	77	62 (80.2 ± 6.2) ^{a,b}	27 (34.7 ± 8.7) ^c	17 (21.6 ± 7.7) ^{g,i}
	D1	91	70	69	68 (98.3 ± 1.7) ^b	55 (79.8 ± 3.2) ^d	44 (62.3 ± 7.2) ^h
	(αMEM/KSOM)	95	73	73	67 (91.5 ± 4.7) ^b	31 (41.2 ± 10.9) ^{c,f}	20 (26.7 ± 5.4) ^{g,i}
<i>In vivo</i> fertilized embryos	M16	/	38	38	38	38	37 (97.2 ± 2.8) ^k
	KSOM	/	85	85	85	84	75 (92.6 ± 2.9) ^k
	D1	/	60	60	60	59	57 (94.9 ± 2.9) ^k

Values with different superscripts in one column are significantly different by one-way ANOVA. $P < 0.05$ for a versus b, e versus f, and i versus j; $P < 0.01$ for c versus d and g versus h.

as well as the regional distribution of POU5F1(Oct4)⁺ cells in cloned blastocysts after cumulus cell NT, differed from that of fertilized embryos (Boiani *et al.* 2005). As the underlying reprogramming processes could be very different between NT and *in vivo* fertilized embryos, different media systems may be warranted.

We report the optimization of culture media to improve the developmental capacity of SCNT embryos. We found that the D media method, which uses a sequential culture media, supports early embryonic development in SCNT embryos far better than most of the common media in use today.

Results

D culture method improves preimplantation development of mouse SCNT embryos

Embryonic development and physiology change throughout preimplantation stages (Zeng *et al.* 2004), thus their culture requirements are naturally different at each stage with different embryo composition. Thus, we developed a sequential culture method, which we named 'D media' that combines the most often used media for efficient NT as well as for normal preimplantation development. The original D media are M16 for the NT process, followed by KSOM, one of the initial 'computer-optimized media' (Ho *et al.* 1995) extensively studied and widely used for culturing preimplantation embryos over the past 15 years. The embryos are transferred to KSOM at the late two-cell stage because these embryos were reported to be the most sensitive to culture conditions during the first and second cell cycles (Doherty *et al.* 2000), and KSOM does not support adequate NT embryo development prior to the two-cell stage (Chung *et al.* 2002).

SCNT were cultured in various media, including CZB, αMEM, M16, KSOM, D1 (M16/KSOM), and αMEM followed by KSOM (αMEM/KSOM), while *in vivo* fertilized embryos were cultured in KSOM, M16, and D1. These media were selected because they previously

showed better results in our laboratory for culturing preimplantation embryos. In addition to M16, we also found that αMEM supports the development of NT embryos well, in agreement with previous reports for this medium (Boiani *et al.* 2005); thus, we also studied sequential media effects of αMEM followed by KSOM (αMEM/KSOM). There were no significant differences in blastocyst formation for *in vivo* fertilized embryos cultured in KSOM, M16, and D1; and for SCNT embryos, no significant differences in the number of reconstructed embryos, pronucleus (PN) formation, or the number of two-cell embryos for all six media tested (Table 1). Notably, the rates of morula and blastocyst formation were significantly higher when the SCNT embryos were cultured in D1 media (Table 1, $P < 0.01$). The blastocyst rate of over 60% is among the highest developmental rate for SCNT embryos ever reported.

Embryo quality assessment by differential staining and TUNEL assay

The D medium supports SCNT development, resulting in a much higher rate of blastocyst formation than other media. We further assessed the quality of SCNT embryos cultured in three media using two commonly used parameters, morula/blastocyst cell number, and the level of apoptosis (van Soom *et al.* 1997).

Total cell number per morula was compared at the same time point (~73 h post-activation) for SCNT embryos cultured in KSOM, D1, or αMEM media using

Table 2 Influence of culture medium on cell number of cloned morula.

Medium	Number of morula	Total cells per morula*	POU5F1-positive cells:total cells [†]
αMEM	19	7.5 ± 0.6 ^a	0.80 ± 0.04 ^d
KSOM	17	12.2 ± 1.1 ^b	1.5 ± 0.6 ^e
D1	15	16.2 ± 1.4 ^c	0.90 ± 0.03 ^{d,e}

Values with different superscripts are significantly different in one column by one-way ANOVA. * $P < 0.01$; [†] $P < 0.05$.

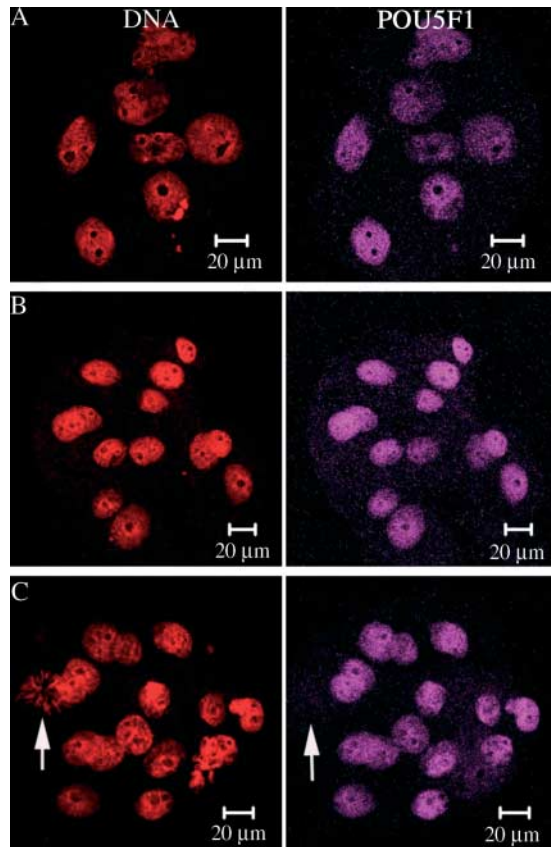


Figure 1 The effect of culture media on cell number and POU5F1 (Oct4) expression of SCNT morula. Immunofluorescent staining using POU5F1 antibody was performed on the morula cultured in different media: α MEM (A), KSOM (B), and D1 (C), Bar, 20 μ m. DNA was stained with PI (red) and POU5F1 expression revealed by a cy5-conjugated secondary antibody (purple staining). Arrows indicate the prometaphase cells that are negative for POU5F1 immunofluorescent staining.

differential staining. Morulas cultured in D1 media showed the highest total cell numbers (Table 2), and more POU5F1-positive cells were found in embryos cultured in D1 media and KSOM, when compared with those embryos cultured in α MEM (Table 2 and Fig. 1).

At 96 h post-activation, blastocysts cultured in KSOM, D1, or α MEM were compared for number of inner cell mass (ICM) cells, trophoctoderm (TE) cells, or total cells. While blastocysts cultured in D1 media showed slightly higher cell numbers, no statistically significant differences were found (Table 3).

Table 3 Influence of culture medium on cell number of cloned blastocysts.

Medium	Number of blastocysts	ICM cells	TE cells	Total cells	ICM:total
α MEM	12	10.8 \pm 1.3 ^a	31.8 \pm 2.9 ^b	42.7 \pm 3.7 ^c	0.257 \pm 0.018 ^d
KSOM	10	11.5 \pm 0.6 ^a	36.7 \pm 4.4 ^b	48.6 \pm 4.7 ^c	0.252 \pm 0.021 ^d
D1	14	13.4 \pm 0.9 ^a	37.6 \pm 3.1 ^b	50.9 \pm 3.5 ^c	0.269 \pm 0.018 ^d

Values with same superscripts indicate that numbers in the same column are not significantly different by one-way ANOVA ($P > 0.05$).

TUNEL assays were used to test for apoptosis of SCNT blastocysts. As shown in Table 4 and in Fig. 2, the three media appeared to cause no significant differences in apoptosis, as calculated by percentage of apoptotic cells per blastocyst.

These results demonstrate that D1 media better support blastocyst formation, do not compromise blastocyst quality, and result in normal number of cells and normal rates of apoptosis per embryo.

Post-implantation development following D and KSOM culture

To investigate the post-implantation effects of D1 media, SCNT blastocysts developed in D1 media or KSOM were transferred into E2.5 pseudo-pregnant recipients, and the pups were delivered by cesarian section at E19.5 (Table 5). The percentage of live births, average pup weights, and average placental weights were similar between the two groups. However, consistent with the improved developmental capacity shown in Table 1, almost twice as many live born pups can be obtained from reconstructed embryos cultured in D1 media. Therefore, the efficiency of SCNT was dramatically improved.

Establishment of NT-ES cells from SCNT cloned blastocysts using D media

To further test the effect of D media on somatic cell reprogramming, NT-ESC lines were established from cloned blastocysts. Blastocysts cultured in D1 media showed a significantly lower degeneration rate, and a higher percentage of ES lines derived from attachments (Table 6).

Effects of EDTA and/or glutamine on early development of NT embryos

D1 media improved the development of cloned embryos compared with other media tested. We first excluded the possibility that the non-essential amino acids (NEAA) and essential amino acids (EAA) in KSOM media or the osmolarity of M16 (data not shown) were the causative agents. We cultured the cloned embryos in CZB that include NEAA and EAA either from the very beginning or after the late two-cell stage; these two conditions were also tested for osmolarities similar to either KSOM or

Table 4 Influence of culture medium on apoptosis of cloned blastocysts.

Medium	Number of blastocysts	Number of apoptotic blastocysts* (%)	Total cells	Apoptotic cells (%) [†]
α MEM	9	9 (100.0) ^a	341	36 (10.9 \pm 1.9) ^b
KSOM	24	21 (87.5) ^a	967	76 (8.2 \pm 1.2) ^b
D1	16	15 (93.8) ^a	829	64 (7.9 \pm 1.2) ^b

Values with same superscripts indicate that numbers in the same column are not significantly different by one-way ANOVA (*) or by Fisher's exact test ([†]), $P > 0.05$.

M16. No marked differences in development efficiency were seen with these culture conditions. We then focused on the fundamental composition of these media. The absence of EDTA and glutamine (Gln) in the M16 component of D1 was noted, so CZB and M16 with different combinations of EDTA and Gln were tested as D media variations.

SCNT embryos cultured in CZB without EDTA or Gln followed by KSOM (CZB–Gln–EDTA/KSOM, 'D2') showed dramatically improved two-cell to morula and blastocyst development rates, when compared to CZB/KSOM (Table 7). The additive effects of removing EDTA and Gln from CZB are most noteworthy, leading to a developmental efficiency closest to that of D1 media (M16/KSOM).

Adding EDTA and Gln to the M16 component of D1 media greatly reduced the developmental capacity of NT embryos (Table 8). A significantly lower proportion of embryos developed to four-cell, morula, and blastocyst stages in M16 (+EDTA+Gln)/KOSM, compared to those cultured in D1. This confirmed our hypothesis that EDTA and Gln may harm early preimplantation development of NT embryos.

Discussion

In this study, we described the establishment of a modified sequential media method, named D media (M16/KSOM and CZB-EG/KSOM) that significantly improves SCNT embryo development. The use of various media as well as the benefit of single versus sequential media protocols has been extensively debated, in the hope of establishing systems that mimic the *in vivo*

physiological environment and allow maximum accommodation of different energy needs of embryos at different preimplantation stages (Summers & Biggers 2003, Biggers & Summers 2008). Sequential culture media can promote appropriate development at different gestational stages (Gardner & Lane 1993, Bavister 1995, Gardner *et al.* 1996), especially for NT-reconstructed embryos (Chung *et al.* 2002).

Our result demonstrated that while lacking any adverse effect on *in vivo* fertilized embryos, compared with other commonly used media, including KSOM, M16, CZB, and α MEM, the D media dramatically improve the blastocyst development of SCNT embryos with normal number of cells and normal rates of apoptosis per embryo. In addition, post-implantation development of SCNT embryos in D media was improved, and higher percentage of NT–ES cells' establishments from SCNT-cloned blastocyst using D media was also observed.

A careful analysis of media components indicated that the omission of EDTA and Gln from the D media could be beneficial for early SCNT embryo development. A synergistic alleviation of two-cell blockage by EDTA and Gln was previously reported for fertilized embryos. EDTA significantly improved preimplantation development of mouse embryos, and Gln was particularly beneficial when provided prior to the four-cell stage (Chatot *et al.* 1989, 1990, Nasr-Esfahani *et al.* 1992, Lane & Gardner 1997). Our results, however, demonstrate that the absence of EDTA and Gln in culture media prior to the four-cell stage is critically important for the preimplantation development of SCNT embryos, consistent with what Devreker & Hardy (1997) reported regarding the deleterious effect of Gln on mouse preimplantation embryos *in vitro*.

It was observed that NT embryos are more sensitive to the culture media than parthenogenetic embryos or *in vivo* fertilized embryos, and that alteration of the *in vitro* culture environment has a significant influence on the development of NT embryos (Heindryckx *et al.* 2001). Embryos have different energy requirements from single-cell zygote through blastocysts' stages, so the optimal culture condition may differ for embryo from different developmental stages and with different nuclear

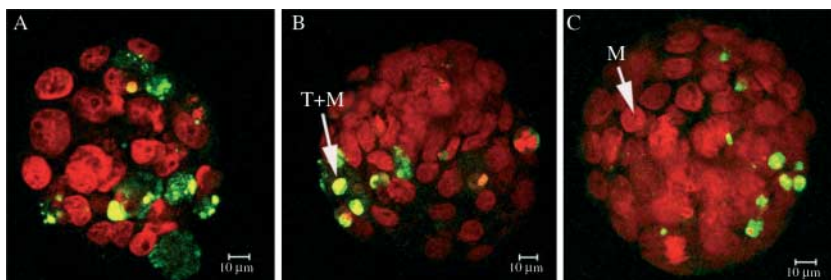


Figure 2 Effect of culture media on apoptosis of SCNT blastocysts. Fluorescence micrographs demonstrating apoptotic morphology in SCNT embryos cultured in different media are shown for α MEM (A), KSOM (B), and D1 (C). PI stains nuclei (red). Apoptotic cells are green after TUNEL while condensed red nuclei mark all cells (M), and yellow (T+M) indicates overlapping signals. Bar, 10 μ m.

Table 5 Influence of D culture method on the full-term development of nuclear transfer embryos.

Medium	Number of two-cell embryos	Blastocysts transferred	Number of live offspring (% transferred)*	Percentage of live offspring (from two-cell)	Av. (g) fetus wt (S.E.M.) [†]	Av. (g) placenta wt (S.E.M.) [†]
KSOM	350	95	3 (3.2) ^a	0.86	1.7 ± 0.02 ^b	0.24 ± 0.01 ^c
D1	200	102	3 (2.9) ^a	1.5	1.7 ± 0.2 ^b	0.19 ± 0.05 ^c

Values with different superscripts are significantly different in one column. a versus a; b versus b; c versus c; $P > 0.05$. Values with same superscripts indicate that numbers in the same column are not significantly different by Fisher's exact test (*) and one-way ANOVA ([†]), $P > 0.05$.

compositions (Gardner & Lane 1993, Bavister 1995, Gardner *et al.* 1996, Summers & Biggers 2003, Biggers & Summers 2008). Thus, the medium that is highly suitable for fertilization stage embryos may not support NT embryos development as efficiently (Chung *et al.* 2002). Differences in culture requirements between NT and fertilized embryos were thought to be related to genomic reprogramming efficiency (Chung *et al.* 2002), but the detailed reprogramming mechanisms by which NT embryo's development was improved by eliminations of EDTA and Gln are not known.

Gln has been the subject of consideration for improving culture conditions for preimplantation embryos because it has a complex role in preimplantation development (Summers & Biggers 2003). It was thought to be an energy substrate in place of glucose in early stages to support the embryo's development through a critical period when lactate and pyruvate fail to supply enough energy (Reitzer *et al.* 1979, Chatot *et al.* 1989, 1990), and may enhance the nucleotide pool as an important amino acid that can be used to synthesize purine and pyrimidine nucleotides critical for rapidly division (Salzman *et al.* 1958, Nomura & Rubin 1988). However, Gln was shown to be the principal contributor to the toxic breakdown of amino acids that affect *in vitro* culturing of preimplantation embryos (Gardner & Lane 1993, Nakazawa *et al.* 1997, Summers & Biggers 2003). Gln-free medium could lead to increases in the numbers of cells that developed in the ICM and TE (Lawitts & Biggers 1993). On the other hand, EDTA was shown to affect embryo development by inhibiting glycolysis at the two-cell stage, thereby preventing the premature stimulation of glycolysis (Gardner & Lane 1993, Lane & Gardner 2001) whereas its chelating effect was controversial (Halliwell & Gutteridge 1985, Kim *et al.* 2006). Thus, NT-reconstructed embryos may have different

energy requirement and tolerance to Gln, as well as different responses to EDTA at early preimplantation stages, leading to the specific requirement for culture media for SCNT embryos.

In summary, we investigated the effects of different culture media on mouse embryonic development. When compared with other commonly used media, the D sequential culture method significantly improves SCNT embryo development, and can result in at least twice the number of live-born cloned embryos. We conclude that the D culture method is by far the most suitable for SCNT embryo development and can be used with M16 or CZB media without EDTA and Gln, for the initial stage of embryo reconstruction after activation. KSOM medium is appropriate for subsequent culturing from the late two-cell stage throughout development to the blastocyst stage. We demonstrate that optimization of *in vitro* culture conditions for SCNT embryos is important for improved cloning efficiency. This work also provides tools to explore underlying differences in reprogramming mechanisms and metabolic characteristics of SCNT and *in vivo* fertilized embryos.

Materials and Methods

Embryo and oocyte collection

Zygotes were collected at 20 h post-hCG treatment from superovulated C57BL/6 female mice (8–12-week-old) mated to a DBA male. Cumulus–oocyte complexes were collected from superovulated B6D2F1 mice (C57BL/6 × DBA/2, 8–12-week-old) 15 h post-hCG treatment, and cumulus cells were removed with hyaluronidase (ICN Pharmaceuticals, Costa Mesa, CA, USA). Before micromanipulation, oocytes were cultured in CZB medium supplemented with 3 mg/ml BSA at 37 °C/5% CO₂.

Table 6 Influence of culture medium on the establishment of nuclear transfer-ESCs lines.

Medium	Number of blastocysts	Number of (%) attachment	Number of (%) degeneration	Number of ES lines (% blastocyst)	Percentage of ES lines (from attachment)
αMEM	10	10 (100.0) ^a	9 (90.0) ^c	1 (10.0) ^e	10.0 ^f
KSOM	10	8 (80.0) ^{a,b}	6 (75.0) ^{c,d}	2 (20.0) ^e	25.0 ^g
D1	19	13 (68.4) ^b	6 (46.2) ^d	5 (26.3) ^e	38.5 ^g

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

Table 7 Influence of CZB without EDTA and/or glutamine on the *in vitro* development of cloned embryos.

Medium	Number of reconstructed embryos	Number of PN formation	Number of two-cell embryos	Number of (%) four-cell embryos	Number of (%) morula	Number of (%) blastocysts
KSOM	161	122	122	99	54 (42.9±4.5) ^a	36 (28.8±2.9) ^c
D1(M16/KSOM)	162	125	125	121	90 (71.7±6.8) ^b	66 (52.9±5.1) ^c
CZB(-Gln)/KSOM	165	136	136	115	53 (38.8±6.5) ^a	36 (26.6±2.2) ^{c,e}
CZB(-EDTA)/KSOM	160	124	124	99	55 (43.6±4.8) ^a	40 (32.4±5.3) ^c
CZB(-Gln-EDTA)/KSOM	163	126	126	112	77 (61.2±3.7) ^b	66 (52.5±3.9) ^d
CZB/KSOM	156	119	119	77	33 (27.4±4.1) ^a	19 (15.9±2.4) ^e

Values with different superscripts are significantly different in one column by one-way ANOVA ($P < 0.05$).

Nuclear transfer

NT was performed using the OSM method as previously described (Zhou *et al.* 2003). The reconstructed embryos were activated 30–60 min later by 10 mM SrCl₂ in calcium-free CZB medium for 5 h and cultured in CZB at 37 °C/5% CO₂ for 4.5 days.

Embryo culture

D1 (M16/KSOM and M16 followed by KSOM) and D2 (CZB-EG/KSOM, CZB without EDTA, and Gln followed by KSOM) media were tested together with CZB supplemented with 3 mg/ml BSA (Sigma), α MEM (Sigma), M16 (Sigma), KSOM containing NEAA and EAA (Chemicon, Billerica, MA, USA), and α MEM/KSOM. For NT embryos, 5 h after the onset of activation, the reconstructed embryos were randomly assigned to one of six groups (CZB, KSOM, M16, α MEM, D, and α MEM/KSOM). At the late two-cell stage (26–29 h after activation), embryos were transferred to a new drop of media; for embryos in the D and α MEM/KSOM groups, the embryos were transferred to KSOM. After 48 h of culture (four-cell stage), the CZB medium was supplemented with D(+) glucose. Zygotes were then cultured in the same media as SCNT embryos.

Differential staining of blastocysts for cell counting and apoptosis assays

The number of cells in the ICM and TE was determined as previously described (Papaioannou & Ebert 1988) for SCNT blastocysts 96 h after activation. Briefly, embryos were exposed to a 1:10 dilution of rabbit anti-mouse whole serum (Sigma) for 30 min, followed by incubation in a 1:10 dilution of guinea pig complement (Sigma) with propidium iodide (PI; Molecular Probes, Carlsbad, OR, USA) and Hoechst 33342 for 30 min at

37 °C, and immediately examined using an inverted NIKON fluorescence microscope (E600, Japan). Cells were counted directly under the light microscope.

Detection and quantification of apoptosis at the single cell level were performed by TUNEL and *In Situ* Cell Death Detection Kit (Roche) according to the manufacturer's instructions. Embryos were observed with a Zeiss LSM 510 META confocal microscope.

Immunofluorescence staining

NT morulas were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100 for 30 min, blocked in 2% BSA for 1 h, and incubated with goat anti-POU5F1 (anti-Oct4; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody overnight at 4 °C followed by cy5-conjugated secondary antibody (Jackson, West Grove, PA, USA) at 37 °C for 1 h. DNA was stained with PI for 15 min at 37 °C, and samples were mounted and observed with the confocal microscope.

Establishment of NT-ES cell lines and stem cell cultures

The NT-ES cell lines were established and ES cells cultured as previously described (Brook & Gardner 1997, Wakayama *et al.* 2003, Zhao *et al.* 2007). DMEM/F12 (1:1, Gibco) with leukemia inhibitory factor (LIF; Chemicon) was used for the establishment of NT-ES cell lines. The concentration of LIF was decreased by half for ES cell culture.

Embryo transfer

SCNT blastocysts were transferred into the uterus of E2.5 pseudo-pregnant CD1 surrogate mothers. All the pregnant recipient females were killed at E19.5 and live pups were gestated by normal CD1 surrogate mothers.

Table 8 Influence of EDTA and glutamine in M16 on *in vitro* development of cloned embryos.

Medium	Number of reconstructed embryos	Number of PN formation	Number of two-cell embryos	Number of (%) four-cell embryos*	Number of (%) morula [†]	Number of (%) blastocysts [†]
D1(M16/KSOM)	93	76	76	71 (92.8±3.0) ^a	59 (77.6±1.7) ^c	47 (62.3±5.7) ^e
M16(+Gln+EDTA)/KSOM	106	86	86	69 (80.5±3.5) ^b	24 (27.9±0.98) ^d	16 (18.6±0.55) ^f

Values with different superscripts are significantly different in one column by one-way ANOVA. * $P < 0.05$; [†] $P < 0.01$.

Statistical analysis

Data analysis was performed using SPSS 13.0 statistical software. One-way ANOVA and Fisher's exact test were performed. A value of $P < 0.05$ was considered to be statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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