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Cryopreservation of Human Embryonic Stem Cells with a New Bulk Vitrification Method[1]

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ABSTRACT

To increase the manipulation efficiency and storage capability of vitrified human embryonic stem cells, a new bulk vitrification method was established using transformed cryovials. This method vitrified a large number of cell clumps, as opposed to those cryopreserved by a slow-freezing method with conventional cryovials at one time (round). After warming, vitrified human embryonic stem cells exhibited a much higher survival rate than the slow-freezing cells. The vitrified stem cells continued to express markers of pluripotency and formed teratomas in mice with severe combined immunodeficiency, confirming the pluripotency of vitrified-warmed human embryonic stem cell clumps. The new bulk vitrification method is superior to and more practical than the open pulled straw vitrification method and the slow-freezing method for the cryopreservation of human embryonic stem cells.

cell strainer, cryopreservation, cryovial, human embryonic stem cells, vitrification

INTRODUCTION

Human embryonic stem (HES) cells offer tremendous potential not only for research but also for clinical applications as an unlimited source of cells for transplantation and tissue regeneration [1, 2]. To exploit the remarkable potential of HES cells, efficient cryopreservation is increasingly important in stem cell technology [3].

Slow-freezing and rapid-thawing methods are most commonly used for the cryopreservation of cell lines [4]. These standard methods are efficient for the cryopreservation of mouse embryonic stem cells [5], but the survival of HES cells is poor [6]. On the other hand, vitrification methods have become more and more popular in recent years, mainly because of the higher survival rate compared to that with slow freezing, especially for cells that are sensitive to cryoinjury [7, 8]. Reubinoff et al. [9] was the first to report vitrfication of a small number of early passaged HES cell clumps, which had shown an obvious improvement in HES cell survival after thawing.

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With the vitrification approach, a glass-like solidification of the freezing solution is achieved using a high concentration of cryoprotectant and rapid cooling. Faster cooling can lessen the cryoprotectant-induced toxicity and, accordingly, is directly related to the efficiency of cryopreservation. The cooling rate is decided mainly by the structure of vitrification carriers and the manipulation procedures. At present, an increased cooling rate is achieved mostly by plunging small-volume samples held on tiny vitrification carriers, such as cryoleaf, cryoloop, and ministraw [10–13], directly into liquid nitrogen (LN_2). Therefore, conventional vitrification methods have hardly ever been considered for the cryopreservation of a large quantity of vigorously proliferating HES cell lines. Recently, we reported the first bulk vitrification method to cryopreserve HES cells with a much higher survival rate than that with slow-freezing methods [14]. While quite efficient, it is not convenient to manipulate and store HES cells in LN_2 with this method, mainly because of the unnecessarily large size and irregular shape of the vitrification carrier, which significantly limited the practical use of this method.

To further increase the manipulation efficiency and storage capability of HES cells, a new bulk vitrification method was developed using transformed cryovials. Herein, we show that this new bulk vitrification method not only effectively cryopreserved a large quantity of HES cell clumps with a high survival rate but also was more convenient and practical than previous vitrification methods for the cryopreservation of HES cell.

MATERIALS AND METHODS

HES Cell Cultures

The cell lines used in the current study were β-thalassemia and α-thalassemia carrier HES cells (β-HES-2 and α-ES-C, respectively), which were derived from embryos during preimplantation genetic diagnosis and diagnosed with β-thalassemia and α-thalassemia, respectively [15–18]. The use of the cell lines and the present study itself were approved by the Institutional Review Board of Sun Yat-sen University.

Fabrication of Vitrification Cryovial

A 2-ml cryovial (Corning 430488, Cryogenic Vial; Corning, Inc.) was cut transversely into two halves. The upper half with the lid was kept for later use. A small piece of stainless-steel mesh (mesh size, 70 μm; Zhenxing Hardware Sifting Screen Factory) was carefully placed on the bottom of the new cryovial. This new cryovial was then sterilized and ready for use (Fig. 1).

Experimental Design

After passage, HES cell clumps were transferred into cell strainers (70-μm nylon; catalog no. 352350; BD Falcon) and washed three times with HES medium. Only those cell clumps with a diameter of larger than 70 μm were collected for cryopreservation. The collected cell clumps were randomly allocated into three experimental treatment groups, and each group was cryopreserved by a different method: 1) the new vitrification method (vide infra), 2) the open pulled straw (OPS) vitrification method [6], and 3) the slow-freezing method [19]. After 2 wk of storage in LN_2, HES cells were thawed and

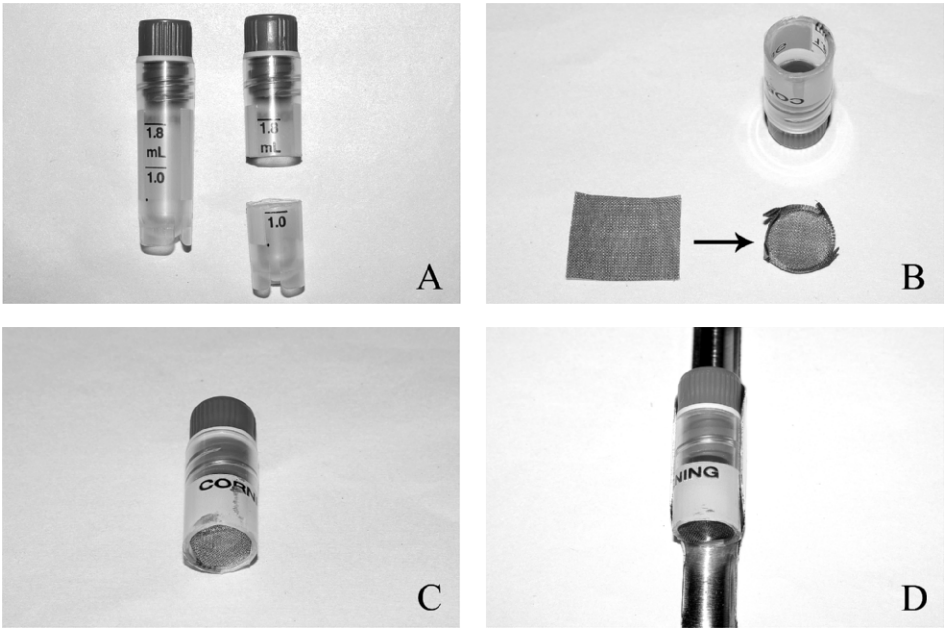

FIG. 1. Photographs illustrating the fabrication of the vitrification cryovial. **A**) A 2-ml cryovial was cut transversely into two halves. The upper half with the lid was kept for later use, and the bottom half was discarded. **B**) A small piece of stainless-steel mesh (mesh size, 70 μm) was molded to be cup-shaped. **C**) The molded steel mesh was carefully placed on the bottom of the new cryovial. **D**) The homemade vitrification cryovials could be quickly set onto canes after being loaded with HES cells.

plated onto fresh feeder layers. The plating efficiency, growth, and differentiation status (colony scoring) of HES cells were compared between the three groups. The experiment was independently replicated five times. After getting a promising result that demonstrated the high efficiency of this new bulk vitrification method, the bulk-vitrified, postwarmed, and further-cultured HES cells were analyzed for the HES pluripotent markers, undifferentiation-specific transcription factors, and teratoma formation to further confirm the effective of this new method.

Methods for Bulk Vitrification and Thawing in Transformed Cryovials

The vitrification solutions (VS) and thawing solutions (TS) have been described previously [14]. All freezing and thawing procedures were performed on a heating stage at 37°C. Before freezing, vitrification medium was added to dishes (catalog no. 3001, BD Bioscience; 3 ml of VS per dish; one medium per dish) and incubated at 37°C in 5% CO_2. Cell clumps were transferred as a suspension of clumps in culture medium to the transformed cryovial. The vitrification cryovial was first transferred to VS1 medium with tweezers and incubated for 1 min, then to VS2 for 25 sec, and then to the LN_2 bowl directly (Fig. 2). Finally, the vitrified cryovials were fixed onto a cane and stored in a LN_2 tank.

After 2 wk in LN_2, the cryovial was retrieved. Ten seconds after removal from LN_2, the cryovial was submerged in TS1 for 1 min, then in TS2 for 5 min, and finally, in HES medium twice for 5 min each time before being plated onto a fresh feeder layer.

Slow-Freezing Protocol and OPS Method

Slow freezing and rapid thawing were performed according to the routine protocol at our laboratory [14, 19]. The OPS vitrification method was carried out as described by Reubinoff et al. [9]

Assessment of HES Cell Survival, Growth, and Differentiation

Cryopreserved HES cells in the three groups were thawed 2 wk after freezing. All of the thawed cell clumps were plated onto fresh feeder layers regardless of the clump size. After 48 h of culture, the cells/cell clumps still floating were regarded as dead cells and discarded. To evaluate the influence of different cryopreservation methods on the survival of HES cells, the plating efficiency or survival rate was calculated for each method (number of attached clumps/number of thawed clumps).

Every postthaw colony firmly attached to the feeder layer was carefully recorded by the same experienced technician, who was blinded to the grouping and study design, after 48 h of culture and medium replacement. The growth and differentiation status of these cell colonies were re-evaluated on the seventh day after plating. Tightly gathered small cells with a high ratio of nucleus to cytoplasm were regarded as undifferentiated HES cells, whereas large cells with abundant cytoplasm were recorded as differentiated cells [1]. The growth and differentiation status of each colony in the three groups were graded by the same technician based on the proportion of undifferentiated cells in each colony as determined by morphological observation [9]. Colonies were regarded as grade A if more than 80% of cells were undifferentiated in one colony, grade B if 50–80% undifferentiated cells were present in colonies, and grade C if less than 50% undifferentiated cells were scattered among differentiated large cells. Grade D colonies included those cell clumps with no growth or an entire colony that was differentiated. Only grade A and B colonies were suitable for additional serial passaging.

Statistical Analysis

The experiment was independently repeated five times, and data were analyzed by one-way ANOVA. The data are presented as the mean ± SD. A probability of $P < 0.05$ was considered to be statistically significant. When ANOVA revealed a significant effect, values between the individual groups

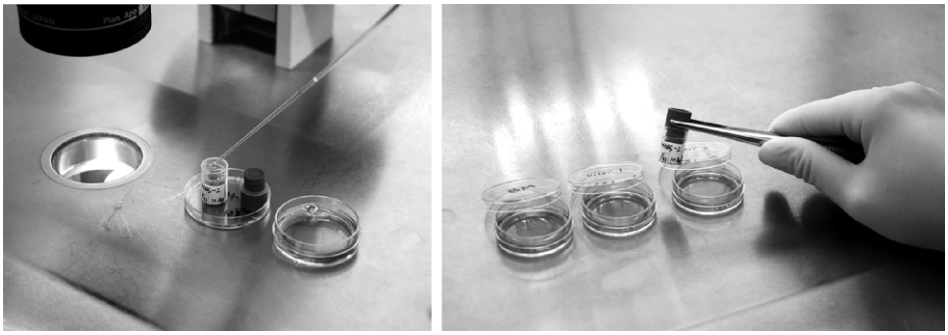

FIG. 2. Bulk vitrification procedures of HES cells with homemade cryovial/vitrification cryovial. **Left**) Cell clumps were transferred as a suspension of clumps in culture medium to the homemade cryovials. **Right**) HES cells loaded in the cryovial could easily be transferred from one vitrification solution to another with tweezers.

TABLE 1. The holding capacity of cryo-containers (CC), recovery rate, and plating efficiency of human embryonic stem (HES) cells cryopreserved by the three different methods.*

Cryopreservation methods†	No. of cell clumps cryopreserved	No. of cell clumps/CC	Recovery rate (%)‡	Plating efficiency (%)§
V-CV	479 ± 14.2	117 ± 21.8	97.1 ± 2.1	91.8 ± 3.9 [c]
OPS	36 ± 4.8	6 ± 1.1	97.6 ± 1.3	93.2 ± 6.1
S-CV	420 ± 18.6	139 ± 34.5	96.5 ± 1.6	40.1 ± 5.3[a,b]

* Data are expressed as mean ± SD.
† V-CV, vitrification method with cryovial; S-CV, slow-freezing method with cryovial.
‡ Recovery rate is indicated by the number of thawed clumps/number of frozen clumps ×100; recovery rate between the three groups, $F = 3.047$, $P = 0.085$.
§ Plating efficiency (survival rate), number of adhered clumps/number of thawed clumps after 48 h of plating ×100; plating efficiency/survival rate between the three groups, $F = 173.7$, $P < 0.001$.
[a] Plating efficiency/survival rate between V-CV and S-CV group, $P < 0.001$.
[b] Plating efficiency/survival rate between OPS and S-CV group, $P < 0.001$.
[c] Plating efficiency/survival rate between V-CV and OPS group, $P = 0.661$.

TABLE 2. Growth and differentiation of post-thawed HES cells on Day 7 with different cryopreservation methods.*

Cell colonies	V-CV (%)	OPS (%)	S-CV (%)
Grade A	56.6 ± 2.5	54.8 ± 3.6	0.0 ± 0.0
Grade B	30.2 ± 2.9	31.0 ± 2.5	8.1 ± 2.5
Grade A+B†	86.4 ± 2.7[c]	85.5 ± 3.2	8.1 ± 2.5[a,b]
Grade C	12.8 ± 3.5	13.2 ± 3.9	26.8 ± 4.3
Grade D	0.8 ± 0.3	1.3 ± 0.2	64.9 ± 5.1
Grade C+D	13.6 ± 1.8	14.5 ± 2.1	91.8 ± 4.7

* V-CV, vitrification method with cryovial; S-CV, Slow-freezing method with cryovial.
† Percentage of grade A plus grade B between the three groups was significantly different, $F = 1308.1$, $P < 0.001$.
[a] Percentage of grade A plus grade B between V-CV and S-CV group, $P < 0.001$.
[b] Percentage of grade A plus grade B between OPS and S-CV group, $P < 0.001$.
[c] Percentage of grade A plus grade B between V-CV and OPS group, $P = 0.610$.

were assessed by least significant difference post hoc test for multiple comparisons.

Characterization of Bulk-Vitrified, Postthawed HES Cells

Before HES cell passaging, glass covers (22 × 22 mm) were put into the bottom of the wells of a six-well plate, and bulk-vitrified HES cells were passaged onto the glass covers after thawing and expansion. With 80% confluence of colonies, at least four wells were used for extraction of total RNA, and the remaining wells were used for the immunofluorescent staining. Four- to six-well plates were used for the experiment, and the experiment was repeated separately three times.

Immunofluorescent staining of stem cell pluripotent markers was performed as follows: At passage 6 after vitrification, embryonic stem cell pluripotent markers, including SSEA1 (MC-480; Developmental Studies Hybridoma Bank), SSEA 3 (MC-631; Developmental Studies Hybridoma Bank), SSEA 4 (MC-813–70; Developmental Studies Hybridoma Bank), TRA 1–80 (MBA4381; Millipore), and TRA 1–60 (MBA4360; Millipore) were determined by immunofluorescent tests.

The RT-PCR analysis was performed as follows: RT was carried out to investigate the presence of undifferentiation-specific transcription factors of HES cells, such as *POU5F1* (previously known as *OCT-4*), *REX1*, and *NANOG*, along with markers of different germ lineages expressed in spontaneously differentiated HES cells (embryo bodies). Imprinted genes were also checked to investigate the effects of vitrification on vitrified HES cells. PCR was carried out using primers as described previously [14].

Teratoma Formation in Mice with Severe Combined Immunodeficiency

Morphologically undifferentiated HES cell colonies were collected from bulk-vitrified, postwarmed, and further-cultured HES cells (propagated from 8 to 14 passages after thawing). Approximately 8×10^6 cells were injected into the rear leg muscles of two mice with severe combined immunodeficiency (SCID) per repeated experiment (in total, 10 SCID mice were injected). Tumors were embedded in paraffin and examined histologically after hematoxylin-and-eosin staining.

RESULTS

The holding capacity of cryocontainers and the recovery rate and plating efficiency of HES cells for the three different freezing methods are summarized in Table 1. The growth and differentiation rates of postthawed HES with the three methods are given in Table 2.

Clearly, conventional slow freezing with the cryovial was the least effective method for HES cell cryopreservation. Only

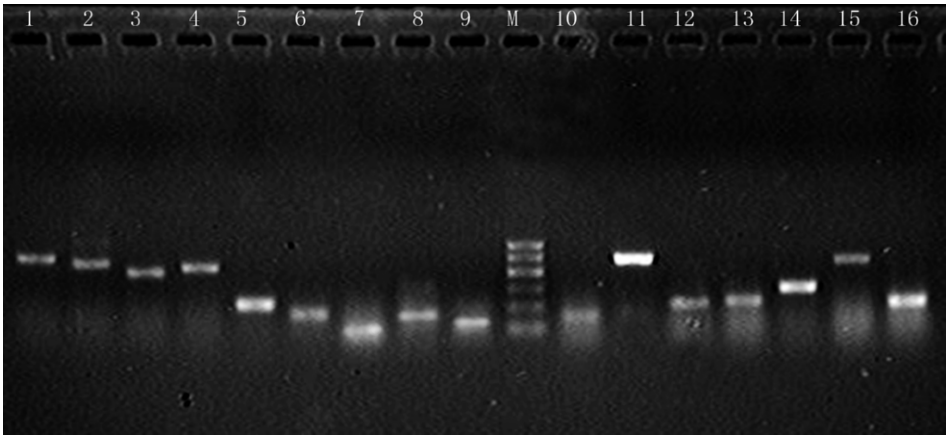

FIG. 3. RT-PCR analysis of gene expressions related to the undifferentiation state, imprinted genes, and differentiation of bulk vitrified HES cells (embryonic bodies [EBs]). Lanes 1 and 2: paternally derived imprinted genes (*IGF2* and *SNRPN*); lanes 3 and 4: maternally derived imprinted genes (*UBE3A* and *H19*); lane 5: *ACTB*; lanes 6–9: markers of the undifferentiated HES cells (*POU5F1*, *NANOG*, *REX1*, and *SOX2*); lanes 10–15: markers of three embryonic germ layers, expressed by EBs derived from frozen-thawed HES cells (*ALB*, *ACTC1*, *AFP*, *GRM2*, *GAPDH*, and *NEFL* [*NF*68]); lane 16: *ACTB*; lane M: marker (50-bp DNA ladder).

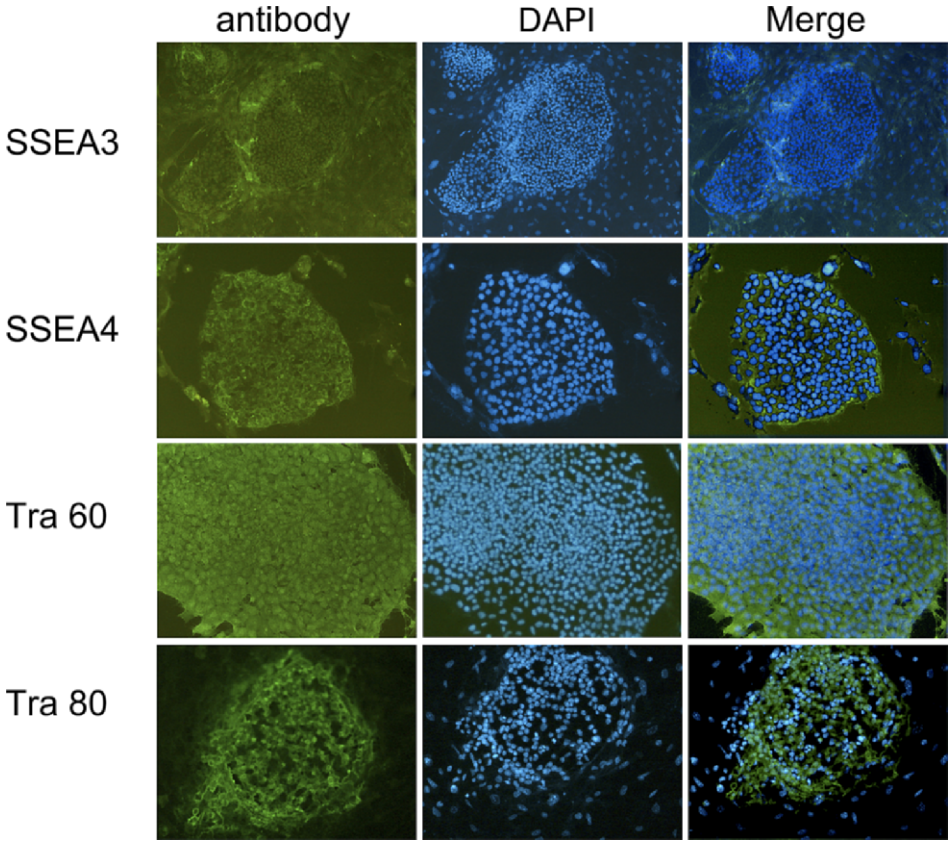

FIG. 4. HES cell marker characterization of thawed (bulk vitrified) HES cell colonies on mitomycin C-inactivated human foreskin fibroblasts. **Left**) Immunofluoresence staining with antibodies to stem cell markers: SSEA3, SSEA4, TRA 1–80 (Tra 80), and TRA 1–60 (Tra 60). **Center**) The nuclei were stained with 4′,6′-diamidino-2-phenyldinole (DAPI). **Right**) The overlay of fluorescein isothiocyanate antibody staining and DAPI signals is shown in the column labeled Merge. Original magnification ×100 (SSEA3, SSEA4, and Tra 80) and ×200 (Tra 60).

40.1% ± 5.3% of the cell clumps survived after thawing on Day 2, which was significantly lower than survival in the cryovial vitrification and OPS groups ($P < 0.001$) (Table 1). Only 8.1% ± 2.5% were undifferentiated (grades A and B), which was significantly lower than in the cryovial vitrification and OPS groups ($P < 0.001$) (Table 2).

Highly effective cryopreservation outcomes were observed when the new bulk vitrification method was used. After warming, the vitrified HES cells exhibited a high survival/plating rate of up to 91.8% ± 3.9%, which was comparable to the OPS method ($P = 0.661$) (Table 1). All attached cell clumps generated HES cell colonies (the majority were undifferenti-

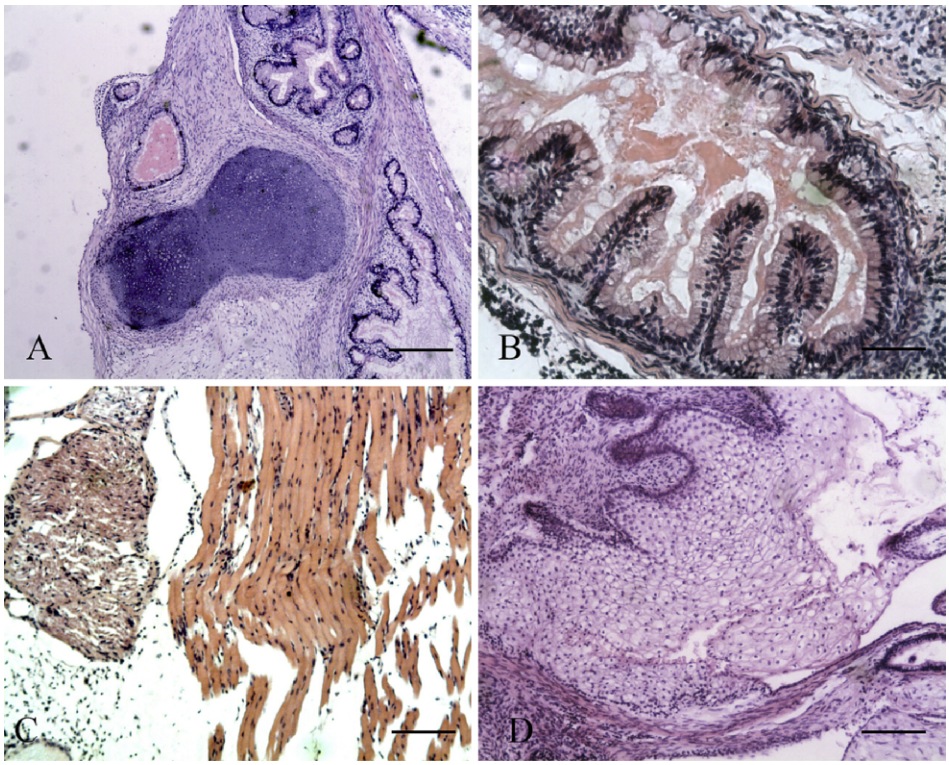

FIG. 5. Histological analysis of teratomas formed from grafted colonies of vitrified-thawed HES cells in SCID mice: cartilage (**A**), higher magnification of mucosa (**B**), muscle (**C**), and adipose cells (**D**). Histological staining: hematoxylin and eosin. Bar = 200 μm (**A**, **C**, and **D**) and 100 μm (**B**).

ated grade A and B colonies). Only grade A and B colonies could be passaged further, and the percentage indicated the efficiency of the different freezing methods. In the present study, grade A plus B colonies in the vitrification cryovial and OPS groups were not significantly different ($P = 0.610$) (Table 2).

Cryovial vitrification was also associated with cell death, which was evident on Day 2 by the presence of dark cells scattered throughout attached colonies and, on Days 4 and 7 after plating, by the significantly reduced mean area of the colonies compared with unfrozen control colonies. After the first passage, no significant difference was found in cell morphology, growth rate, and differentiation status between cryovial-vitrified-thawed HES cells and unfrozen control cells.

In the present study, each vitrification cryovial was loaded with 117 ± 21.8 cell clumps at one time (round), which was similar to the number used in the slow-freezing method with a cryovial (139 ± 34.5) (Table 1). Furthermore, the recovery rate was as high as in the other two groups (Table 1), because no transfer procedures with Pasteur pipettes were needed during the thawing process. After the homemade vitrification cryovials were loaded with HES cells and finally put into a LN_2 bowl, the cryovials were quickly set onto canes and safely stored in a LN_2 tank because of the special design of the cryovial and cane.

Bulk-vitrified, postthawed HES cell colonies maintained expression of undifferentiated cell markers, such as *POU5F1*, *REX1*, and *NANOG*. Embryonic bodies derived from frozen-thawed HES cells expressed markers of their characteristic three germ layers, indicating their differentiation capacity, as determined by TaqMan RT-PCR analysis (Fig. 3). Furthermore, these embryonic bodies also correctly expressed paternally derived imprinted genes (*IGF2*) and maternally derived imprinted genes (*UBE3A* and *H19*) (Fig. 3). Most of the postthawed HES cell colonies were positive for the relevant HES pluripotent markers, such as SSEA3, SSEA4, TRA 1–60, and TRA 1–81 (Fig. 4), predominantly in the undifferentiated colonies (20–30 colonies/glass cover) but not in the differentiated stroma-like cells. The results from the three separate experiments were consistent with each other.

After the injection of 10 different bulk-vitrified/thawed HES colonies into the rear leg muscle of SCID mice, teratomas comprising all three primordial germ layers formed in seven of these mice, confirming the pluripotency of HES cells cryopreserved by this new bulk vitrification method (Fig. 5).

DISCUSSION

Slow freezing is popular for the cryopreservation of most somatic cell types. Millions of cells could be loaded into one cryovial simultaneously and stored in LN_2. Thus, the slow-freezing method with cryovials is a practical choice for vigorously proliferating HES cells. However, use of the slow-freezing method in the present and a previous study [6] has resulted in low thaw survival rates, presumably because of ice crystal formation that disrupted cell-cell adhesions.

Vitrification, on the other hand, circumvents problems associated with ice crystal formation, cell dehydration, and control of freezing rates. A glass-like solidification of the freezing solution is achieved using a high concentration of cryoprotectant and rapid cooling. Currently, a rapid cooling rate is achieved mostly by loading small-volume samples (oocytes and embryos) either onto/into tiny vitrification carriers and then placing them directly into LN_2. It is impossible to vitrify millions of somatic cells with these tiny containers. Furthermore, it is impractical to swiftly transfer millions of cells between different freezing/thawing solutions with Pasteur pipettes during the freezing and thawing process. Finally, it is difficult to quickly load a large number of cells onto/into vitrification carriers along with minimal freezing solutions and put these carriers into LN_2 to maximize cooling rate during the freezing process.

In the present study, we transformed the most commonly used, 2-ml cryovials into a new vitrification carrier. The bottom was made up of a stainless-steel mesh. When cell clumps were loaded onto the relatively large-sized steel mesh with minimal freezing solution and then submerged into LN_2, the cells cooled down very quickly, similar to being vitrified by large electron microscopy grids rounded with a safe wall. Each cryovial was loaded with cell colonies harvested from half of a 35-mm dish, which was 20-fold the number loaded in a pulled straw of the OPS group (Table 1).

The transformed cryovial could easily hold cryopreserved cell clumps when they were incubated in freezing/thawing medium. No need existed to transfer cells from one freezing/thawing medium to another by Pasteur pipettes. The step-by-step transfer of HES cells between different freezing/thawing media could be quickly and efficiently carried out with tweezers. The transfer time and cell exposure to highly toxic cryoprotectants was minimized, which was the prerequisite to achieve a high vitrification survival rate. The recovery rate of HES cell colonies after thawing was excellent with this method; cell clumps could be easily found and picked up from the steel mesh, for transfer to dishes, under a dissecting microscope after thawing.

Unlike vitrification with cell strainers [14], it was quick and easy to fix this new vitrification carrier (transformed cryovial) onto a cane, easily identify it, and handle it in a LN_2 tank, because this is done with a conventional cryovial using the slow-freezing method. Moreover, most of the currently used LN_2 tanks, buckets, canes, and other accessories are all specially designed to facilitate the manipulation and storage of cryovials in LN_2. Thus, this new vitrification method could combine the well-known merits of cryovials as cryocontainers with the high vitrification survival rate. It offers researchers a more practical choice to bulk vitrify HES cells efficiently compared with previous vitrification methods.

The cryovial vitrification method can also be used to cryopreserve most of the cultured somatic cells types, especially for cells sensitive to cryoinjury. The ongoing cryopreserved cells should be prepared as cell clumps by cell scrapers or gentle collagenase treatment before vitrification, because the transformed cryovial can retain only cell clumps of larger than 70 μm during the freezing and thawing process.

Vitrified HES cell colonies continued to express markers of pluripotency and formed teratomas in SCID mice after warming and subsequent culture, confirming that they maintain their bona fide stem cell properties after vitrification and warming. The results were highly reproducible, as reflected by the precision of the replicates.

We note, however, that our vitrification method involved direct contact of HES cells with LN_2, similar to some of the currently used vitrification methods, thus increasing the possibility of contamination and cell infection [20]. Although vitrification with closed, sealed straws has been reported [21], only limited numbers of cells could be cryopreserved in straws. Therefore, to achieve bulk cryopreservation and long-term storage of HES lines in stem cell banks, this bulk vitrification method using transformed cryovials either needs further modifications or must incorporate other techniques.

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