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—Full Paper—

Nuclear Transfer Preserves the Nuclear Genome of Freeze-Dried Mouse Cells

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Abstract. Mouse spermatozoa can be freeze dried without losing genetic integrity and reproductive potential. However, it is not known if freeze-dried mouse cells similarly maintain their genetic integrity and developmental potential following nuclear transfer. Here, we investigated the developmental capacity and embryonic stem (ES) cell derivation of reconstructed oocytes by nuclear transfer using freeze-dried cumulus or ES cells. Cumulus and ES cells were lyophilized overnight and stored at 4 C for up to 1 week. After rehydration, all cells showed membrane damage and were unviable. However, following nuclear transfer, 1–4% of the reconstructed oocytes developed to the blastocyst stage. A total of five nuclear transfer ES (ntES) cell lines were generated from blastocysts and morulae. All ntES cell lines had normal karyotypes and were positive for the ES-cell-specific markers (alkaline phosphatase, Oct3/4 and Nanog). After aggregation of ntES cells with fertilized embryos, chimeric mice with a high level of coat color chimerism were generated. Our findings show that the genomic integrity of cells can be maintained after freeze-drying and that it is possible to produce offspring from the cells using nuclear transfer techniques.

Key words: Clone, Freeze-dry, Nuclear transfer, Nuclear transfer embryonic stem (ntES) cell, Mouse, Reprogramming

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Freeze-dried sperm can support normal development into healthy mice if injected directly into mature oocytes even though the lyophilized spermatozoa are all dead in the conventional sense [1, 2]. It has been shown that the complete sperm DNA can be maintained after freeze-drying in several species [3, 4]. In mammals, spermatozoa are structurally compatible with lyophilisation since they are small cells with a low level of hydration and their transcriptionally inactive DNA are tightly packed with protamines [5]. In contrast to spermatozoa, it has been thought that somatic cells would be less tolerant to the freeze-drying process because they are larger, have a higher water content and their chromatin organization is unpacked, making them vulnerable to the freeze-drying process. It is not known if freeze-dried cell nuclei can generate further generations following nuclear transfer.

It has been shown that when frozen cells are thawed without cryoprotection, nuclear transfer ES (ntES) cell lines can be generated from the dead cell nuclei and live mice can be created via germline transmission of chimeric mice [6]. We have already succeeded in producing live cloned mice from mouse somatic cells frozen for 16 years [7], and this suggests that the viability of donor cells is not important for producing the next generation using nuclear transfer. Recently, Loi *et al*. reported that sheep blastocysts could be generated from freeze-dried somatic cells with a similar success rate as for fresh cells following storage at room temperature for 3 years [8]. This was the first attempt to use freeze-dried somatic cells for nuclear transfer, but that study only demonstrated the developmental potential to the blastocyst stage rather than the preservation of nuclear function. Blastocyst development per se cannot demonstrate the nuclear integrity because most oocytes fertilized with damaged or dead freeze-dried spermatozoa can develop into morphologically normal blastocysts [9].

Trehalose protects organisms against various stresses such as dehydration, freezing and osmotic pressure. Previous studies have shown that trehalose protects mammalian cells, and the presence of trehalose on both sides of the cell membrane can maximize the protective effect of trehalose during cell freezing and drying [10, 11]. Trehalose also has a protective effect on evaporatively dried mouse spermatozoa [12, 13].

We investigated the nuclear integrity of freeze-dried cells with and without trehalose. Following rehydration of dead freeze-dried cells, cloned blastocysts were obtained by nuclear transfer. We established ntES cell lines from morulae and blastocysts, and nuclear integrity was then examined by production of chimeric mice. The results indicate that freeze-drying of mouse cells as well as spermatozoa can be used to maintain the complete mouse genome for future production of offspring.

Materials and Methods

Animals

Adult female B6D2F1 mice (2–3 months old), pCX-eGFP C57BL/6 Tg females, DBA/2 males and adult ICR mice (2–6 months old) were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). We then produced pCX-eGFP-B6D2F1 mice by mating C57BL/6 Tg females with DBA/2 males. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation of the RIKEN Kobe Institute.

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Preparation of ES or cumulus cells and freeze drying

We used an ES cell line derived from previously established GFP-129B6F1 Tg mouse lines [14]. Non-GFP ES cells (C14) [14] were used for viability testing. ES cells were detached from their dishes by treatment with 0.25% trypsin and 0.75 mM EDTA. The cell suspension was pipetted for a few minutes to release the cells from the dish surface and centrifuged (300 G, 5 min). The supernatant was removed, and the cells were washed with modified EGTA solution (mEGTA: 50 mM EGTA, 100 mM Tris-HCl, pH 8.0) [15] with or without 0.5 M trehalose. Mature pCX-eGFP-B6D2F1 mice were superovulated by administration of 5 IU equine chorionic gonadotropin (eCG; Teikokuzoki, Tokyo, Japan) followed 48 h later by 5 IU human chorionic gonadotropin (hCG, Teikokuzoki). Cumulus-oocyte complexes were collected from the oviducts about 16 h after injection of hCG. After collection, cumulus cells were dispersed with 0.1% bovine testicular hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) in droplets of HEPES-buffered CZB medium (HEPES-CZB). After several minutes, the cumulus cells were transferred to 1.5-ml tubes and centrifuged (300 G, 5 min). The supernatant was removed, and the cells were washed with mEGTA solution with or without 0.5 M trehalose. Ten to 50 μl of each cell suspension prepared as above was put in 1.5-ml tubes (overnight) or vials (one-week storage). Each tube was put in a freezer at –30 C and kept there for 3 h before connection to the lyophilizer. After drying in a vacuum for more than 5 h, each vial was stored at 4 C for 6 days.

Viability of cells

The viability of lyophilized cells was examined using a LIVE/DEAD Reduced Biohazard Viability/Cytotoxicity kit (Molecular Probes™; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. SYTO 10, a green fluorescent nucleic acid stain, is a highly membrane-permeant dye that labels all cells, including those with intact plasma membranes. DEAD Red™ (ethidium homodimer-2) is a cell-impermeant red fluorescent nucleic acid stain that labels only cells with compromised membranes. In this experiment, non-GFP ES cells (C14) were used, and the experiment was repeated three times to obtain more than 1,000 cells. We also examined the viability of rehydrated cells by culturing them in ES cell medium and investigated cell attachment and growth in culture dishes.

Nuclear transfer and ntES cell establishment

Oocyte collection and nuclear transfer were performed as previously described [16, 17] with slight modifications. The donor cells were suspended in 1% PVA-NIM or 4% PVP-NIM solution [18, 19] instead of 10% PVP-HEPES HTF solution (Irvine Scientific, Santa Ana, CA, USA). The oocytes reconstructed with cumulus cells were activated [20] with 5 nM TSA at 37 °C under 5% CO_2 in air for 6 h [21, 22], and then the oocytes were cultured in CZB medium containing 5 nM trichostatin A (TSA) for 3 h. After TSA treatment, cloned embryos were transferred to CZB medium and cultured for up to 4 days. If ES donor cells were used, the reconstructed oocytes were activated without using TSA because TSA only improves the cloning success rate of somatic donor cells [21].

After the cloned embryos developed to the morula or blastocyst stage, they were used to establish ntES cell lines as previously described [14, 23] except that 20% knockout serum replacement and 0.1 mg/ml adrenocorticotropic hormone (American Peptide Company, Sunnyvale, CA, USA) were added to the ES cell medium instead of fetal calf serum [24].

Production of chimeric mice

For production of diploid chimeric mice, ntES cells were introduced into the perivitelline spaces of fertilized 4–8-cell stage embryos collected from ICR strain mice using a piezo-actuated microinjection pipette. Following incubation of these aggregates in CZB medium for 24 h and development to the morula or blastocyst stage, embryos were transferred to a pseudopregnant ICR female (2.5 dpc) and examined at 9.5 dpc or 19.5 dpc through natural birth or Caesarian section. At birth, the GFP expression was noted, and at adulthood, black eyes and chimeric contribution to more than half the coat color was considered to indicate "high" chimerism.

Immunofluorescence staining, alkaline phosphatase staining and karyotype analysis by Giemsa

Established ES cell lines were tested for pluripotency by alkaline phosphatase staining, according to the manufacturer's protocol (Sigma-Aldrich). To test the normality of established ntES cell lines, the expression levels of Oct3/4 and Nanog were examined using immunofluorescence staining. Initially, ntES cell lines were cultured for 2 days on gelatin-coated 8-well chamber slides (Nalge Nunc International, Rochester, NY, USA). After removing the culture medium, the slides were air dried for 15 min and fixed in 4% paraformaldehyde for 20 min. The fixed cells were then washed twice in phosphate buffered saline (PBS, Sigma-Aldrich), stored overnight in PBS with 1% bovine serum and 0.1% (v/v) Triton X-100 at 4 C and incubated with antibodies at room temperature. The primary antibodies used were mouse monoclonal anti-Oct3/4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal anti-Nanog (ReproCELL, Tokyo, Japan). After being washed three times in PBS, the cells were incubated with the following secondary antibodies: Alexa Fluor 568-labeled goat antimouse IgG and Alexa Fluor 568-labeled goat antirabbit IgG (both from Molecular Probes™). After three washes in PBS, the chromosomes were stained with 4,6-diamidino-2-phenylindole (DAPI; 2 μg/ml: Molecular Probes™). Following complete washing, the cells were coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

Chromosomes from the ntES cells were stained using Giemsa as previously described [14, 23, 25].

Statistically analysis

Outcomes were evaluated using χ-square tests, and $P<0.01$ was assumed to be statistically significant.

Results and Discussion

We previously showed that mouse spermatozoa can be freeze-dried without loss of genetic integrity and reproductive potential and that reconstituted lyophilized sperm can support normal development when injected into mature oocytes [1]. Here, we

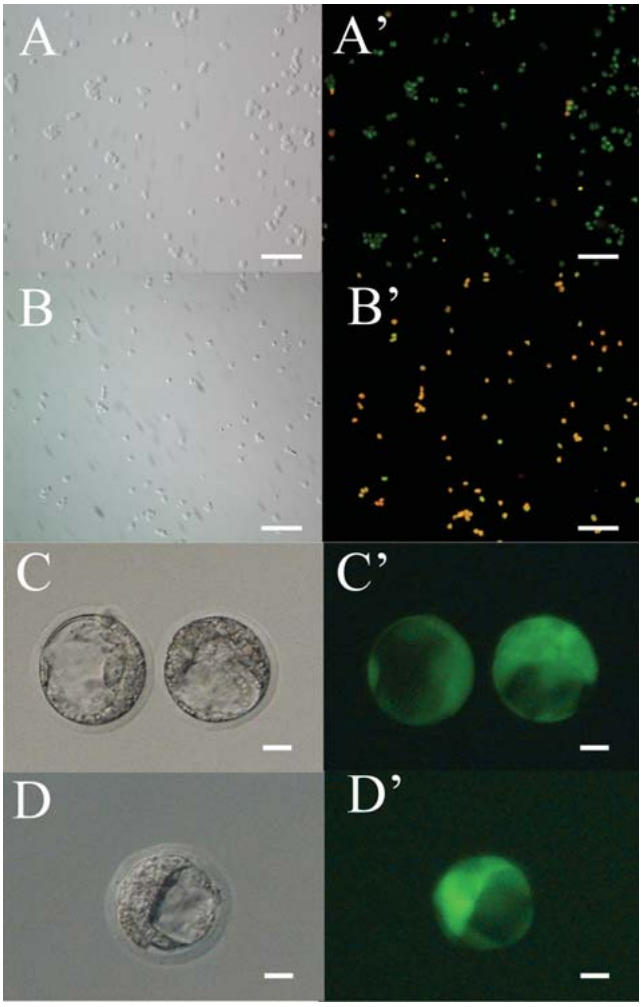

Fig. 1. Freeze-dried cells and cloned blastocysts. A–A': LIVE/DEAD staining of fresh ES cells. A: Bright field. A': Merge image. Fresh cells exhibit green staining but no red staining, indicating that the cells were live. B–B': LIVE/DEAD staining of freeze-dried ES cells. B: Bright field. B': Merge image. All ES cells exhibit green and red staining, indicating that the cells were dead following freeze-drying. C–C': Expression of GFP in a cloned blastocyst derived from freeze-dried ES cells. C: Bright field. C': GFP expression. D–D': Expression of GFP in a cloned blastocyst derived from freeze-dried cumulus cells. D: Bright field. D': GFP expression. Scale bars: A–B'=100 μm, C–D'=20 μm.

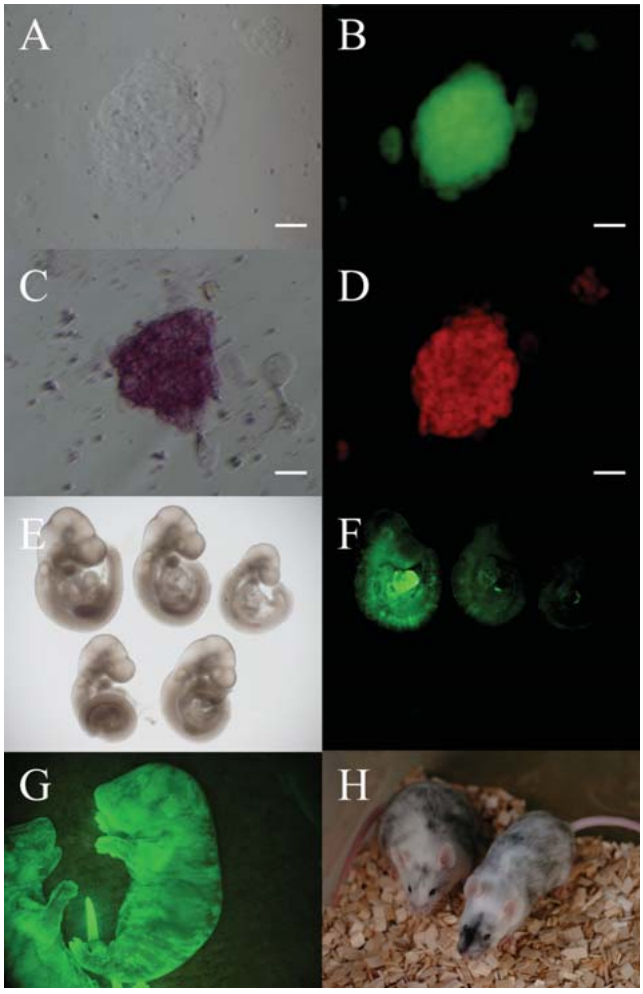

Fig. 2. Generation of ntES cell and chimeric mice. A–D: ntES cell line derived from freeze-dried ES cells. A: Bright field. B: GFP expression. C: Alkaline phosphatase staining. D: Immunofluorescent staining for Oct3/4. E–F: Chimeric mice (9.5 dpc) were produced by injecting ntES cells derived from freeze-dried somatic cell nuclei. E: Bright field. F: GFP expression. G–H: Chimeric mice were produced by injecting ntES cells derived from freeze-dried ES cell nuclei. Scale bars=40 μm.

investigate the nuclear integrity of freeze-dried mouse cells by generating cloned embryos, ntES cell derivation and production of chimeric mice.

Mouse cumulus and ES cells were lyophilized according to the protocol described in our previous report [1]. After rehydration, the viability of more than 1,000 freeze-dried cells was determined using LIVE/DEAD staining. Ninety-three percent of fresh cells were live, whereas all rehydrated cells (100%) were judged to be dead based on membrane impermeable staining (Fig. 1A–B'). We also checked if any cells survived after freeze-drying and rehydration by culturing them in culture media, but none attached to the dish or grew, irrespective of the cell type (data not shown). These results show that cells do not survive after freeze-drying and rehydration. When these dead cells were placed in 10% PVP-HEPES HTF solution as for live cell nuclear transfer [16], they were shrunken and hard, probably because of cell membrane damage (data not shown). To overcome this problem, we modified the nuclear transfer method by using 1% PVA-NIM or 4% PVP-NIM solution instead of the 10% PVP-HEPES HTF solution. This did not result in any morphological changes in the donor cells.

Using this modified method, we injected rehydrated freeze-dried donor cells into enucleated oocytes and attempted to establish ntES cell lines when the cloned embryos developed to morulae or blastocysts. We did not try to produce cloned mice by embryo transfer into recipient females because the generation rate of ntES cell lines

Table 1. Preimplantation development of reconstructed oocytes after nuclear transfer using freeze-dried cells

Storage period	Cell type (strain)	Trehalose	No. enucleated oocyte	No. surviving after NT (%)	No. pronuclear formation (%)	No. (%) embryo developed[*1]			
						24 h	48 h	72 h	96 h
						2-cell	4–8-cell	Morula/ Blastocyst	Blastocyst
Fresh[*2]	ES (B6D2F1)	–	193	–	108	–	–	20 (19)	–
	Somatic (B6D2F1)	–	198	–	153	–	–	107 (70)	–
Over-night	ES (129B6F1)	–	376	207 (55)	192 (93)	62 (32)[a]	4 (2)	2 (1)	2 (1)
		+	315	141 (45)	132 (94)	54 (41)[a]	11 (8)	5 (4)	5 (4)
	Somatic (B6D2F1)	–	168	99 (59)	84 (85)	71 (85)[b]	11 (13)	8 (10)	1 (1)
		+	186	101 (54)	92 (91)	69 (75)[b]	13 (14)	7 (8)	1 (1)
1 week	ES (129B6F1)	–	161	93 (58)	88 (95)	22 (25)[a]	2 (2)	2 (2)	2 (2)
		+	163	99 (61)	97 (98)	34 (35)[a]	1 (1)	1 (1)	1 (1)

[*1]: Percentage based on the number of pronuclear embryos. [*2]: These data are from reference #28. Values with different superscripts in the same column are siginificantly different (P<0.01).

Table 2. Establishment of ntES cell lines from freeze-dried cells

Storage period	Cell type (strain)	Trehalose	No. of enucleated oocyte	No. of cloned morulae or blastocysts used	No. of established ntES cell lines (%)[*1]	Name of cell line
Fresh[*2]	ES (B6D2F1)	–	193	20	6 (30)	–
	Somatic (B6D2F1)	–	198	107	22 (21)	–
Over-night	ES (129B6F1)	–	376	2	0	–
		+	315	9	2 (22)	E1D1, 2
	Somatic (B6D2F1)	–	168	5	0	–
		+	186	4	2 (50)	S1D1, 2
1 week	ES (129B6F1)	–	161	1	1 (100)	E1W1
		+	163	1	0	–

[*1] Percentage based on the number of cloned morulae or blastocysts used. [*2] These data are from reference #28.

is higher than the rate of cloned mice production [14, 16, 23, 26, 27]. Approximately 90% of the oocytes formed pronuclei following nuclear transfer, irrespective of donor cell type and storage period (Table 1). After culturing the nuclear transfer embryos, 70–80% of the embryos derived from freeze-dried cumulus cells developed to the two-cell stage at 24 h. This percentage was significantly higher than the production of embryos from freeze-dried ES cells (30–40%, P<0.01). When embryos were cultured for up to 96 h, at least one embryo (1–4%) reached the blastocyst stage in all groups, irrespective of donor cell type or preservation period (Table 1). The morula or blastocyst stage embryos were then used to generate ntES cell lines, and we were able to successfully obtain five ntES cell lines from these embryos (Table 2). All morula, blastocyst and established ntES cell lines expressed GFP genes, indicating that the nuclei of reconstructed oocytes originated from the donor cells (Fig. 1C–D', 2B).

We used trehalose in some experiments during freeze-drying of donor cells. Trehalose is found in the cells of many organisms that naturally survive dehydration, a phenomenon known as anhydrobiosis. The mechanism by which trehalose confers tolerance to desiccation is unclear, although trehalose is thought to replace the shell of water from the surfaces of macromolecules. However, all our cells were dead after rehydration even with trehalose treatment, and the rate of development from the oocyte to blastocyst stage was the same with or without trehalose (Table 1). These results suggest trehalose did not improve the survival rate of the freeze-dried mouse cells. Once the cells were dead, later developmental viability depended on nuclear integrity, which is affected by factors other than protection by trehalose.

The newly established ntES cells were analyzed for pluripotency by alkaline phosphatase and immunofluorescence staining for Oct3/4 and Nanog, and their *in vitro* differentiation potential was assessed by embryoid body formation. All ntES cell lines stained positive for the ES-cell-specific markers (Fig. 2A–D, Nanog is not shown) and formed embryoid bodies in a pattern similar to that observed in normally fertilized ES cells (data not shown). In addi-

Table 3. Contribution of ntES cells in chimeric mice

Origin of ntES cell line					No. of used embryos (recipients)	No. of offspring	
Line ID	Mouse strain	Donor cell	Storage period	Normal karyotype %		Total[*1]	High (> 50%)[*2]
E1D1	129B6F1	ES	1 day	19%	192 (13)	19	7
E1D2	129B6F1	ES	1 day	72%	143 (11)	31	7
E1W1	129B6F1	ES	1 week	80%	74 (6)	9	3
S1D1	B6D2F1	Somatic	1 day	82%	157 (11)	31	4
S1D2	B6D2F1	Somatic	1 day	60%	144 (10)	22	4

[*1] Some pups died soon after birth. [*2] The contribution of ntES cells to each chimera was scored as high (> 50% of the coat color or GFP expression was derived from ntES cells).

tion, chromosome analysis revealed that all ntES cell lines had the normal range of karyotypes. To determine the possible pluripotency of these ntES cells, chimeric mice were produced by injecting the ntES cells into the perivitelline spaces of 4–8-cell stage embryos derived from the ICR albino mouse strain. These embryos were then transferred into pseudopregnant recipients. The chimerism or ntES cell contribution rate was identified by the presence of black eyes, coat color and GFP expression. As shown in Table 3 and Fig. 2, chimeric mice (with more than half the target coat color) were obtained from all cell lines. These results suggest ntES cell lines derived from freeze-dried cells are pluripotent and have the potential to generate the next generation via germ cells in chimeric mice.

The developmental rate to morula or blastocyst stage was extremely low compared with our previous results using fresh cells as donors (20–70% *vs*. 1–10%) [16, 23, 27, 28]. Possible explanations for this low success rate include the possibility that 1) nonviable cells lose nuclear integrity within a short time or 2) the freeze-drying process damages donor nuclei. It has been shown that even dead cells after freeze-thawing can generate ntES cell lines within the normal range [6]. This indicates that the freeze-drying process is the more likely cause of the low success rate. However, Loi *et al*. reported that blastocysts could be produced from freeze-dried sheep somatic cells with a success rate equivalent to that for fresh cells [8]. Even after storage for 3 years at room temperature, freeze-dried somatic cells still had the developmental potential for blastocyst formation following nuclear transfer. Their study suggests that the freeze-drying process does not prevent the development of donor cells after nuclear transfer. Although the mouse is the first species in which offspring from freeze-dried spermatozoa were produced with a high success rate, mouse cells may be less tolerant to freeze-drying than other species.

Although the establish rate was low, we were able to generate several ntES cell lines that differentiated into the organs in chimeric mice. This is strong evidence for the nuclear integrity of freeze-dried cells. If donor nuclei had sustaine damage during freeze-drying, it appears that minor damage can be repaired during the course of ES cell generation [29]. Loi *et al*. only demonstrated developmental potential to the blastocyst stage and did not show nuclear integrity because most oocytes fertilized with damaged freeze-dried spermatozoa developed into morphologically normal blastocysts [9]. We have demonstrated, for the first time, that the complete nuclear genome can be preserved after freeze-drying and that chimeric mice can be produced from these cell nuclei using ntES cell technology.

In conclusion, we have demonstrated successful reprogramming of nuclei from freeze-dried cells in mice. Genomic integrity can be maintained in the freeze-dried condition, and chimeras can be produced from ntES cells. In this study, although we did not try to produce cloned mice using freeze-dried cells because the generation rate of cloned mice is low, it is likely that tetraploid chimera or cloned mice can be produced using these ntES cell lines.

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