—Mini Review—

Embryonic Stem Cell Lines from Somatic Cell Nuclei Via Nuclear Transfer

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Abstract: *The progress of nuclear transfer technology, we can create cloned animals. And recently from this same technology we can possible to establish nuclear transfer embryonic stem cells (ntES cell). From our experiments we can establish mouse ntES cells, which any kind of cell type and strain and sex. And ntES cell has very similar ability like ES cells that has capacities for in vitro differentiation and in vivo germline transmission. The ntES cell made from donor somatic cells, which are very useful for therapeutic cloning Because of no immune rejection. Especially for human, it have high expectation from this new opportunity for rejuvenation of the ageing or diseased body. However, even for cloned animals the efficiency is so low and that have many problems. So, it necessary to do more analysis of ntES cell can work normal and safe before clinic.*

Key words: *ntES cell, Nuclear transfer, Clone*

Introduction

Recent years have seen active research into the possible applications of human embryonic stem (ES) cells in regenerative medicine, in which ES cells are envisioned as potential sources for cells to be used in cell replacement therapies. However, as with any allogeneic material, ES cells derived from fertilized blastocysts, and the progeny of such cells, inevitably face the risk of immunodeficiency on transplantation. It has been proposed that ES cells derived from embryos cloned from the host patient's own cells represent a potential solution to the problem of rejection in transplantation, as any replacement cells would be genetically identical to the host's own somatic cell nuclei [1–3]. We have shown that ntES cells can be generated relatively easily from a variety of mouse genotypes and cell types of both sexes, even though it may be more difficult to generate clones directly. Several reports have already demonstrated that ntES cells can be used in regenerative medicine in order to rescue immune deficient or infertile phenotypes. However it is still unclear if ntES cells possess the same abnormalities as cloned animals. This review seeks to describe the phenotype and possible abnormalities of ntES cell lines.

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Background of the ntES Cell

There has been a great deal of interest in the possible application of human embryonic stem (ES) cells in regenerative medicine, as ES cells are envisioned as potential sources for cell replacement therapies. In contrast, nuclear transfer can be used to generate embryonic stem cell lines from a patient's own somatic cells. These ES-like cell lines generated from somatic cells via nuclear transfer (ntES cells) were first reported in the cow [4]. Production of ntES cells was also reported in the mouse [5–7] and human [8]. We have previously shown that ntES cell lines are capable of differentiating into all three germ layers *in vitro* or into spermatozoa and oocytes in chimeric mice [7]. This was the first demonstration that ntES cells have the same developmental potential as ES cells from fertilized blastocysts and that cloned mice can be obtained from these ntES cell lines using a second nuclear transfer [7]. These techniques have now been applied to not only preliminary medical research [9], but also basic biological research. For example, irreversible changes were demonstrated in the DNA of adult lymphocytes [10], but not of olfactory neurons [11, 12]. Cancer cell types have also been characterized [13, 14]. We also demonstrated that this ntES technique is also applicable

Table 1. Establishment of ntES cell lines from different mouse strains and mutant mouse

Mouse strain	Source of donor cells	gender	Number (%) of eggs/oocytes				Established ntES cell lines		
			Enucleated	Survived	Activated	Developed to morulae/blastocysts	Total no.	% from 1-cell embryo	% from morula/ blastocyst
$B6D2F_1$	Tail	Male	417	341 (81.8)	246 (59.0)	41 (16.7)	11	4.5	26.8
	Cumulus	Female	109	104 (95.4)	88 (80.7)	49 (55.7)	8	9.1	16.3
$B6C3F_1$	Tail	Male	215	150 (69.8)	67 (31.1)	23 (34.3)	5	7.5	21.7
	Tail	Female	100	65 (65.0)	61 (61.0)	3 (5.0)	1	1.6	33.3
	Cumulus	Female	328	263 (80.2)	257 (78.4)	140 (54.5)	27	10.5	19.3
$BD129F_1$	Tail	Male	120	108 (90.0)	89 (74.2)	53 (59.6)	31	34.8	58.5
	Cumulus	Female	47	40 (85.1)	26 (55.3)	16 (61.5)	12	46.2	75
C3H/He	Tail	Male	88	34 (38.6)	22 (25.0)	3 (13.6)	1	4.5	33.3
	Tail	Female	210	110 (52.4)	53 (25.2)	12 (23.0)	2	3.8	16.7
	Cumulus	Female	92	44 (47.8)	30 (32.6)	17 (56.6)	1	3.3	5.9
C57BL/6	Tail	Male	703	520 (74.0)	307 (43.7)	27 (8.8)	5	1.6	18.5
	Cumulus	Female	270	205 (76.0)	191 (70.7)	51 (26.7)	5	2.6	9.8
DBA/2	Tail	Male	230	207 (90.0)	144 (62.6)	11 (7.6)	2	1.4	18.2
	Cumulus	Female	95	50 (52.6)	39 (41.1)	20 (51.2)	2	5.1	10
FVB	Tail	Male	140	86 (61.5)	36 (25.7)	13 (36.1)	3	8.3	23.1
ICR	Tail	Male	282	201 (71.3)	114 (40.4)	11 (10.0)	1	0.9	9.1
	Tail	Female	218	178 (81.7)	136 (62.4)	13 (10.0)	3	2.2	23.1
$Tg\text{-}BDF_1$	Tail	Male	79	60 (75.9)	50 (63.3)	15 (30.0)	3	6	20
	Cumulus	Female	58	56 (96.6)	53 (91.4)	25 (47.2)	3	5.7	12
$129B6F_1$	Sertoli	Male	80	55 (68.8)	49 (61.3)	15 (31.0)	4	8.2	26.7
Total			3,881	2,877 (74.1)	2,058 (53.0)	558 (27.1)	130	6.3	23.3

to the preservation of genetic resources of mouse strains instead of the embryo, oocyte, or spermatozoa. At present, this technique is the only available method for the preservation of valuable genetic resources from mutant mice without the use of germ cells [15].

However, the rate of successful production of cloned animals is still very low. Cloned embryos can develop to full term, although most animals show abnormalities of gene expression in the embryo [16, 17] and placenta [18, 19], obesity [20, 21] and early embryonic death [22]. These are thought to be due to failures in genomic reprogramming, but the molecular mechanism is not got fully understood. In contrast, we confirmation ntES cell lines showed an almost identical phenotype to normal ES cells, i.e. positive Oct3/4 and SSEA-1 expression (unpublished observation).

Establishment of ntES Cell Lines

Hwang *et al.* [8] successfully derived ES cells from a human embryo cloned from a somatic cell nucleus using nuclear transplantation technology. They reported that only one line of ntES cells was established from a set of 30 original blastocysts (establishment rate, 3.3%), and that the donor somatic cell and oocyte were taken from the same individual. The low efficiency raises the reasonable question of whether only female patients who possess a supply of healthy oocytes will be able to take advantage of the potential benefits of therapeutic cloning. In the mouse, the genotype of the animal strain or the sex of the donor nucleus often affects the successful full-term development of cloned offspring [23, 24]. For example, hybrid genotypes are more tolerant of cloning than inbred genotypes, such as C57BL/6 and C3H/He, which are commonly used in mouse genetic studies but have never been cloned successfully [23, 24].

In order to determine the efficiency of ntES cell line establishment, we have tested several different male and female mouse genotypes as sources of nuclei (Table 1). Nuclei of ntES cells were injected into recipient enucleated oocytes from adult hybrid mice, and ntES cell lines were successfully established from all mouse genotypes of both males and females. However, when inbred and F_1 genotypes were compared, the rate of development to the blastocyst stage and the frequency of ntES cell derivation were significantly higher when F_1 cumulus cells were used as the donor nucleus (Fig. 1a). However, this difference was only observed when the data were compared from reconstructed oocytes (Fig. 1b). No strain- or gender-dependent differences were detected in tail-tip cells

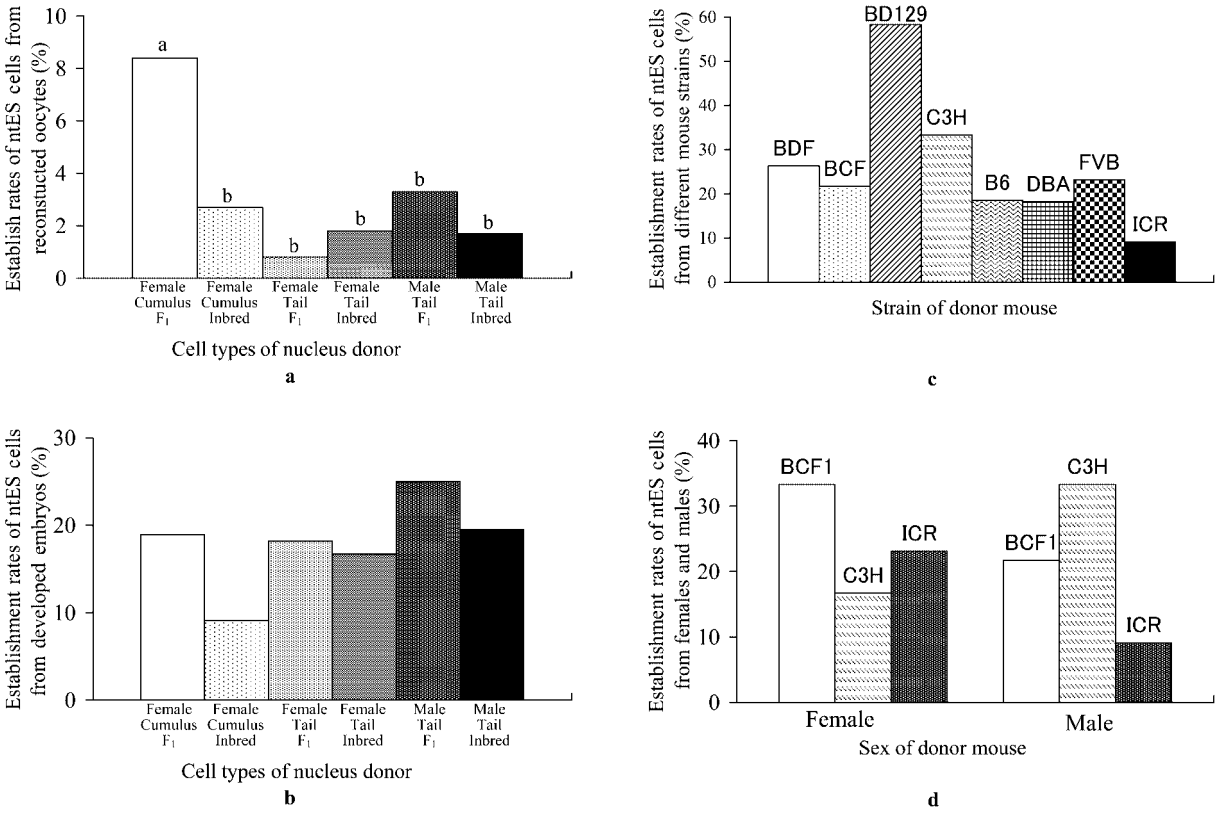

Fig. 1. Establish rate of various ntES cell lines. The rates of establishment of ntES cell lines were compared using female cumulus cells, female tail-tip fibroblast nuclei and male tail tip fibroblast nuclei as donors. The ntES cells establish rates from reconstructed oocytes (a); Cumulus cells from F_1 female mice had better rate than the others, however, that difference disappeared when the establish rate was compared among morula/blastocyst developed embryos (b). ntES cells could be established from all examined mouse strains and without significant difference (c). There was no significant difference between sexes when tail-tip fibroblasts were used as donor nuclei (d).

(Fig. 1c, d) [25].

In the first reports of cloning in mice [26] and cows [27], female cumulus cells were used as the donor nucleus. In addition, the first human ntES cell line was also established from cumulus cell nuclei [8]. Thus, cumulus cells have predominated in nuclear transfer experiments, but cumulus cells are available only in the case of a healthy and fertile female. On the other hand, fibroblastic cells are easy to obtain from both females and males, and even unhealthy animals. So, we compared the efficiency of ntES cell establishment between fibroblast and cumulus cells and male and female fibroblasts. There was no significant difference between cell types or between sexes [25]. Surprisingly, it has been shown that even differentiated neuron and lymphocyte cells can be used to establish an ntES cell line (0.1–2%), although in previous studies these cells have failed to produce any cloned offspring [10–12, 23].

Therapeutic Application of ntES Cell Techniques

ntES cells are genetically identical to the donor and are potentially useful for therapeutic application. Therefore, therapeutic cloning may improve the treatment of neurodegenerative diseases, blood disorders, or diabetes, since therapy for these diseases is currently limited by the availability and immunocompatibility of tissue transplants [1–3]. We have demonstrated that dopaminergic and serotonergic neurons can be generated from ntES cells derived from tail-tip cells [7]. Rideout *et al*. [9] reported that therapeutic cloning combined with gene therapy was able to treat a form of combined immune deficiency in mice. They made one ntES cell line from an immune deficient mutant mouse. First, the ntES cell with mutated alleles was repaired by homologous recombination, thereby restoring the normal gene

structure. The ntES cells were transplanted to the immune deficient mouse [9]. In addition, Barberi *et al.* reported an interesting study that showed that mouse tail or cumulus cell derived ntES cells could be differentiated into neural cells at even higher efficiencies than fertilization derived ES cells [28].

Application of ntES Cell Techniques to Basic Biological Science

ntES techniques can also be applied to biological science. We have demonstrated that cloned mice can be generated from the nucleus of ntES cells by a second nuclear transfer [7]. However, the success rate was not improved when comparable to that of various kind of somatic cell cloning. If difficulty was experienced in the production of cloned mice from the donor cell, difficulty was also experienced while attempting to produce cloned mice by the second nuclear transfer from ntES cell nuclei [7, 15]. To overcome this low efficiency in the production of cloned animals, complementation with tetraploid embryos was employed, in which ntES cells were injected into the tetraploid blastocyst. As a result, almost all parts of the chimeric offspring, including germ cells, originated from ntES cells. The offspring were referred to as ES mice [29, 30]. Using this technique, monoclonal mice have been generated from ntES cells derived from B and T lymphocyte nuclei [10]. The ntES cell techniques can also be used for characterization of very rare cells in the body. If ntES cells from these rare cell nuclei are established once, the cells can proliferate infinitely. It was hypothesized that an odorant receptor gene chosen from over a thousand is controlled by DNA rearrangements in olfactory sensory neurons, such as lymphocyte nuclei. However, this could not be demonstrated due to the very low number of specific differentiated cells. Li *et al.* and Eggan *et al.* generated ntES cells from the nucleus of a single olfactory sensory neuron and demonstrated that odorant receptor gene choice is reset by nuclear transfer and is not accompanied by genomic alterations [11, 12]. ntES cell techniques can also be used to assess tumorigenic and developmental potential. ntES cell lines are established from embryonal carcinoma (EC) cells or melanoma cell nuclei. But chimeric mice from the ntES cells developed cancer with higher frequency, and it has been demonstrated that non-reprogrammable genetic modifications define the tumorigenic potential [13, 14].

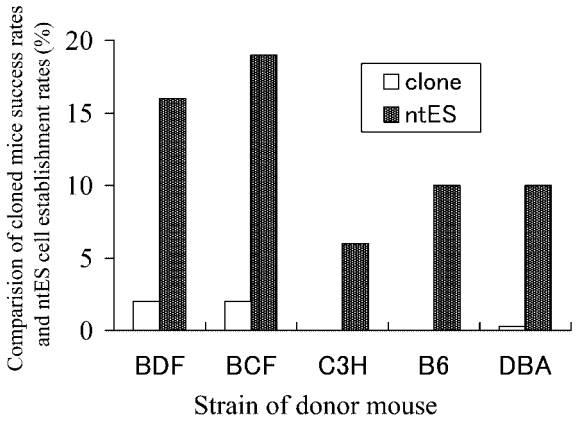

Fig. 2. Comparison between full-term development of cloned mice and the rates of establishment of ntES cell lines. Clones were produced by cumulus cell nuclear transfer and morula/blastocyst transfer into recipient females, whereas the cell lines were established from morula or blastocysts derived from cumulus cell donor nuclei. The success rate in establishing ntES cell lines is much higher than that for conventional cloning in al thel mouse strains studied.

Preservation of Genes from Infertile Mice without the Use of Germ Cells by ntES Technology

Infertility is listed as a phenotype in more than half of the mutants described in one large-scale ethyl-nitrosourea (ENU) mutagenesis study [31, 32]. The ability to maintain such types of mutant mice as genetic resources will offer numerous advantages in the investigation of the molecular mechanisms of human infertility and the biology of reproduction. Unfortunately, the success rate of somatic cell cloning is very low [26]. Even in cases in which the cloning of a sterile mouse is successful, because of the non-reproductive nature of the phenotype, it is still necessary to clone all subsequent generations. This represents another significant potential barrier to the maintenance of the strain, as the success rate of cloning from cloned mice decreases with each successive generation after the first nuclear transfer [33].

In recently studies, ntES cells could maintain the mutant gene, but neither cloning nor tetraploid complementation chimera construction could rescue the lineage of the original infertile hermaphrodite male [15, 25]. These result suggested that ntES technology might allow us to maintain some interesting genes from mouse strains even without the use of germ cells.

Are ntES Cells Substantially Similar to ES Cells?

The success rates for the establishment of ntES cell lines have been much higher than full term developed cloned mice. This is particularly noted in the case of the inbred strains, C57BL/6 and C3H/He, which are commonly used in research [7, 15, 25], but have never been successfully used to produce cloned mice (Fig. 2) [23]. The establishment rate of ntES cell lines is comparable to those of fertilized ES cells [34]. This raises the question of why the success rate for the establishment of ntES cell lines is higher than that for the full-term development of a cloned mouse. On average, 30–50% of nuclear transfer embryos develop to the blastocyst stage. However, most cloned embryos die immediately after implantation [35], which is thought to be attributable to incomplete reprogramming. This suggests that even when reprogramming is incomplete, a significant percentage of cloned embryos are able to develop to the blastocyst stage, indicating that complete reprogramming is not required for preimplantation development. If it is true that the high rates of developmental failure of cloned mice are due to incomplete reprogramming, two possible explanations for the discrepancy between the rates of blastocyst and full-term development can be entertained. The first possibility is that the majority of ntES cells are inherently abnormal. ntES cell lines are established from blastocyst stage embryos, and probably have limited gene expression requirements at this stage. Therefore, even if some embryonic genes that are essential for full-term development are not expressed, the embryos can develop to blastocysts [16, 17]. If this is true, some ntES cell lines are established from incompletely reprogrammed cloned blastocysts. It will be necessary to determine the nature and extent of such aberrations in ntES cells before considering their application in regenerative medicine. However, it is possible to use even incompletely reprogrammed ntES cells as an alternative to embryos or gametes in the preservation of genetic resources, because the abnormalities observed in cloned mice are not transmitted to their offspring [21].

The second possible explanation for the different developmental competencies of cloned embryos is that the genomic reprogramming of somatic cells after nuclear transfer may occur gradually. To establish an ntES cell line, the cloned blastocyst must be cultured for more than one month *in vitro*. During the culture period, survival of the cloned embryo is independent of autonomous gene expression and placental development, which in any case always shows some abnormality [16–19, 36, 37], and reprogramming is able to proceed gradually. However, in cloning, the cloned embryos must express all appropriate genes and exhibit relatively normal placental development immediately after implantation. This more stringent set of criteria may account for the higher developmental failure rates of presumably incompletely reprogrammed cloned embryos following implantation [3].

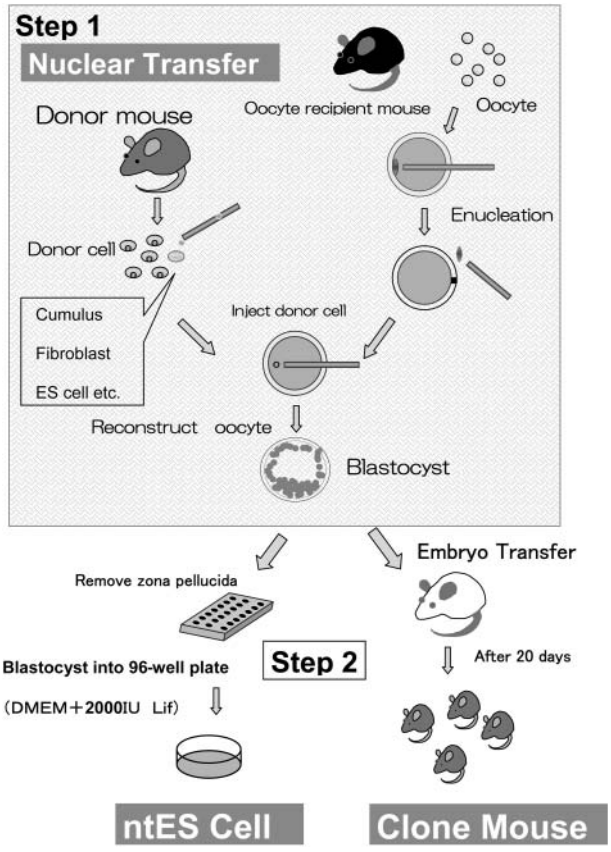

Fig. 3. The method of ntES cell establishment requires two independent steps. The first step is nuclear transfer which generates cloned morula/blastocysts. This is the usual method for producing cloned mice. When cloned morula/blastocysts were transferred into surrogate mothers, 1–2% of embryos could develop to full term. For ntES cell establishment, second step is required. Cloned morula/blastocysts embryos are treated with acetic tyrode solution to remove zonae pellucida. The cloned blastocyst are washed several times and then plated into a 96 well dish containing a performed feeder layer and culture about 10 days. During this period, the cloned blastocysts attach to the surface of the feeder layer and the inner cell mass (ICM) can be seen to grow.

Perspective

As reviewed above, ntES cell techniques have great potential for use both in therapy and biological science. However, current methods required the cytoplasm of fresh oocytes from a healthy female. Recently, the ability of ES cells to generate gametes or inter-species oocytes has shown great promise toward addressing the chronic scarcity of human oocytes for use in nuclear transfer studies. These studies can be accomplished either through the artificial creation of oocytes by *in vitro* differentiation [38] or through non-human oocytes, such as cow or rabbit [39, 40], which have the potential to be reprogrammed using human somatic cell nuclei to establish human ntES cell lines. Fundamental questions remain as to whether reconstructed oocytes have the same developmental potential as natural oocytes, and whether ntES cells and their rabbit mitochondria containing derivatives will be tolerated by a human host's immune system on transplantation. If these doubts can be laid to rest, these techniques may one day offer an unrestricted supply of precious biological resources for use in the establishment of new ntES lines without requiring the participation of healthy, fertile women as oocyte donors [3].

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