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Comparative pluripotency analysis of mouse embryonic stem cells derived from wild-type and infertile hermaphrodite somatic cell nuclear transfer blastocysts

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Therapeutic cloning, whereby embryonic stem cells (ESCs) are derived from patient-specific cloned blastocysts via somatic cell nuclear transfer (SCNT), holds great promise for treating many human diseases using regenerative medicine. Teratoma formation and germline transmission have been used to confirm the pluripotency of mouse stem cells, but human embryonic stem cells (hESCs) have not been proven to be fully pluripotent owing to the ethical impossibility of testing for germ line transmission, which would be the strongest evidence for full pluripotency. Therefore, formation of differentiated cells from the three somatic germ layers within a teratoma is taken as the best indicator of pluripotency in hESC lines. The possibility that these lines lack full multi- or pluripotency has not yet been evaluated. In this study, we established 16 mouse ESC lines, including 3 genetically defective nuclear transfer-ESC (ntESC) lines derived from SCNT blastocysts of infertile hermaphrodite F_1 mice and 13 ntESC lines derived from SCNT blastocysts of normal F_1 mice. We found that the defective ntESCs expressed all *in vitro* markers of pluripotency and could form teratomas that included derivatives from all three germ layers, but could not be transmitted via the germ line, in contrast with normal ntESCs. Our results indicate that teratoma formation assays with hESCs might be an insufficient standard to assess full pluripotency, although they do define multipotency to some degree. More rigorous standards are required to assess the safety of hESCs for therapeutic cloning.

somatic cell nuclear transfer, embryonic stem cells, therapeutic cloning, hermaphrodite

Cloning by nuclear transfer is the process of using adult somatic cells to generate cloned embryos. When cultured, these embryos can give rise to ESCs that have the potential to become any (or almost any) type of cell present in the adult body. Because ESCs generated by nuclear transfer are genetically identical to the donor and are thus potentially useful for therapeutic applications, this process is also called "therapeutic cloning"[1]. In humans, patient-specific ntESC lines derived from cloned embryos have great potential for the field of re-

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generative medicine. Recently, fully pluripotent ESC lines have been derived from nuclear transfer embryos by therapeutic cloning in mice in several labs[2,3]. ntESCs have been shown to differentiate both *in vitro* and *in vivo*[4], and several reports have shown the ability of ESCs or ntESCs to rescue immunodeficient or degenerative phenotypes[5,6]. For example, differentiated dopaminergic neurons from ntESCs have improved symptoms in Parkinsonian mice[7]. ntESCs can even differentiate into spermatozoa and oocytes in chimeric mice[8]. Moreover, mice can be cloned from ntESC lines by nuclear transfer[8,9]. Other evidence indicates that ntESC lines are broadly similar to conventional ESCs and are indistinguishable from their conventional counterparts at the molecular level[2,3].

Despite the high level of interest in its potential applications, nuclear transfer is extremely inefficient. Most cloned embryos die soon after implantation, and only a small fraction of clones survive beyond birth. Survivors are often afflicted with abnormalities, such as obesity, premature death and dysfunctional placentas[10−12]. In addition, genetic and epigenetic defects, such as DNA rearrangements are frequently found in mature central nervous system neurons and in stem cells during long-term cultivation[13]. Several reports have demonstrated that broadly multipotent ESCs can be derived from embryos cloned from defective mice. Thus, many safety issues must be examined and resolved before therapeutic cloning can be applied to cell therapies and preclinical trials for disease treatment are carried out.

In this study, we established 16 ntESC lines from infertile hermaphrodite and normal F_1 mouse somatic cells and evaluated the pluripotency of these lines using established criteria, including morphology, surface makers, differentiation potential *in vitro* and *in vivo*, and developmental potency by nuclear transfer or tetraploid blastocyst complementation.

1 Materials and methods

1.1 Mature oocyte collection and donor cell preparation

The hermaphrodite sterile mouse used here was discovered in our B6D2 F_1 (C57BL/6×DBA/2 hybrid) mouse-breeding colony when it was 12 weeks old. B6D2 (C57BL/6×DBA/2) F_1 females (8−10 weeks old) were superovulated by separate injections of PMSG and HCG that were 48 h apart. Cumulus-oocyte complexes (COC) were collected 13−14 h after HCG injection. Cumulus cells were removed by treatment with 300 U/mL hyaluronidase (ICN Pharmaceuticals, Costa Mesa, CA), and oocytes were cultured in CZB medium before micromanipulation in a humidified atmosphere at 37℃ with 5% CO_2.

The heart of the hermaphrodite sterile mouse was isolated, cut into small pieces, and incubated in 5 mL DMEM supplemented with 10% fetal calf serum. After incubation for 5−7 d at 37℃ under 5% CO_2 in air, many fibroblast cells were seen spreading over the surface of the dish. These cells were detached from the dish by treatment for 5 min with Ca-Mg-free PBS medium containing 0.25% trypsin and 0.75 mmol/L EDTA. The medium was pipetted up and down several times to release the cells from the dish surface, collected and centrifuged to sediment the cells. The cells were then washed with DMEM and sedimented by centrifugation 3 times[14]. Prior to microinjection, the cell suspension was mixed with HEPES-CZB and stored at 4℃.

1.2 Nuclear transfer and embryo transfer

Nuclear transfer was performed as previously described[15]. Donor nuclei or chromosomes were removed from donor cells by gently aspirating in and out of the injection pipette and injected into recipient oocytes. The meiotic metaphase plate was withdrawn while removing the pipette from the cytoplasm after injection. Twenty to thirty oocytes were placed into a 1-mL chamber containing HEPES-CZB medium with 5 μg/mL cytochalasin B (CB) covered by mineral oil. Pipettes with an internal diameter of 10−12 μm were used for injection of the donor nucleus using a piezoelectric device (P150, PrimeTech). One to 2 h after injection, the reconstructed embryos were activated by 5 h incubation in calcium-free CZB supplemented with 10 mmol/L $SrCl_2$ and 5 μg/mL cytochalasin B. One portion of the reconstructed embryos was cultured for 4 d to evaluate their development potential *in vitro*, and another portion was transferred at the two cell stage into the oviducts of 0.5 d pseudopregnant Imprinting Control Region (ICR) surrogate mice. Transferred embryos were recovered by Caesarian section at day 19.5.

1.3 Establishment of ntESC lines

The zona pellucida was removed from cloned blastocysts using Acidic Tyrode's solution. Blastocysts were placed in 4-well dishes pre-coated with inactivated

mouse embryonic fibroblasts (MEFs). Proliferating outgrowths were dissociated by manual pipetting or 0.25% trypsin treatment, and then replated on feeder cells (MEFs) until stable ntESC lines grew.

1.4 Identification of embryonic stem cells

The pluripotency of established ntESC lines was determined by alkaline phosphatase (ALP) staining, immunostaining and embryoid body (EB) formation. Cultured confluent ES cells were fixed with formalin for 15 min before ALP staining as described with 100 mmol/L Tris-HCl (pH 9.5) plus NBT (Sigma N-5514) and BCIP (Sigma B-0274)[16,17]. Immunostaining was performed with the following monoclonal antibodies: SSEA-1 (stage-specific embryonic antigen-1) (Chemicon MAB4301), SSEA-3 (Chemicon MAB4303), SSEA-4 (Chemicon MAB4304), Oct-4 (Santa Cruz SC-8628), and Nanog (Millipore AB9220). DNA was stained with propidium iodide (10 μg/mL) and/or 0.1 μg/mL Hoechst 33342 at room temperature for 5－10 min. Observations were performed on a fluorescence microscope (Leica) or confocal microscope (Zeiss LSM 510 META). Karyotyping and EB formation in mouse ntESCs were performed as described[3,18,19].

1.5 Production of chimeric mice and confirmation of germ line transmission

ICR blastocysts were flushed from the ovaries of female mice 3.5 d after mating. ntESCs were introduced into the blastocoel of ICR blastocysts by piezo-assisted microinjection. Immediately after injection, the blastocysts were transferred into 2.5 d pseudo-pregnant ICR strain surrogate mothers. Chimeric offspring with dark or gray coat colors were selected at 8 weeks and mated with ICR mice. ntESCs were scored as capable of germline transmission if pups with fully black coat colors identical to B6D2F1 mice were produced.

1.6 Teratoma formation

ntESCs maintained for ⩽15 passages on MEF feeder layers were harvested in the absence of feeder cells, and approximately 5×10^6 cells were injected into 5 week old NOD-SCID mice. In 3－4 weeks, teratomas were dissected, fixed in 4% paraformaldehyde and examined histologically after hematoxylin and eosin staining.

1.7 Histological analysis of testes, ovaries and teratomas

Gonads of the infertile hermaphrodite mouse and teratomas were fixed in 4% buffered formalin and embedded in paraffin. Samples were cut into 3－4 μm sections. Serial sections were mounted on slides, stained with hematoxylin/eosin and observed by light microscopy.

1.8 PCR analysis of genomic DNA and RT-PCR analysis of EB differentiation

The Y chromosome-specific genes *Sry*, *Zfy1*, *Rbmy*, *Ssty1* and *Ssty2* and the euchromosomal genes *Myog* and *Gapdh* were amplified using primer pair sequences described in refs. [20, 21][22,23]. DNA was extracted from ntESCs. Total RNA from EB samples was extracted using trizole (invitrogen, Grand Island, NY). cDNA was synthesized from about 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY). cDNA samples were subjected to PCR amplication with primers selective for *Sox1*, *Nes*, *T*, *Myod1*, *Gata6*, *Afp*. *Gapdh* was assayed using the same RT-PCR method as a control for mRNA quality. The PCR primers are described in Table 1. PCR was carried out for 35 cycles, and products were separated on 2% agarose gels stained with ethidium bromide before visualization.

1.9 Statistical analysis

Results were analyzed statistically using one-way ANOVAs and SPSS software.

2 Results

2.1 Histological analysis of hermaphrodite mouse gonads

The infertile hermaphrodite F_1 mouse displayed an outwardly male phenotype. After dissection, we detected an ovary and a small testis. Histological analysis revealed that the ovary of this mouse had growing oocytes and that the testis had no multiplying spermatogenic cells (Figure 1).

2.2 *In vitro* developmental potential of nuclear transfer embryos derived from hermaphrodite mouse somatic cells

We examined the early developmental capacity of embryos derived from hermaphrodite mouse heart fibroblast cells and normal mouse tail tip fibroblasts. Our results indicated that there was no significant difference in the three key stages of mouse preimplantation development, including embryo activation, cleavage and the morula/blastocyst stage, between embryos derived from the two types of donor cells (Table 2).

Table 1 PCR primers used to detect the presence of Y chromosome-specific genes and EB differentiation

Gene	Primer	Size (bp)	GenBank accession No.
Sry	sense, 5′-TGGGACTGGTGACAATTGTC-3′ antisense, 5′-GAGTACAGGTGTGCAGCTCT-3′	402	21674
Zfy1	sense, 5′-GACTAGACATGTCTTAACATCTGTCC-3′ antisense, 5′- CCTATTGCATGGACAGCAGCTTATG-3′	187	22767
Rbmy	sense, 5′-CTCAA TCTTC TGGAA GGGCA G-3′ antisense, 5′-ATATT TACTC TGAAG AGACA T-3′	250	19657
Ssty1	sense, 5′-CTGGAGCTCTACAGTGATGA-3′ antisense, 5′-CAGTTACCAATCAACACATCAC-3′	342	20611
Ssty2	sense, 5′-GTTTT TCCTC AGGTG AGGGA-3′ antisense, 5′-CAGAG GGGTC TCTGG AATGT-3′	237	70009
Myog	sense, 5′-TTACGTCCATCGTGGACAGCAT-3′ antisense, 5′-TGGGCTGGGTGTTAGTCTTAT-3′	245	17928
Sox1	sense, 5′- TGGCCCAGGAAAACCCCAAG-3′ antisense, 5′- GTCTCTTGGCCTCGTCGATG-3′	114	009233
Nes	sense, 5′- GGACAGGACCAAGAGGAACA-3′ antisense, 5′- TCCCACCTCTGTTGACTTCC-3′	599	016701
T	sense, 5′- CCCGAGACCCAGTTCATAG-3′ antisense, 5′-:ATTACATCTTTGTGGTCGTTTC-3′	127	009309
Myod1	sense, 5′- CCTGAGCAAAGTGAATGAG-3′ antisense, 5′- ACCTTCGATGTAGCGGATG-3′	115	010866
Gata6	sense, 5′-TCAGGGGTAGGGGCATCAG-3′ antisense, 5′- GAGGACAGACTGACACCTATG-3′	113	010258
Afp	sense, 5′- GCTCACACCAAAGCGTCAAC-3′ antisense, 5′- CCTGTGAACTCTGGTATCAG-3′	410	007423
Gapdh	sense, 5′-CTCGTCTCATAGACAAGATGGTGAAG-3′ antisense, 5′-AGACTCCACGACATACTCAGCACC-3′	150	14433

Table 2 Effect of different donor cells on preimplantation development of embryos[a)]

Donor cells	RE	Activated (%)	Two-cell (%)	Morulae/blastocysts (%)
Hermaphrodite heart fibroblast cells	284	237 (83.5%)[a]	195 (68.7%)[a)]	43 (18.1%)[a]
Normal tail tip cells	126	103 (81.7%)[a]	72 (69.9%)[a)]	16 (15.5%)[a]

a) RE, reconstructed embryos. Numbers with superscripts denote values that differ significantly within a column (P<0.05).

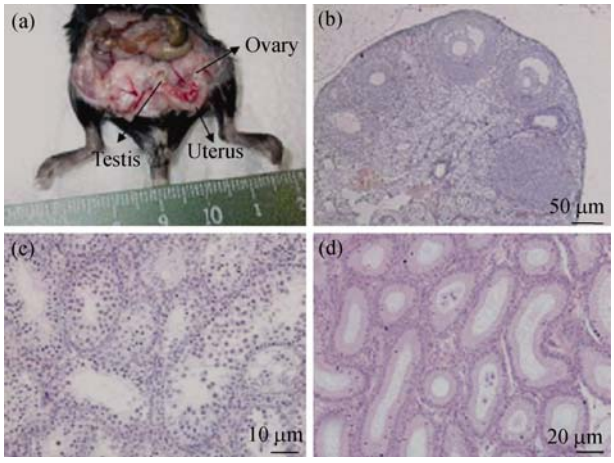

Figure 1 Identification of a hermaphrodite F_1 mouse. (a) Testis, epididymis, and seminal vesicle, as well as ovary, oviduct and uterus; (b) light micrograph of a section of the ovary, showing mesenchymal cells, differentiating oocytes and luteinizing cells; (c) light micrograph of a section of the testis showing Sertoli cells but no differentiated male germ cells in the seminiferous tubule; (d) light micrograph of a section of the epididymis.

2.3 ntESC line derivation

Thirteen ES cell lines were generated from 95 cumulus cell nuclear transfer blastocysts, and 3 ES cell lines were generated from 14 hermaphrodite heart fibroblast cell nuclear transfer blastocysts. The efficiency of ESC derivation did not differ significantly between the 2 donor cell types (Table 3). PCR analysis demonstrated that some Y chromosome-specific genes were absent from the genomic DNA of the hermaphrodite mouse-derived ntESC lines (Figure 2(b)).

2.4 ESC marker expression and differentiation capacity

Mouse ESCs are known to express Oct4, Nanog, SSEA-1 and ALP, but not SSEA-3 and SSEA-4. Immunostaining of these positive and negative ESC makers showed expected expression patterns as shown in Figures 3—5. Table 4 summarizes the expression profiles of

Table 3 Derivation efficiency of ESC lines from cloned blastocysts derived from different donor cells[a)]

Type of ESC	Donor cells	Blastocysts	Derived ESCs (% of blastocyst)
Normal ntESC	cumulus cells	95	13 (14%)[a)]
Hermaphrodite ntESC	heart fibroblast cells	14	3 (21%)[a)]

a) Numbers with superscripts denote values that differ significantly with a column (P<0.05).

Table 4 Expression of ESC markers in derived ES cell lines[a)]

Cell line	Donor cells	ntESC lines	ALP	EB	Immunohistochemistry				
					Oct4	Nanog	SSEA-1	SSEA-3	SSEA-4
R1	*in vivo*	1	+	+	+	+	+	–	–
ntESC	cumulus cells	13	+	+	+	+	+	–	–
Hermaphrodite ntESC	heart fibroblast cells	3	+	+	+	+	+	–	–

a) + indicates positive staining for the antigen; – indicates negative negative staining for the antigen.

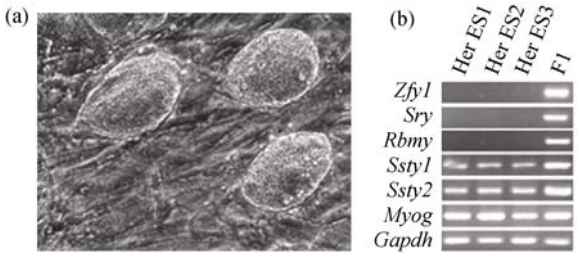

Figure 2 Morphology of a hermaphrodite-derived ntESC colony (a) and PCR analysis of Y chromosome-specific genes from genomic DNA of hermaphrodite-derived ntESC lines (b).

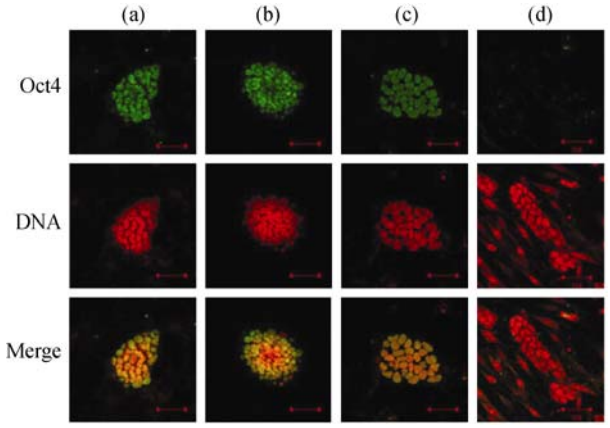

Figure 3 Expression of Oct4 in various mouse ESC lines. Immunostaining with Oct4 antibody was performed on ESC derived from fertilized embryos (a) or cloned embryos ((b) and (c)). Immunostaining with secondary antibody only is shown in (d). DNA was counterstained with Propidium Iodide (Red). (a) R1; (b) ntES-1; (c) Hermaphrodite ntES-1; (d) Negative control. Bars = 50 μm

these markers in our ESC lines. We examined the differentiation capacity of these cell lines by testing for formation of embryoid bodies (EB) (Figure 6(a)) in suspension culture and obtained positive results for all 16 ntESC lines and . RT-PCR results showed that genes from three embryonic germ layers expressed in EBs,

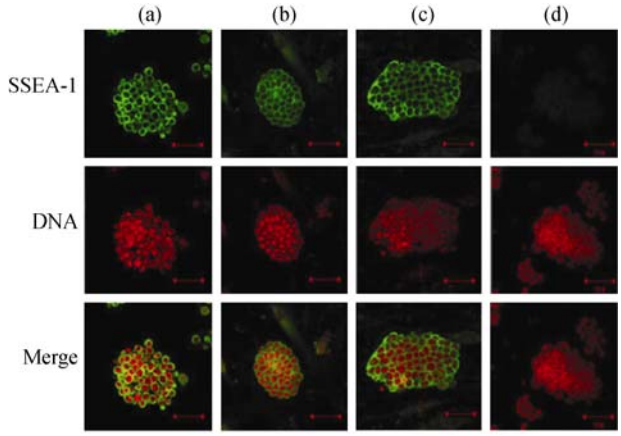

Figure 4 Expression of SSEA-1 in various mouse ESC lines. Immunostaining with SSEA-1 antibody was performed on ESC derivedfrom fertilized embryos (a) or cloned embryos ((b) and (c)). Immunostaining with secondary antibody only is shown in (d). DNA was counterstained with Propidium Iodide (Red). (a) R1; (b) ntES-1; (c) Hermaphrodite ntES-1; (d) Negative control. Bars = 50 μm

including *Sox1*, *Nes*, *T*, *Myod1*, *Gata6*, *Afp* (Figure 6(b)). Teratoma formation assays were preformed, and the results demonstrated that all 3 hermaphrodite ntESC lines were capable of giving rise to cell and tissue types of the three primary germ layers (Figure 7).

2.5 Karyotypic analysis of ntESC lines

One of the properties of ES cells is their ability to exhibit and maintain a stable, diploid and normal complement of chromosomes (karyotype). More than 50 metaphase nuclei were examined for each ntESC line. The ntESC lines had normal karyotypes in 50%—75% of the cells when examined using Giemsa staining (Figure 8(a)). These results are within the normal range for mouse ESC lines[3].

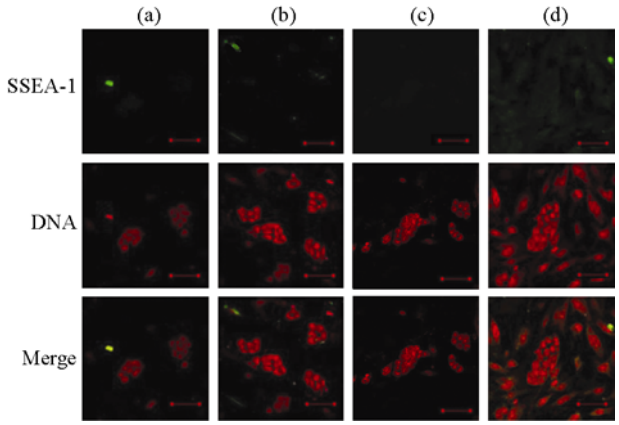

Figure 5 Expression of SSEA-3 in various mouse ESC lines. Immunostaining with SSEA-3 antibody was performed on ESC derived from fertilized embryos (a) or cloned embryos ((b) and (c)). Immunostaining with secondary antibody only is shown in (d). DNA was counterstained with Propidium Iodide (Red). (a) R1; (b) ntES-1; (c) Hermaphrodite ntES-1; (d) Negative control. Bars = 50 μm

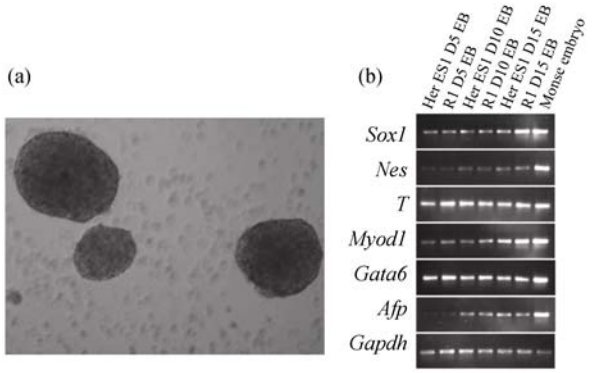

Figure 6 Formation of embryoid bodies (a) and RT-PCR analysis for the expression of representative genes of the three germ layers of EB (day 5, 10 and 15) differentiated cells (b). Mouse embryo as a positive control.

2.6 Production of ntESC-derived offspring using diploid and tetraploid chimeras

Table 5 summarizes a series of experiments in which ntESCs derived from the hermaphrodite mouse were transplanted into the blastocoel of diploid and tetraploid blastocysts to produce chimeric mice. When ESCs are injected into a tetraploid blastocyst, all cells in the resulting mouse are derived from the ESCs, and the tetraploid cells can only develop into the placenta. We obtained 16 chimeric mice (Figure 8(b)) from diploid blastocysts but failed to obtain chimeric mice from tetraploid blastocysts. When the chimeric mice were mated with ICR males or females to test for germ line transmission, none of the chimeric mice showed germ line transmission after several matings. This result suggests that ntESCs derived from the infertile hermaphrodite mouse are unable to contribute to gametogenesis. In a control experiment, chimeric mice produced from cumulus cell ntESCs displayed germ line transmission after being mated with ICR males.

3 Discussion

In this study, we used an infertile hermaphrodite mouse as a model to evaluate the pluripotency of ntESCs. Genetically, the hermaphrodite mouse was a male with a Y chromosome. PCR analysis of genomic DNA from hermaphrodite mouse-derived ntESC lines showed that the sex-determining Y chromosome gene *Sry* was absent (Figure 2(b)), which might have led to the hermaphro-

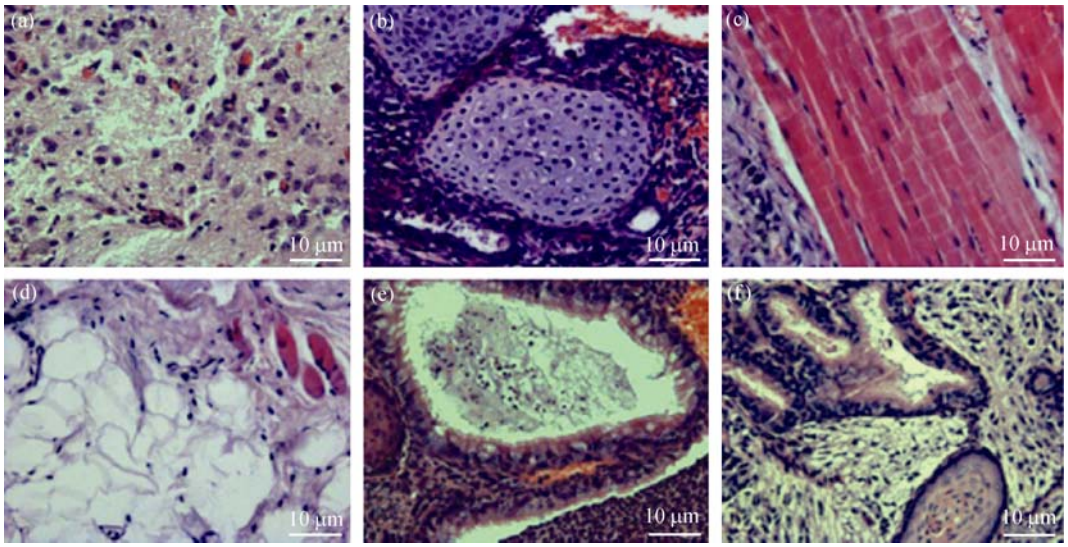

Figure 7 Derivatives from all three germ layers are present in teratomas produced by hermaphrodite-derived ntESCs. (a) Mature central nervous system tissue (ectoderm); (b) and (d) cartilage, muscle cells, fat cells (mesoderm); (e) and (f) gland, squamous epithelium with keratin (endoderm). Bars = 10 μm.

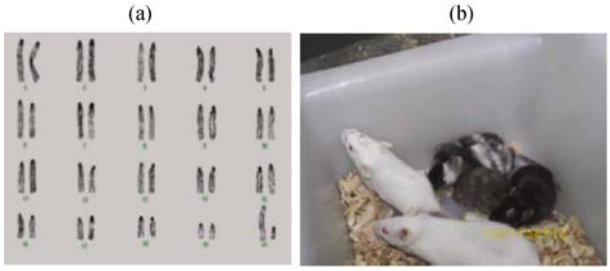

Figure 8 Karyotype analysis of hermaphrodite ntESCs (a) and diploid chimeric mice from hermaphrodite ntESCs (black and white coat color) (b).

dite mouse having growing oocytes and no multiplying spermatogenic cells in its gonads. A previous report showed that an infertile hermaphrodite ICR mouse lacking germ cells was propagated by injecting ntESCs derived from the hermaphrodite donor into diploid or tetraploid blastocysts. The contribution of a tetraploid embryo that receives embryonic stem cells is restricted to extraembryonic tissues such as the placenta, making the transferred ES cells the sole contributors to somatic development. Unfortunately, the authors were unable to clone the hermaphrodite mouse using conventional somatic cell or ESC nuclear transfer[20]. Genetic background is an important factor in cloning efficiency. Cloning mice from somatic cells has been successful in hybrid F_1 strains. Until recently, cloning outbred ICR mice was achieved by trichostatin A treatment after somatic cell nuclear transfer[21]. As with somatic cells, ESCs from hybrid mice are more efficient donors for nuclear transfer than ESCs from inbred 129 mice and R1 ES cells from out-crossed 129 mice: up to 10%—20% of cloned blastocysts produced from F_1 hybrid ESC nuclei develop to full-term mice.

We successfully derived 3 ntESC lines from hermaphrodite F_1 mouse somatic cells. We produced cloned mice from the ntESC nuclei, but failed to obtain live pups (except for placentas) directly from ntESCs derived from the hermaphrodite mouse (data not shown). Similar failure to generate full-term development of embryos from ntESCs derived from a hermaphrodite ICR mouse has been reported[9]. Our results suggest that due to genetic defects, a hermaphrodite F_1 mouse might not be cloned when ntESC nuclei derived from it are injected into enucleated oocytes. We also attempted to generate chimeric mice using diploid or tetraploid embryos. When ntESCs were injected into the blastocoels of diploid embryos, many chimeric mice were produced, but none of the chimeric mice demonstrated germ line transmission after mating with ICR mice. When ntESCs were injected into the blastocoels of tetraploid embryos, placentas were obtained but living pups were not. All three hermaphrodite-derived ntESC lines produced teratomas after injection into severe combined immunodeficient (SCID) mice. In these teratomas, cell types from all three germ layers were identified using histochemical methods. These results suggest that compared with germline transmission, teratoma formation might only partially confirm multipotency in ESCs.

Several reports have indicated that the process of ntESC derivation rigorously selects for immortal cells that have been completely reprogrammed to pluripotency and that therefore, ESC lines derived from cloned or fertilized blastocysts should have identical therapeutic potentials[2,24]. This is indeed true in mice because many ntESC lines differentiate into germ cells in chimeric mice, providing the strongest evidence to date that these cells are fully pluripotent. Since the derivation of the first human embryonic stem cell (hESC) lines, many hESC lines have been established due to their potential as sources of cells for regenerative medicine and as valuable tools for drug discovery and for understanding human development and disease[25—27]. Currently, hESCs are evaluated by a set of makers and their capacity to differentiate. For ethical and practical reasons, the ability of hESCs to contribute to the germ line in chimeras is not a testable property, but this criterion is the most stringent for ESC pluripotency[28]. To date, the formation of differentiated cells from the three somatic germ layers within a teratoma is taken as the best indicator of pluripotency in hESC lines[29]. Our experiments clearly

Table 5 ntESC contributions to chimeras after injection into normal fertilization-derived blastocysts

Origin of ntESC line	Recipient blastocysts		Blastocysts transferred (recipients)	Chimeric contribution in coat (F/M)[a]			Germ line transmission	
	Strain	Ploidy		Total no. of chimeras	High (>50%)[b]	Low (<50%)[b]	Mated with ICR female	Germline transmission (black pups)
F1hermaphrodite ntESCs	ICR	2n	118(9)	16(1/15)	15(4/11)	1(1/0)	2	(0/417)
		4n	298(17)	0	0	0	0	0
F1 cumulus cell ntESCs	ICR	2n	45(3)	7(2/5)	3(1/2)	4(2/2)	1	1(1/63)

a) F, female; M, male. b) Contribution was scored as either high (>50% of coat color derived from ntESCs) or low(<50%).

demonstrate that in mice, ntESC lines with genetic defects can be established from a hermaphrodite F1 mouse. These ntESCs display typical ESC morphology, express ESC markers and form teratomas in SCID mice, but cannot be transmitted through the germ line. This situation may also exist in hESC lines, which might present a potential risk for their clinical use. It has been recognized that there is a need for more comprehensive characterization of hESC lines than is currently being undertaken to assess their true potential. To provide a more comprehensive assessment of hESC pluripotency, a set of validated makers should be identified. For example, the fact that differentiation of hESCs into embryoid bodies *in vitro* results in formation of cells that express makers, such as VASA, which are specific to gonocytes could be used as an indicator of pluripotency[30].

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