はなかなか進展がみられていない。最近，神経堤細胞の異常によって発症する先天性の致死性疾患である家族性自律神経失調症(familial dysautonomia：FD)の疾患特異的iPS細胞を用いたHTS解析が報告された[52]。Leeらは，以前に報告したFD患者由来iPS細胞を神経堤細胞に分化させる方法[53]を用いて，原因遺伝子であるIKBKAPの正常スプライシング産物が増加する化合物をスクリーニングしたところ，6,912種類の化合物のうち8個の化合物が有効であった，と報告している。Leeらは，これらの化合物の効果は他の細胞種(iPS細胞，線維芽細胞やリンパ球)では十分認められなかったことから，疾患に関連する細胞種を分化させてスクリーニングすることの重要性を強調している。

疾患特異的iPS細胞の課題

疾患特異的iPS細胞には多くの可能性があるが，前述のように，現状では多くの疾患特異的iPS細胞の報告は“疾患モデリング”に留まっている。iPS細胞技術を用いて，疾患の病態生理を深く解析するためには，解決すべき課題が存在する。最初の問題は，ある患者から樹立した複数iPS細胞クローンについて，クローン間表現型のばらつきが存在しうることであり，これが大きいと疾患に関連した真の表現型の解析が困難になる。このようなクローン間のばらつきは，ゲノム変異[54]，エピジェネティック修飾[55]，ソースとなる体細胞の種類，残存トランスジーン活性，および女性由来の細胞ではX染色体不活化状態がクローンによって異なること[56)-58]などにより生じる。さらに，いくつかの疾患，特にFanconi貧血などのDNA修復異常症などではiPS細胞の樹立自体が難しいことがある。このような場合は，一旦遺伝子修復を行ってiPS細胞を樹立し，その後導入遺伝子を除去するなど，特殊な方法が必要になるかもしれない。

また，前述の通り，疾患特異的iPS細胞の対照群をどのようにとるか，ということも問題である。原因遺伝子が判明している場合，患者由来iPS細胞の原因遺伝子を修復して対照クローンとすれば，ゲノムバックグランドの差異による表現型のばらつきなどを最小限に抑えて解析することができる。ヒト多能性幹細胞の相同組み換え効率は極めて低かったが，最近，ヘルパー依存性アデノウイルスを用いる手法[59]や，Zinc finger nuclease[60]やTALEN[61]といった配列特異的ヌクレアーゼを用いる方法により高効率かつ簡便に遺伝子ターゲティングが行えるようになってきた。今後はこのような手法を用いて，十分に機能評価がされた“正常”iPS細胞クローンやES細胞に疾患特異的変異を導入し，疾患モデルして用いることも可能になると思われる[62)63]。この場合，①iPS細胞クローンの評価に時間と労力を割く必要がない，②臨床研究に関する研究計画書を整備し，患者から同意を取得するという手順が省略できる，③分化能・分化した細胞の機能などが十

分に解析されたクローンを使用できる，といった利点があるため，単一遺伝子疾患の解析にはこちらが主流となっていくかもしれない。

終わりに

以上のように，課題もあるものの，疾患iPS細胞を用いることにより，疾患の表現型の少なくとも一部は試験管内で再現可能であることが次々と証明されている。iPS細胞を用いた治療薬開発や病態解明が進み，これまで治療法のなかったさまざまな難治性疾患に対するアプローチが少しでも早く進展することを期待したい。

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TECHNOLOGY REPORT

Optimization of Slow Cooling Cryopreservation for Human Pluripotent Stem Cells

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Summary: Human pluripotent stem cells (hPSCs) have the potential for unlimited expansion and differentiation into cell types of all three germ layers. Cryopreservation is a key process for successful application of hPSCs. However, the current conventional method leads to poor recovery of hPSCs after thawing. Here, we demonstrate a highly efficient recovery method for hPSC cryopreservation by slow freezing and single-cell dissociation. After confirming hPSC survivability after freeze-thawing, we found that hPSCs that were freeze-thawed as colonies showed markedly decreased survival, whereas freeze-thawed single hPSCs retained the majority of their viability. These observations indicated that hPSCs should be cryopreserved as single cells. Freeze-thawed single hPSCs efficiently adhered and survived in the absence of a ROCK inhibitor by optimization of the seeding density. The high recovery rate enabled conventional colony passaging for subculture within 3 days post-thawing. The improved method was also adapted to a xeno-free culture system. Moreover, the cell recovery postcryopreservation was highly supported by coating culture surfaces with human laminin-521 that promotes adhesion of dissociated single hPSCs. This simplified but highly efficient cryopreservation method allows easy handling of cells and bulk storage of high-quality hPSCs. genesis 52:49–55, 2014. © 2013 Wiley Periodicals, Inc.

Key words: ectoderm; tissue endoderm; tissue mesoderm; tissue other; tissue germ layer; process; early development; single cell dissociation; slow freezing

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have an infinite proliferative potential and capacity for differentiation into all cells of the three germ layers. For successful application of hPSCs in transplantation therapy or drug discovery, it is necessary to prepare large numbers of hPSCs with various genetic backgrounds (Serra *et al.*, 2012). There are several approaches to improve the yield of hPSCs, but cryopreservation is a key operation because it enables long-term preservation and easy transportation of cells. Currently, hPSCs are cryopreserved by vitrification or slow cooling (Heng *et al.*, 2005; Reubinoff *et al.*, 2001). Vitrification involves flash cooling directly in liquid nitrogen, and some studies have recovered 20–90% of undifferentiated hPSC colonies postcryopreservation (Li *et al.*, 2010; Suemori *et al.*, 2006). However, vitrification requires skilled manipulation and strict temperature control during storage and transportation, because of the small volumes of cryoprotectant used, making it impractical for large-scale storage of hPSCs. In contrast, conventional slow cooling controls the cooling rate at 1°C/min in freezing medium that

Additional Supporting Information may be found in the online version of this article.

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typically contains 10% dimethylsulfoxide (DMSO). The slow cooling method is widely used because it is applicable to various cell types including mouse embryonic stem cells. However, for cryopreservation of hPSCs, the slow cooling method results in less than 10% survivability (Wagh *et al.*, 2011). Nevertheless, for bulk storage and easy handling, slow cooling is desirable for cryopreservation of hPSCs. In this study, we aimed to optimize the slow cooling method for hPSC cryopreservation.

Cryopreservation involves a series of processes including cell detachment, freezing, storage, thawing, and reseeding. During these processes, hPSCs may suffer necrotic cell death from cell detachment to thawing because of physical damage, and apoptotic cell death at reseeding because of adhesion failure. Therefore, hPSC survivability should be verified at each step. However, previous studies have reported different timings for their analyses, various methods of measurement, and counting either colonies or individual cells (Li and Ma, 2012). In particular, counting of colonies postseeding, although it has been often performed, is inaccurate because it leads to overestimation because of an increase of colony formation caused by fragmentation of the original colonies during freeze-thaw processes. For correct evaluation of the survival efficiency, in this study, we counted individual hPSCs in a stepwise manner.

To evaluate the effect of different methods of cell detachment from culture vessels, we first compared cell survival after conventional colony dissociation or single-cell dissociation. While the viability of cells detached by conventional colony dissociation before freezing were approximately halved (42.3–59.4% survival), the majority of hPSCs cryopreserved under a colony state were already dead immediately after freeze-thawing (15.5–32.5% survival) (Fig. 1a, c, e–h). Although the conventional dissociation method maintains the colony state to prevent apoptosis caused by failure to adhere at reseeding, hPSCs had already died by necrosis before attachment. This observation suggests that it is necessary to avoid cell death mainly caused by physical damage during cell dissociation and freeze-thawing processes. Next, we performed freeze-thawing of dissociated hPSCs. The majority of dissociated hPSCs showed higher viability before cryopreservation (68.1–77.7% survival). More importantly, single hPSCs were still alive just after thawing (59.7–66.1% survival), corresponding to 80–90% viability just after dissociation (Fig. 1b, d, e–h).

Although we found that single-cell dissociation of hPSCs is advantageous for freeze-thawing, it is necessary to avoid apoptotic cell death caused by dissociation (Amit *et al.*, 2000). Because we previously showed that the seeding density significantly affects the survivability of single hPSCs (Miyazaki *et al.*, 2012), we next assessed the effect of seeding density on the viability of freeze-thawed single hPSCs. The number of hPSCs

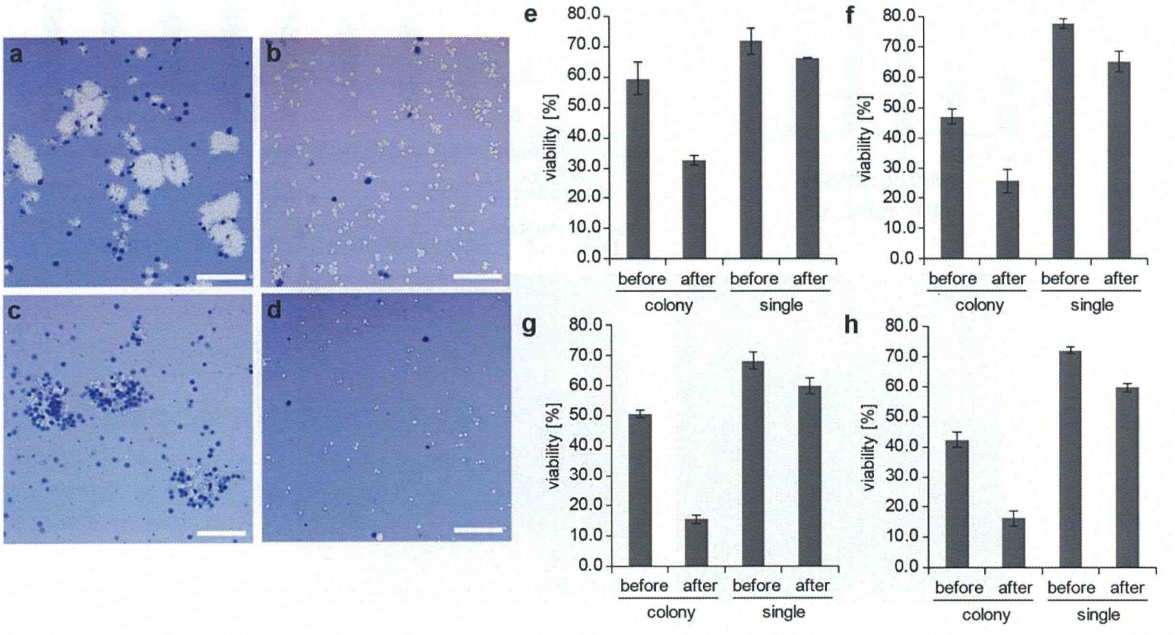

FIG. 1. hPSC survivability is influenced by the cellular state during cryopreservation. (**a–d**) Estimation of cellular state-dependent hPSC survivability by trypan blue exclusion. Representative bright field images of colony-dissociated H9 hESCs before (a) and after (b) cryopreservation and single dissociated H9 hESCs before (c) and after (d) cryopreservation. (**e–h**) Flow cytometric analysis of cellular state-dependent hPSC survivability during cryopreservation by fluorescent dye influx. e: H9 hESCs; f: 253G1 hiPSCs; g: KhES-1 hESCs; h: iPS(IMR90)-1 hiPSCs. Error bars indicate the standard deviation (SD) of three experiments. Scale bars: 200 μm.

attached to the substrate gradually decreases to the minimum at 12 h postseeding (Chen *et al.*, 2010). To evaluate survival correctly, we measured the viability of freeze-thawed single hPSCs at 12 h postseeding. On Matrigel in mTeSR1 medium, freeze-thawed single H9 hESCs seeded at 3×10^5 cells/cm^2 showed maximum survivability (36.2 ± 6.0% survival) (Fig. 2a, c), indicating the optimal condition for recovery of cryopreserved hPSCs. It is well known that administration of a Rho-associated protein kinase (ROCK) inhibitor dramatically improves the survival of single hPSCs (Watanabe *et al.*, 2007). However, ROCK inhibitor treatment did not significantly improve the survival of single hPSCs seeded at the optimal density (Fig. 2b). An effect of the ROCK inhibitor was only observed when single hPSCs were seeded at a lower cell density. These results indicate that a ROCK inhibitor is unnecessary for the optimized seeding density, whereas it should be administrated when single hPSCs are seeded at a lower seeding density. For stable survival of hPSCs, cell-cell contact is a key element (Chen *et al.*, 2010). Therefore, the seeding density of single hPSCs should be as high as possible for rapid formation of cell-cell contacts. However, seeding at very high densities leads to less efficient survival because of depletion of the adhesion surface. It is therefore important for freeze-thawed single hPSCs to be seeded at an appropriate density.

After adherence of single hPSCs, the majority of single hPSCs immediately formed cell-cell contacts as small clusters (Fig. 2c). By 3 days post-thawing, the cells had grown as colonies and reached confluency for passaging (Fig. 2d). Immediately after freeze-thawing, flow cytometric analysis showed that hPSCs remained in an undifferentiated state and maintained the undifferentiated status following subculture (Fig. 3a). After the first passage, hPSCs grew as colonies (Fig. 3b) and sustained an undifferentiated state (Fig. 3a, c) and potential for differentiation (Fig. 3c) without karyotypic abnormalities

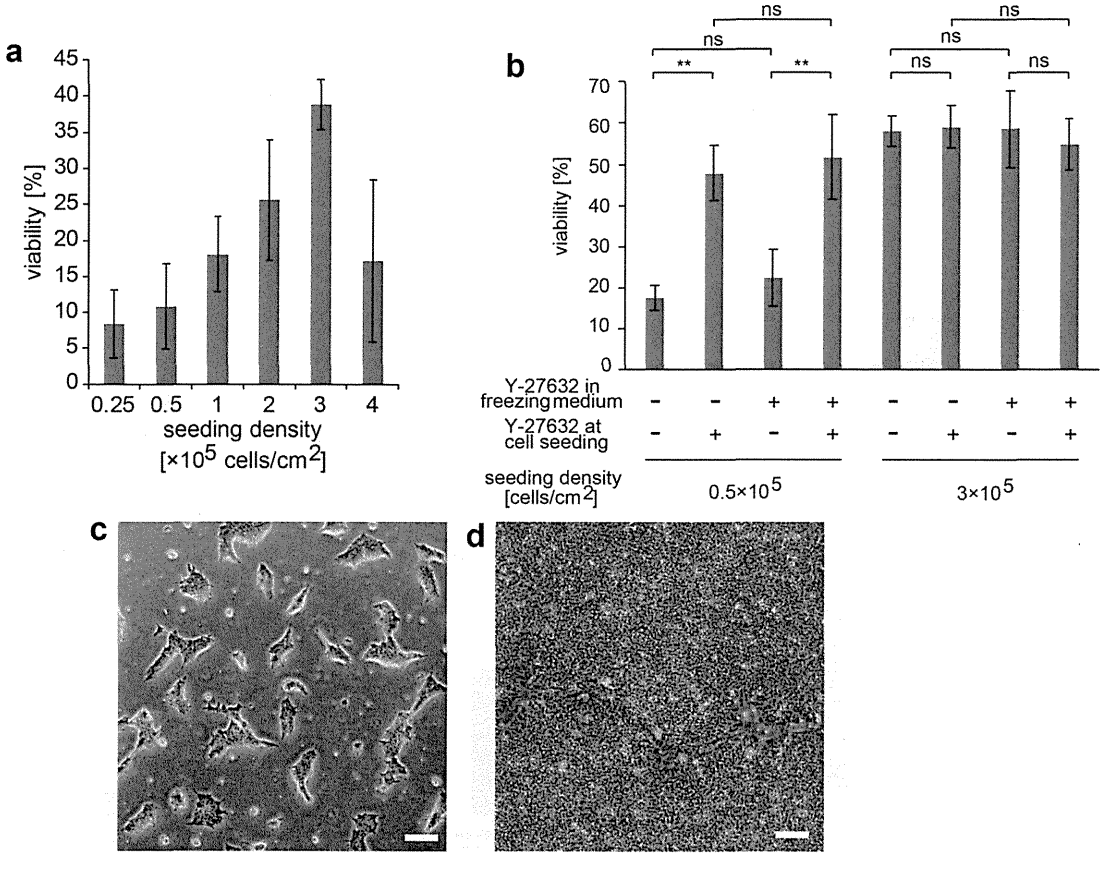

FIG. 2. Recovery of freeze-thawed single hPSCs. (**a**) Seeding density-dependent adhesion of freeze-thawed single H9 hESCs at 12 h postseeding. Cell viability is expressed as the ratio of the attached cell number to live H9 hESCs at seeding. Data represent the means ± SD of three freeze-thawing experiments of one cryopreserved batch of cells. (**b**) Effect of the ROCK inhibitor Y-27632 on adhesion of freeze-thawed single H9 hESCs at 12 h postseeding. Error bars indicate the SD. of three experiments. $^{**}P < 0.05$; ns, not significant, two-tailed Student's *t*-test. (**c**, **d**) Phase contrast images of freeze-thawed single H9 hESCs at 12 h (c) and 3 days (d) postseeding at 3×10^5 cells/cm^2. Scale bars: 200 μm.

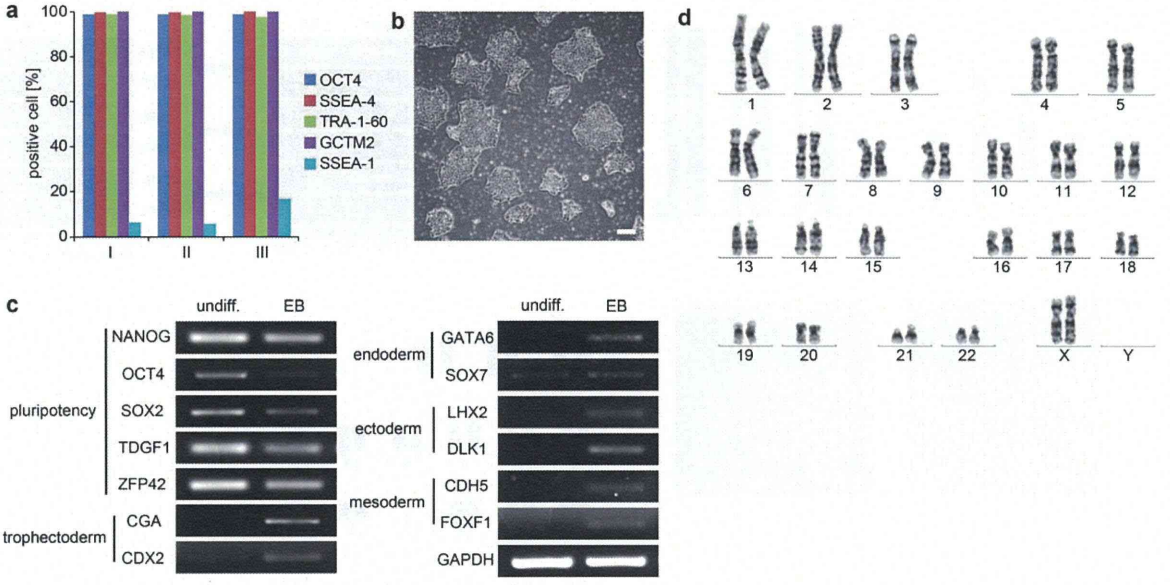

FIG. 3. Characterization of freeze-thawed hPSCs. (**a**) Flow cytometric analysis of undifferentiated markers before cryopreservation (I), at 3 days postseeding (II), and at subculture (III). (**b**) Phase contrast image of H9 hESCs at subculture. (**c**) Reverse transcription-PCR analysis of differentiation marker gene expression in embryoid bodies. Total RNA was extracted from day 14 embryoid bodies generated from cells after subculture. undiff. indicates cells at subculture. (**d**) G-banding analysis of H9 hESCs after subculture. Data for subcultured cells were obtained at two passages after freeze-thawing. Scale bars: 200 μm.

(Fig. 3d). Thus, hPSCs can be rapidly and efficiently cryopreserved by slow cooling while maintaining their pluripotent state.

Because hPSCs used for transplantation therapy would be expanded under xeno-free culture conditions, we evaluated xeno-free TeSR2 medium and recombinant human laminin-521 as the culture substrate. Laminin-521 is a laminin isoform used to improve the adhesion of dissociated hPSCs. Freeze-thawed single hPSCs in TeSR2 medium showed high survivability (86.8 ± 3.1% survival) immediately after freeze-thawing. Considering the promotion of adhesive by laminin-521, we re-evaluated the seeding density and found that freeze-thawed single hPSCs seeded at 1×10^5 cells/cm^2 showed maximal survival (59.7 ± 15.5% survival for H9 hESCs and 43.2 ± 11.2% survival for 253G1 iPSCs) (Fig. 4a and Supporting Information Fig. 1a). Moreover, on the laminin-521-coated culture surface, a higher number of single hPSCs were able to adhere and survive even at a lower seeding density (36.2 ± 6.1% survival for H9 hESCs and 32.5 ± 8.7% for 253G1 iPSCs at 0.25×10^5 cells/cm^2). After reseeding, the freeze-thawed hPSCs rapidly formed colonies on laminin-521 (Fig. 4b) and could be passaged within 3 days (Fig. 4c). The freeze-thawed hPSCs maintained their undifferentiated state (Fig. 4d, e, and Supporting Information Fig.4b), potential for differentiation (Fig. 4e), and a normal karyotype after passaging on laminin-521 in TeSR2 medium (Fig. 4f). Therefore, freeze-thawing of single hPSCs is applicable to xeno-free culture conditions, and human laminin-521 enhances the survival of single hPSCs after thawing.

In the present study, we obtained a high recovery rate following cryopreservation by optimizing the cellular state during freezing and the seeding density after thawing. These modifications achieved 80–90% survival of hPSCs post-thawing and around 60% survival following subculture on laminin-521, even in the absence of a ROCK inhibitor. hPSCs are delicate cells as compared with other types of cultured cells. Therefore, hPSCs require specific conditions not only for maintenance in culture but also for cryopreservation. Most previous approaches for effective hPSC cryopreservation have focused on development of a cryoprotectant specific for hPSCs (Ha *et al.*, 2005; T'Joen *et al.*, 2012). However, our study demonstrated that a fundamental solution for cryopreservation of hPSCs is their cellular state. It is therefore necessary to consider the cellular state for further investigation of hPSC cryopreservation. Moreover, our examination of the survivability of individual cells in a stepwise manner will contribute to estimation of the precise survival efficiency of hPSC cryopreservation.

It is still unclear why hPSC colonies are susceptible to injury after freeze-thawing. One risk is that hPSC colonies undergo unavoidable physical damage. Because cell scraping and pipetting for cell detachment is usually associated with cell rupture, freeze-thawed colonies of hPSCs may still be damaged following cell detachment. In addition, cell colonies have poor penetration of cryoprotectant. During cryopreservation, cells are

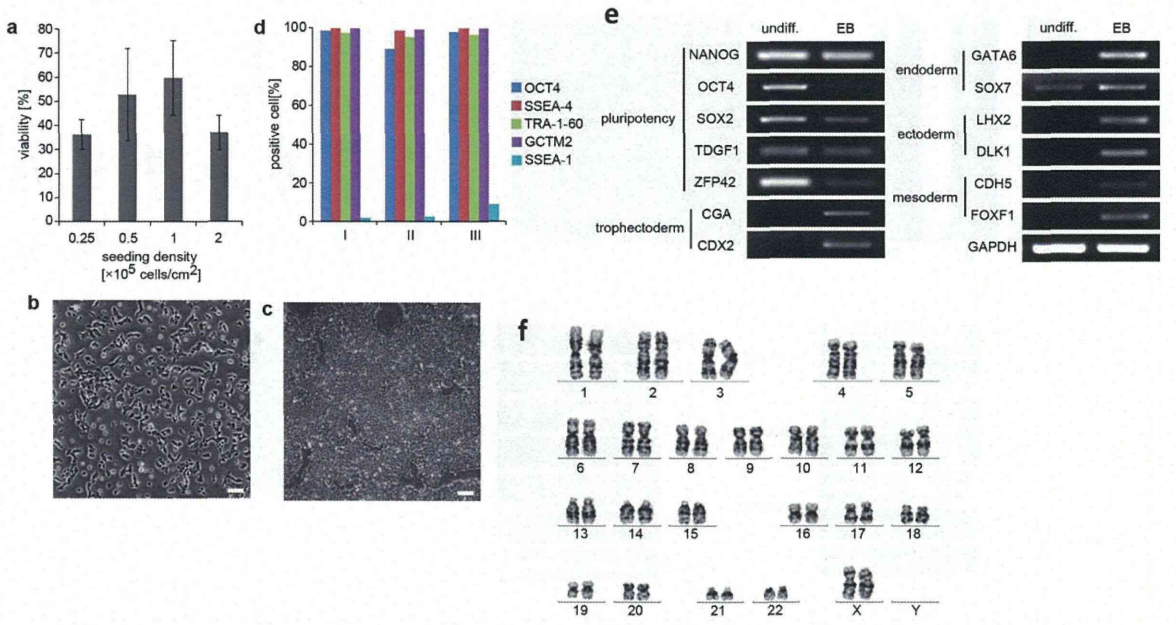

FIG. 4. Cryopreservation of single hPSCs under xeno-free culture conditions. (**a**) Seeding density-dependent adhesion of freeze-thawed single H9 hESCs on laminin-521 in TeSR2 medium at 12 h postseeding. Cell viability is expressed as the ratio of the attached cell number to live H9 hESCs at seeding. Data represent the means ± SD of three freeze-thawing experiments of one cryopreserved batch of cells. (**b**, **c**) Phase contrast images of freeze-thawed single H9 hESCs at 12 h (b) and 3 days (c) postseeding at 1×10^5 cells/cm^2. (**d**) Flow cytometric analysis of undifferentiated markers before cryopreservation (I), at 3 days postseeding (II), and at subculture (III). (**e**) Reverse transcription-PCR analysis of differentiation marker gene expression in embryoid bodies. Total RNA was extracted from day 14 embryoid bodies generated from cells after subculture. (**f**) G-banding analysis of H9 hESCs after subculture. Data for subcultured cells were obtained at two passages after freeze-thawing. Scale bars: 200 μm.

often ruptured by rapid osmotic changes caused by the cryoprotectant (Hunt, 2011). hPSC colonies are tightly connected by cell–cell interactions such as adherence junctions, and their formation generates a heterogeneous cellular state for cryoprotectant penetration. When compared with a complex cellular structure, single cells may be more suitable for cryopreservation in terms of cryoprotectant penetration. In addition, dissociation to single cells may minimize cellular damage at detachment because physical manipulation is unnecessary. Here, we used TrypLE Select as the dissociation solution, but other dissociation solutions used for single cell passaging, such as accutase and trypsin, would provide similar results.

In conclusion, our method enables highly efficient slow cooling of hPSCs. The cellular manipulation is simple and very similar to commonly used techniques for culturing most mammalian cell lines. Furthermore, our method is suitable for large-scale cryopreservation of pluripotent stem cell lines and machine-aided automation.

METHODS

Matrix Coating

Six-well flat-bottomed plates (BD Falcon, 353046) and 12-well flat-bottomed plates (BD Falcon, 353043) were coated with Matrigel (BD Biosciences, 354230) at 25 μg/cm^2 or laminin-521 (Bio Lamina, BLA-LN521-02) at 3 μg/cm^2 for 3 h at room temperature (RT) just prior to use. Matrigel and laminin-521 were diluted in DMEM/F12 or D-PBS, respectively.

Maintenance and Passaging of hPSCs

hESC lines, H9 and KhES-1, and hiPSC lines, iPS(IMR90) clone 1, and 253G1 were maintained on mitomycin C-treated mouse embryonic fibroblasts as described previously (Suemori *et al.*, 2006). hPSCs were transferred onto Matrigel-coated culture vessels in mTeSR1 medium (StemCell Technologies, ST-05850) or laminin-521-coated culture vessels in TeSR2 medium (StemCell Technologies, ST-05860) following the manufacturer's instructions.

Subculture was performed by colony dissociation as follows. Semi-confluent hPSCs were incubated with 2 mg/ml dispase in DMEM/F12 at 37°C for 3 min, and then rinsed twice with DMEM/F12. After addition of culture medium, weakly adherent colonies were detached using a cell scraper. The cells were collected and centrifuged at 200*g* for 3 min at 4°C. Small colonies were passaged onto fresh matrix-coated culture vessels at a ratio of 1:4. The cells were cultured at 37°C with 3% CO_2 in a humidified incubator. Medium changes were performed daily.