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難病・がん等の疾患分野の医療の実用化研究事業
（再生医療関係研究分野）

ヒト胚性幹細胞を用いた臨床利用の安全性検証のための試料保存と分析システムの構築

平成２５年度　総括研究報告書

研究代表者　　末盛　博文

平成２６年（２０１４）年　４月

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平成２６年（２０１４）年　4月

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Ⅰ．総括研究報告書

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ヒト胚性幹細胞を用いた臨床利用の安全性検証のための試料保存と分析システムの構築

研究代表者　　末盛　博文　　　京都大学　再生医科学研究所　准教授

研究要旨： ヒトES細胞を用いた再生医療の安全性を長期間にわたって検証可能とする基盤構築をめざし、臨床研究に関わる細胞試料やデータを主な対象とした保存システムの構築と、有害事象の原因究明に関連する分析技術およびこれらにかかわるノウハウを蓄積することにより、移植医療の安全性向上に資することを目標とする。これまでに整備された、試料の保存設備・検体管理システム・分析機器等を活用し、まず試料を安定的に保存するために必要となるモニタリングシステムの検証を行い、冷凍保存設備の温度記録や異常時の警報などが正常に動作することを確認した。またこれと並行して、細胞バンク事業において実施している品質管理技術を臨床研究機関に適切に技術移転する仕組みの構築を開始した。

A．研究目的

本研究はES細胞を用いた臨床研究において特に安全性を担保する上で必要となる研究の各段階での試料の保存と分析を行うシステムを確立し、将来的により安全な再生医療を実現する研究基盤を構築することを目的とする。

ヒトES細胞を用いた再生医療はすでに欧米では臨床試験が開始されており、今後より広範な医療領域での利用が期待されている。しかしながら、従来の細胞移植医療と異なり、ES細胞を用いた移植医療では、１ドナーに由来する細胞を様々な疾患を対象として多数の患者に適用することになる。そのため移植組織細胞の腫瘍化や、これに由来する感染性因子の伝搬は、被験者/患者のみならず公衆衛生上の問題を生じうるのではないかとの懸念がもたれており、これらの課題

への対応は、ES細胞を含めて幹細胞医療の普及を促進する上で、重要性・必要性が非常に高い。

従来のヒト幹細胞の臨床利用に関わる指針に代わり、再生医療安全確保法に基づく政省令により、ヒトES細胞の臨床利用にかかわる各種の基準が明らかにされると予想されている。これに適合した、ES細胞作成研究の申請にむけての作業が26年にも開始されると思われる。本研究では提供医療機関と連携し、ドナー情報等の取扱/保存について検討、また、実施が想定される細胞の検査法の感度や精度について検証を行うことにより、将来のヒトES細胞を用いた再生医療の安全性の担保を推進する他の事業に資することができると考えられる。

B．研究方法

現在ヒトES/iPS細胞の臨床利用を目指し多くの機関が基礎/前臨床研究を進めており、我々は早期の再生医療の実現を目指して臨床用ES細胞作製の基盤研究を他の研究経費により進めている。本研究計画と別途進めている臨床用ES細胞株の樹立と分配では、臨床研究機関に細胞を分配するためのセルバンクが構築される。本事業ではこの時、臨床研究機関へ供給されるものと同一ロットの複数サンプルを将来の検証が必要になった場合に備え保存する。また、保存が適切に行われるように、その検証システムを整備する。

臨床研究機関での未分化細胞の増幅/凍結保存が行われることも想定されるが、その場合は培養履歴とともに一部試料の寄託を求めるよう調整に努める。分化誘導後移植に用いる機能細胞が作出されるが、この途中段階で組織幹細胞や前駆細胞が作出される場合は必要に応じてその一部の寄託を求める。

臨床試験において有害事象が発生した場合に、品質管理上の問題の有無について、必要に応じて寄託された試料のウイルス等の検査や遺伝子解析を行えるよう解析技術の開発検証を行う。

（倫理面への配慮）

ヒトES細胞を用いる研究に関しては、文部科学省「ヒトES細胞の樹立および分配に関する指針」「ヒトES細胞の使用に関する指針」に従い実施された。

C．研究結果

本研究を実施する京都大学再生医科学研究所では、基礎研究用のヒトES細胞株の樹立と特性解析を行ってきた。このような研究の過程で蓄積した培養/解析技術をもとに、臨床用ヒトES細胞株を作成・利用する上で必要となる細胞バンク構築を別途実施している。本事業はこの臨床用ヒトES細胞樹立研究及び医療機関等での臨床研究/治験と連動して行われるものである。

ヒトES細胞の臨床研究指針は平成25年度中の施行が期待されていたが、依然として沭現されていない。さらにES細胞を用いた臨床応用が再

生医療法のもと行われることとなり、関連する規定等の施行にはさらに時間がかかると思われる。詳細が明らかになり次第、臨床用ヒトES細胞株作製研究の申請準備が行われるが、現時点では想定される基準等への対応のため、臨床用細胞バンクの構築と連携して、本事業の一貫として、試料保存システムの運用の規則、SOPなどの整備を行うとともに、品質管理の手法、規格の検証を行った。これらと並行して試料保存システムの適切な稼働を担保するため、機能の確認を順次行っている。

バンキングにおける安全性試験にかかわる基準の検証については、別途進められている臨床用ヒトES細胞のバンキングシステムの実証試験のなかで開発が進められている。安全性にかかわる試験では、大きく12の大項目をもうけている。外部機関との連携を考えた場合これらについて方法、規格値の妥当性や技術移転の方法について検討が必要であり、これを順次進めている。今年度は無菌試験、エンドトキシン試験についてSOPや規格について検討をおこなった。これらの検査は方法が確立され、また規格も明確であり、現時点でバンクの品質管理として実施する上での問題は見いだされなかった。

また、特性解析試験基準の検証を同様に進めている。特性解析についてはInternational Stem Cell Banking Initiativeから提起されている特性試験内容に基づき行われている。これは、大きく7項目に分類されるが、形態および細胞増殖速度の2項目について、バンキング時のSOP案に従い実施解析した。その結果、従前の試験と同様のデータが得られそのプロトコールの妥当性について一定の検証ができた。

これらに加え、凍結保存容器のモニタリングシステムの検証を順次実施している。臨床研究に関わる試料は長期間にわたり保存が必要である。24年度補助事業において整備された保存・培養システムについて、温度等の運転状況のモニタリングシステムを導入したが、これをもちいて監視を実施している機器のなかから、超低温保存容器と、細胞培養装置についてシステムの動作を検証した。その結果、必要な仕様を満たした記録が行われていることを確認した。

D．考察

本研究事業では臨床用ヒトES細胞バンキング事業において実施されている品質管理をもとに、これを標準的な技法として提供することを目的としている。品質解析技術の多くはすでに確立されているものも多いが、適切な実施には相応のノウハウが要求されると思われる。これを様々な機関において再現性良く実施することが重要である。

また、再生医療で移植される細胞製品やその原材料の保存に関してはその安定性などは実証されておらず今後長期にわたり検証を続ける必

要がある。

E．結論

バンキングにおける品質管理を基本とし、規格化された試験方法などをバンクから細胞を供与する臨床研究機関にスムーズに移行し共通の基準で細胞の品質管理を行うことを目指す。これは長期的な細胞の利用が前提となるES細胞の臨床応用の実用化において重要である。確立された試験法について、臨床研究機関においても同様の基準で適切に実施されることが、長期的な評価には有用であると考えられるため、これを研究機関との連携のもと実施する方法論を確立する必要がある。

F．健康危険情報

該当なし。

G．研究発表

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(2) Miyazaki T., Nakatsuji, N., Suemori, H. Optimization of slow cooling cryopreservation for human pluripotent stem cells. *Genesis* 52, 49-55. (2014)

2．学会発表

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(4) 再生医療製品の中間体としての ES/iPS 細胞とその製造関連材料の品質
末盛　博文

バイオロジクスフォーラム第１１回
学術集会　　（1/24,　東京）

H．　知的財産権の出願・登録状況
（予定を含む。）
該当なし

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Miyazaki T., Nakatsuji, N., Suemori, H.	Optimization of slow cooling cryopreservation for human pluripotent stem cells.	Genesis	52	49-55	2014
Hirata N, Nakagawa M, Fujibayashi Y, Yamauchi K, Murata A, Minami I, Tomioka M, Kondo T, Kuo TF, Endo H, Inoue H, Sato SI, Ando S, Kawazoe Y, Aiba K, Nagata K, Kawase E, Chang YT, Suemori H, Eto K, Nakauchi H, Yamanaka S, Nakatsuji N, Ueda K, Uesugi M.	A Chemical Probe that Labels Human Pluripotent Stem Cells.	Cell Reports	6	1165-1174	2014

Ⅲ．研究成果の刊行物・別刷り

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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

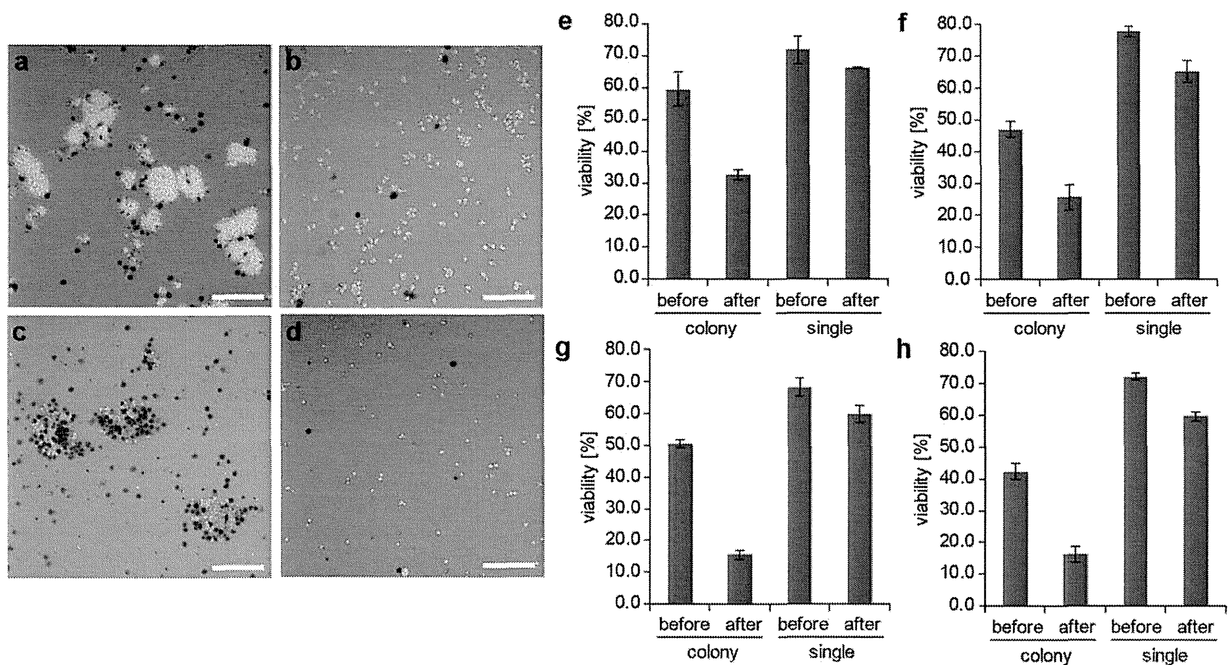

FIG. 1. hPSC survivability is influenced by the cellular state during cryopreservation. **(a–d)** Estimation of cellular state-dependent hPSC survivability by trypan blue exclusion. Representative bright field images of colony-dissociated H9 hESCs before (a) and after (b) cryopreservation and single 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

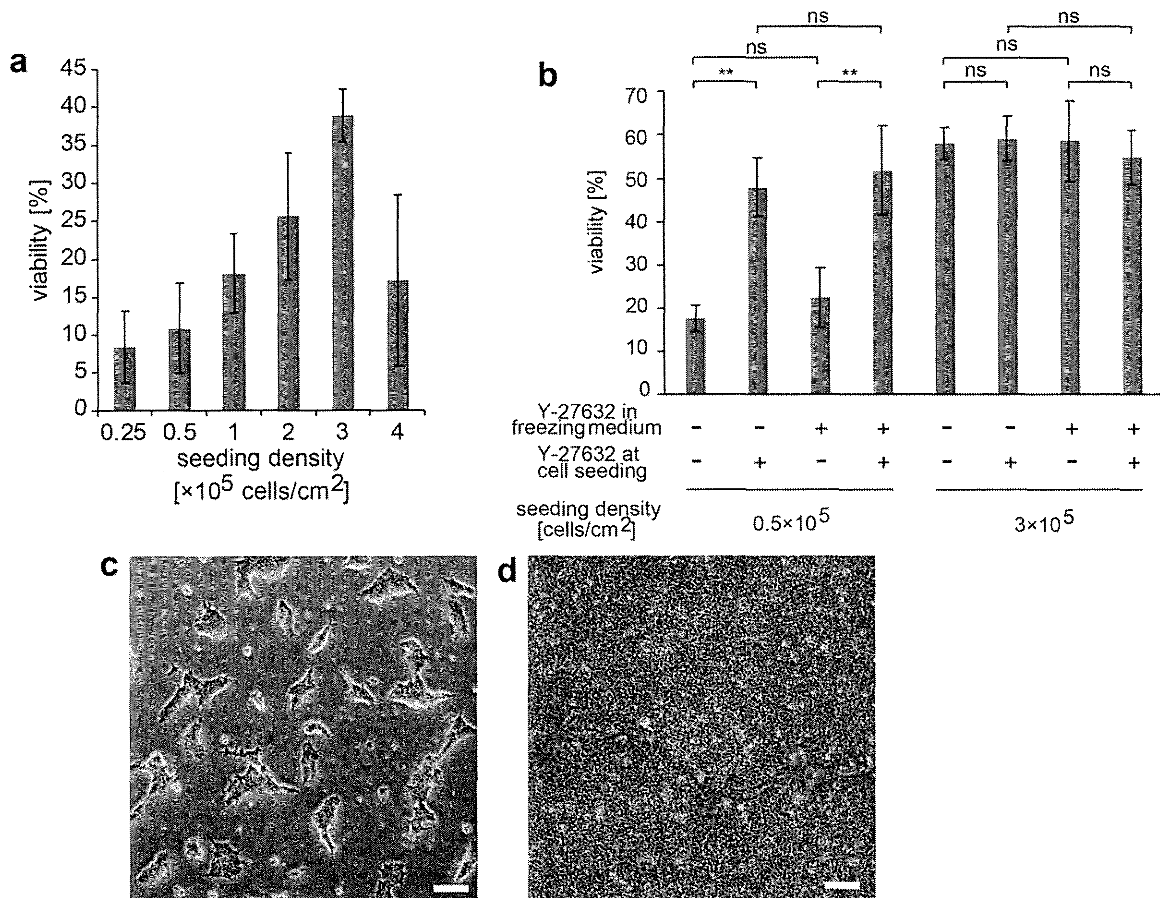

FIG. 2. Recovery of freeze-thawed single hPSCs. (**a**) Seeding density-dependent adhesion of freeze-thawed single H9 hESCs at 12 h postseeding. Cell viability is expressed as the ratio of the attached cell number to live H9 hESCs at seeding. Data represent the means ± SD of three freeze-thawing experiments 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.

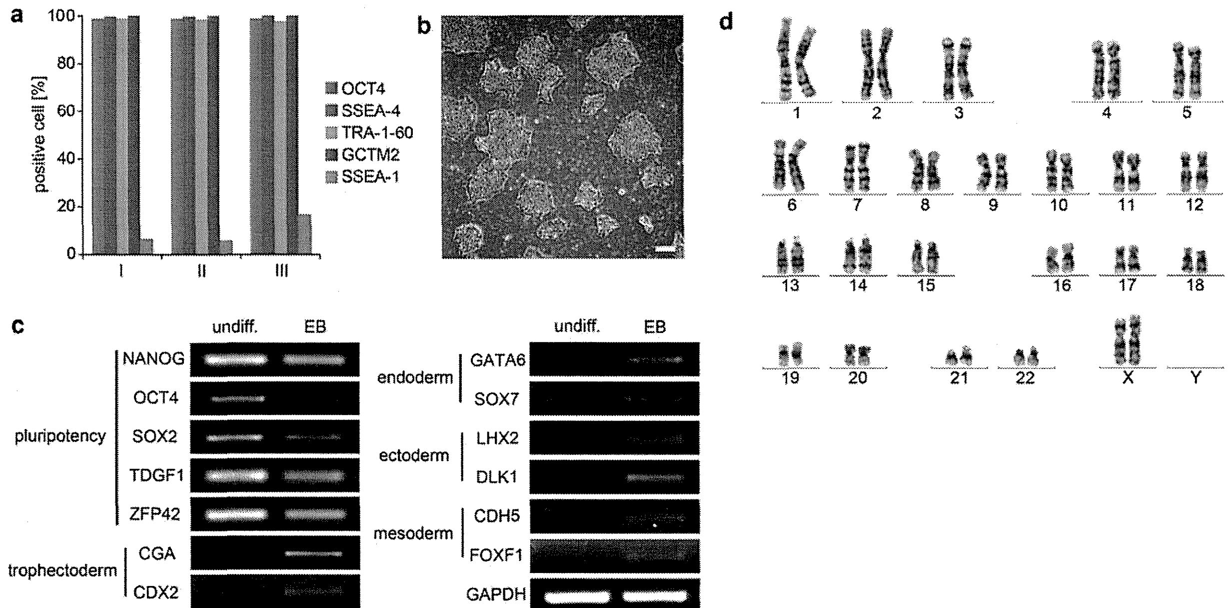

FIG. 3. Characterization of freeze-thawed hPSCs. (**a**) Flow cytometric analysis of undifferentiated markers before cryopreservation (I), at 3 days postseeding (II), and at subculture (III). (**b**) Phase contrast image of H9 hESCs at subculture. (**c**) Reverse transcription-PCR analysis of differentiation 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 ($86.8 \pm 3.1\%$ 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 ($59.7 \pm 15.5\%$ survival for H9 hESCs and $43.2 \pm 11.2\%$ 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 ($36.2 \pm 6.1\%$ survival for H9 hESCs and $32.5 \pm 8.7\%$ 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

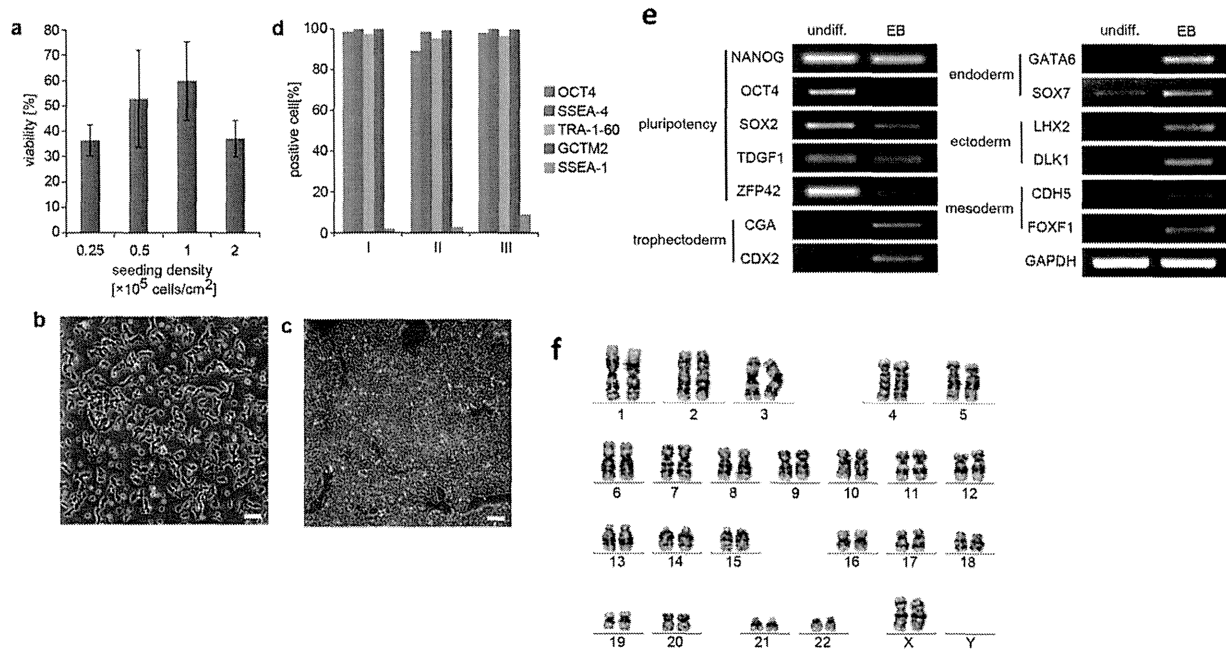

FIG. 4. Cryopreservation of single hPSCs under xeno-free culture conditions. (**a**) Seeding density-dependent adhesion of freeze-thawed single H9 hESCs on laminin-521 in TeSR2 medium at 12 h postseeding. Cell viability is expressed as the ratio of the attached cell number to live H9 hESCs at seeding. 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.

Cell Freezing and Thawing

For cryopreservation, hPSCs were dissociated as follows. Semiconfluent hPSCs were treated with 4.8 m*M* EDTA/PBS for 3 min at RT and then TrypLE Select (Invitrogen, 12563011) for 1 min at 37°C. The cells were collected and pipetted for complete dispersal and then centrifuged at 200*g* for 3 min at 4°C.

For freezing, 2.0–2.2 × 10^6 hPSCs were resuspended in 0.5 ml prechilled culture medium, gently mixed with the same volume of 2× prechilled freezing medium containing 20% DMSO (final concentration: 10% DMSO), and then transferred to cryovials (NUNC, 377224). The cryovials were placed in a Mr. Frosty Freezing Container (NUNC, 5100-0001) and stored at −80°C for 24 h. The cryovials were then transferred to liquid nitrogen and stored for at least 1 week before analysis.

For thawing, the cells were warmed at 37°C in a water bath and then diluted with cold culture medium. The cells were collected by centrifugation at 200*g* for 3 min and seeded onto matrix-coated culture vessels at the indicated seeding densities.

Cell Viability Assays

The viability of hPSCs cultured with mTeSR1 medium was estimated by trypan blue exclusion or flow cytometric detection of fluorescent dye influx. To obtain accurate data of cell viability before and after cryopreservation, cell preparation for viability assays was performed by centrifugation at 500*g* for 5 min to ensure maximum recovery of more than 90% of the frozen cells. For trypan blue staining, hPSCs were incubated with a 0.2% trypan blue solution (Wako, 207-17081) for 1 min at RT, and then observed under an optical microscope in a bright field. For flow cytometric detection, hPSCs were rinsed with D-PBS by centrifugation at 500*g* for 5 min at 4°C, and then completely dissociated by treatment with 4.8 m*M* EDTA/D-PBS for 2 min at RT. The cells were rinsed with culture medium and resuspended in D-PBS. The cell suspension was incubated with 105 n*M* thiazole orange for 5 min and then 11 μ*M* propidium iodide for 2 min just prior to analysis. Considering cell damage during preparation for FACS analysis, single hPSCs were additionally treated by a dissociation process similar to that for hPSC colonies.

Cell Adhesion Assays

Freeze-thawed single hPSCs were seeded at several cell densities in 24-well plates coated with Matrigel or laminin-521. After 12 h of incubation, the cells were rinsed with prewarmed D-PBS, and the remaining live cells were detached with 0.25% trypsin/EDTA and counted using a hemocytometer.

To evaluate the effect of Y-27632 treatment, freeze-thawed single hPSCs were seeded at two cell densities and then incubated with 10 μ*M* Y-27632 (Wako, 253-00513). After 12 h, adherent live cells were counted as described above.

Karyotype Analysis

hPSCs were treated with 100 ng/ml colcemid (Life Technologies, 15212-012) for 2–3 h. After dissociation in 0.25% trypsin/EDTA, the cells were treated with a hypotonic solution and then fixed in Carnoy's solution. Cells were spread onto glass slides and stained with Giemsa. Chromosome spreads were then analyzed by randomly counting 50 cells using the Ikaros Karyotyping System (META system).

Flow Cytometric Analysis

Cells were dissociated by treatment with 4.8 m*M* EDTA/PBS for 2 min and then TrypLE Select for 1 min. To detect surface markers, the cells were rinsed twice with 10% FBS/DMEM, and 1 × 10^5 cells were incubated with primary antibodies diluted in staining buffer (0.1% BSA/D-PBS) for 30 min at 4°C. The cells were rinsed twice with staining buffer and then incubated for 30 min at 4°C with the secondary antibody diluted in staining buffer. After rinsing twice with staining buffer, the cells were resuspended in D-PBS and stained with propidium iodide just prior to analysis. To detect intracellular markers, dissociated cells were fixed with 4% formaldehyde/PBS for 15 min at 4°C. After rinsing with D-PBS, cells were incubated with saponin permeabilization buffer (SPB) (1 mg/ml saponin and 1% BSA in D-PBS) for 15 min, and then the primary antibody for 30 min at 4°C. Cells were rinsed twice with SPB, and then incubated for 30 min at 4°C with the secondary antibody diluted in SPB. After three rinses with SPB, the cells were resuspended in staining buffer for analysis by a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software. Antibodies against the following markers used were SSEA-4 (Developmental Studies Hybridoma Bank (DSHB), MC-813-70, 1 μg/ml), Tra-1-60 (Millipore, MAB4360, 1 μg/ml), GCTM2 (Millipore, MABD90, 1 μg/ml), TRA-2-54 (DSHB, 1 μg/ml), and SSEA-1 (DSHB, MC-480, 2 μg/ml) as primary antibodies and a mouse anti-Ig/FITC (Becton Dickinson, 731735, 1 μg/ml) as the secondary antibody.

Differentiation Assay

Differentiation potential was estimated by semi-quantitative polymerase chain reaction (PCR) analysis of differentiation marker genes expressed in embryoid bodies. Freeze-thawed hPSCs subcultured for two passages were detached by treatment with CTK solution (ReproCell, RCHETP002) and using a cell scraper. The cell clumps were cultured as a suspension in petri dishes with hESC medium without FGF-2. Medium changes were performed every 2 days. After 14 days of

culture, total RNA was extracted using an RNeasy Mini Kit (Qiagen, 74104), and cDNA was synthesized from 1 to 2 μg RNA using an Omniscript™ RT Kit (Qiagen, 205111) according to the manufacturer's instructions. PCR conditions were optimized to facilitate semiquantitative comparison with the log phase of amplification. The gene-specific primers are described elsewhere (Kumagai *et al.*, 2013; Miyazaki *et al.*, 2012). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Statistical Analysis

The statistical significance of differences was determined by the two-tailed Student's *t*-test. Differences with a value of $P < 0.05$ were considered significant.

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A Chemical Probe that Labels Human Pluripotent Stem Cells

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SUMMARY

A small-molecule fluorescent probe specific for human pluripotent stem cells would serve as a useful tool for basic cell biology research and stem cell therapy. Screening of fluorescent chemical libraries with human induced pluripotent stem cells (iPSCs) and subsequent evaluation of hit molecules identified a fluorescent compound (Kyoto probe 1 [KP-1]) that selectively labels human pluripotent stem cells. Our analyses indicated that the selectivity results primarily from a distinct expression pattern of ABC transporters in human pluripotent stem cells and from the transporter selectivity of KP-1. Expression of ABCB1 (MDR1) and ABCG2 (BCRP), both of which cause the efflux of KP-1, is repressed in human pluripotent stem cells. Although KP-1, like other pluripotent markers, is not absolutely specific for pluripotent stem cells, the identified chemical probe may be used in conjunction with other reagents.

INTRODUCTION

Human embryonic stem cells (hESCs) (Thomson et al., 1998) and induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007) have been serving as valuable tools for basic biological research and as promising resources for regeneration therapy. Despite advances, substantial challenges remain for the clinical application of stem cells. One safety concern has been posed by the appearance of teratomas in animal models transplanted with cell samples containing a small number of undifferentiated stem cells. Methods of detecting and ablating undifferentiated stem cells are required for safer stem cell therapy.

Antibodies against stage-specific embryonic antigens 4 and 5 (SSEA-4 and SSEA-5) have been used extensively to detect human pluripotent stem cells (Henderson et al., 2002; Tang et al., 2011; Thomson et al., 1998). SSEA-4 is a glycolipid that is expressed in early embryos and, for unknown reasons, is presented selectively on the surface of hESCs and embryonic carcinoma (EC) cells (Henderson et al., 2002). SSEA-5, which is classified as an H-type 1 glycan, is a recently identified antigen specifically expressed in human pluripotent stem cells (Tang et al., 2011). Other markers of human stem cells include Oct3/Oct4 and Nanog, which are transcription factors required for the maintenance of undifferentiated states of stem cells and are downregulated upon differentiation (Chambers et al., 2003; Mitsui et al., 2003; Niwa et al., 2000; Pesce and Schöler, 2001; Rosner et al., 1990). Although their antibodies are highly useful for detecting pluripotent cells, these unstable protein tools suffer from high cost and often require fixation and permeabilization of cells. Alkaline phosphatase is another routinely used marker of human stem cells (Shamblott et al., 1998; Thomson et al., 1995). Although the assay for its enzymatic activity provides a simple method for detecting stem cells, this housekeeping enzyme is expressed in a number of other cell types, and its specificity to pluripotent stem cells is a major concern. A small molecule fluorescent probe specific for human pluripotent stem cells would permit their rapid detection and separation. Furthermore, small molecule probes provide reversible detection that can be tuned by varying the dose. Stable, chemically defined, and cost-effective synthetic probes would offer significant advantages as tools for basic research and for lowering the risk of tumor formation in stem cell therapy.

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