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Improving single-cell cloning workflow for gene editing in human pluripotent stem cells

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ABSTRACT

The availability of human pluripotent stem cells (hPSCs) and progress in genome engineering technology have altered the way we approach scientific research and drug development screens. Unfortunately, the procedures for genome editing of hPSCs often subject cells to harsh conditions that compromise viability: a major problem that is compounded by the innate challenge of single-cell culture. Here we describe a generally applicable workflow that supports single-cell cloning and expansion of hPSCs after genome editing and single-cell sorting. Stem-Flex and RevitaCell supplement, in combination with Geltrex or Vitronectin (VN), promote reliable single-cell growth in a feeder-free and defined environment. Characterization of final genome-edited clones reveals that pluripotency and normal karyotype are retained following this single-cell culture protocol. This time-efficient and simplified culture method paves the way for high-throughput hPSC culture and will be valuable for both basic research and clinical applications.

1. Introduction

Human pluripotent stem cells, including induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs), hold great promise for cell research and clinical applications (Kiskinis and Eggan, 2010; Park et al., 2008; Saha and Jaenisch, 2009). Recent advances in genome engineering and specifically the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR associated 9 (Cas9) endonuclease system makes the human genome more amenable than ever to genetic research. By combining these two technologies, scientists are now able to correct disease-associated mutations in patient-derived iPSCs enabling researchers to avoid confounding, complex genetic background effects via the creation of isogeneic control iPSCs. Alternatively, genome engineering allows the introduction of disease-associated mutations into 'normal' iPSC lines such that genetic disorders can be modeled without the need to obtain patient cells harboring the specific disease-causing mutations. Additionally, genome engineering can be used to modify endogenous loci such that endogenous proteins can be tagged with fluorescent or other protein domains, creating reporter lines that can be used for high throughput screening of small molecule libraries to search for therapeutic compounds or for cell tracing experiments (Doudna and Charpentier, 2014; Hsu et al., 2014).

Traditional hPSC expansion requires feeder cells and serum-containing media to maintain the "stemness" of the hPSCs (Stojkovic et al., 2004). Recently, the need for pharmacological and medical applications has driven the development of fully defined and xeno-free media for hPSC culture with improved surface matrices to achieve feeder-free culture conditions (Braam et al., 2008; Chen et al., 2011; Ludwig et al., 2006a, b; Rodin et al., 2014). Routine expansion of hPSCs involves passaging of cells as small aggregates or clumps to avoid unwanted selective pressure on cell populations and cell death associated with single-cell dissociation. It is generally not recommended to passage hPSCs as single cells as it can lead to genetic aberrations in the culture (Buzzard et al., 2004). However, for the practice of transfection or identifying singe-cell derived genome edited clones, it is important to dissociate cells in a single-cell manner. Although recent improved chemically defined media have shown excellent performance for routine hPSC culture, poor cell viability and clonogenicity of cultured single hPSCs remains as a major bottleneck after single-cell passaging. Different approaches including extracellular matrices (Higuchi et al., 2015; Rodin et al., 2014), protein inhibitors (Valamehr et al., 2012;

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Watanabe et al., 2007), irradiated mouse embryonic fibroblasts (MEFs) (Yang et al., 2013) and human serum-derive protein (Pijuan-Galito et al., 2016) have shown improved survival of single-cell derived hPSC clones. However, these methods have not been widely implemented, being either too expensive, lacking commercial availability, the need for MEFs or poor reproducibility. Such systems are particular laborious and inefficient for genetically modifying hPSCs with CRISPR-Cas9; especially with low editing rates observed for knock-in modifications. Thus, there is a great need to develop a simplified and robust protocol that can support high-throughput and reliable single-cell derived clonal growth of stable hPSCs.

In the present study, we demonstrate that Stem-Flex media with RevitaCell supplement is ideal for single-cell culture of hPSCs. We also perform an unbiased comparison among different commercially available feeder-free culture systems and supplements on their performance with single-cell cloning and expansion of cloned hPSCs. Herein, we describe a simplified and time-efficient cell culture system for single-cell cloning, supporting expansion of clones while undergoing genome editing and maintaining pluripotency.

2. Material and methods

Several hPSC lines were used in this study including H9 and H1 hESCs (WiCell), WTC11 (Coriell institute), BJFF.6 and other iPSCs (GEiC or collaborators). hPSCs were maintained on Matrigel (Corning) coated plate in E8-Flex/Stem-Flex medium (ThermoFisher) unless otherwise noted. FACS was conducted on a MoFlo cell sorter (Beckman Coulter). hPSCs karyotype (G-banding) analysis was performed by Cell Line Genetics and Cytogenetics core at Washington University in St. Louis. Pluripotency of hPSCs were characterized for SSEA4, OCT4, SOX2, and TRA-1-61. hPSCs differentiation was assessed for AFP, SMA and TUJ1. Immunofluorescent images of the stained cells were captured using the Nikon fluorescence microscope and CCD camera. Statistical analysis was performed using Prism GraphPad 6.0. A p value of < 0.05 was considered statistically significant. Comprehensive information on the experimental procedures is described in the Supplemental Information.

3. Results

3.1. TrypLE-Select and ROCK inhibitor support cell survival and expansion after single-cell passage

Both enzymatic and enzyme-free reagents have been commonly used for routine hPSC passaging. Many dissociation reagents have been designed to gently separate multicellular colonies from the substrate into small cell aggregates, and single-cell suspension can be further achieved by adjusting the dissociation conditions (Fig. 1A). To determine which dissociation reagents can be used to support single-cell culture, single cells were dissociated and maintained in E8-Flex defined media.Three different hPSC lines cells were plated at 10,000 cells/well in 6-well plates (Fig. S1A). After 7 days in culture, wells with surviving cells were counted. While non-enzymatic cell dissociation reagent is reported to be gentler on cells (Beers et al., 2012), it showed poor support on cell survival following single-cell passaging for three different hPSC lines (Fig. 1B). In contrast, a recombinant protease based TrypLE-Select (TrypLE-S) reagent supported nearly 3-fold higher cell survival in single-cell culture conditions for the BJFF.6 hPSC line. In addition, 0.75× TrypLE-S showed the best survival rate following single-cell dissociation compared to other passaging reagents for the BJFF.6 hPSC line. Similar results were obtained using crystal violet staining (Fig. 1C and Fig. S1B). Therefore, 0.75× TrypLE-S was used for single cell dissociation for our subsequent studies.

Small molecular inhibitors of specific signaling pathways have been used in iPSC generation and maintenance (Valamehr et al., 2012; Watanabe et al., 2007). To determine whether small molecule inhibitors further support hPSC single-cell survival and growth; BJFF.6 cells were dissociated into single cells and then seeded in Matrigel-coated plates with E8-Flex plus Y-27632 (traditional ROCK inhibitor: ROCKi), RevitaCell (supplement containing a proprietary ROCKi) or SMC4 (small molecule cocktail of 4 inhibitors, consisting SB431542 (TGF-β), PD0325901 (MEK), CHIR99021 (GSK) and Thiazovivin (ROCK)) supplementation. Viability of cells was evaluated at day 4, 5 and 6 after single-cell dissociation. While TrypLE-S can support cell survival of single cell passage, the addition of ROCK inhibitors enhanced cell viability as previously reported (Watanabe et al., 2007) (Fig. 1D). Cells seeded in Y-27632 or RevitaCell supplemented medium (E8-Flex) exhibited similar viability levels, whereas seeding into SMC4 showed poor survival, in contrast to the previous studies with conventional or mTeSR1 medium (Valamehr et al., 2012; Yang et al., 2013). Moreover, the cells with SMC4 supplement showed flat and scattered morphology as compared to ROCKi containing supplements (Fig. S1C). While Y-27632 and RevitaCell showed similar viability levels in BJFF.6 cells, higher cell survival was observed in H9 and WTC cells using RevitaCell compared to Y-27632 (Fig. S1D). Together, these findings establish 0.75× TrypLE-S support cell survival in single-cell dissociation culture and the addition of ROCK inhibitors enhanced adaption efficiency of single-cell growth.

3.2. RevitaCell and Geltrex/VN support single-cell cloning after sorting by flow cytometry

While both ROCKi-containing additives, Y-27632 and RevitaCell, can enhance single-cell viability after passaging, RevitaCell has been shown to be a more selective ROCK inhibitor (SCIENTIFIC, 2015). To test whether either supplement could support single-cell growth after flow cytometry based cell sorting, various cell densities of BJFF.6 cells were sorted into Matrigel coated 96-well plates and the cloning efficiency was assessed at day 8. Interestingly, the addition of Y-27632 did not support single-cell growth whereas RevitaCell was able to support single cell growth with 12% and 17% clonability in 1 cell/well and 3 cells/well, respectively (Fig. 2A). RevitaCell was therefore used for single-cell sorting in our subsequent studies. While various times of ROCKi pre-treatment of hPSCs have been shown to improve single-cell clonal growth (Pijuan-Galito et al., 2016; Watanabe et al., 2007), we found that at least 1 h pre-treatment with ROCKi is sufficient for single-cell splitting and sorting procedure (data not shown).

Many extracellular matrices have been developed for feeder-free culture of hPSCs (Fig. 2B). In addition, some xeno-free and chemically defined substrates such as VN and COAT-1 can be applied to support xeno-free culture conditions for clinical applications. To determine whether different coating matrices could support clonal growth of single hPSCs, four different hPSC lines were pre-treated with RevitaCell and single cells were sorted into plates coated with different coating reagents. Increased clonal efficiency was observed in COAT-1, Geltrex, VN and Laminin-521 groups as compared to Matrigel across different hPSC lines with Geltrex and VN groups showing up to 40% clonal expansion (Fig. 2C). Similar to BJFF.6, both H1 and H9 hESCs showed coating reagent-dependent effect on clonal efficiency while WTC11 was less sensitive to different matrices (Fig. 2C). While similar clonability was observed in Laminin-521 across different hPSC lines, we continued on with Geltrex and VN since there was no dramatic increase in clonability and the cost was prohibitive.

A number of different culture conditions or additives have been shown to enhance hPSC clonal growth, such as low physiological oxygen condition (Forsyth et al., 2006), the addition of fibronectin (Valamehr et al., 2012) and the usage of conditioned medium (Yumlu et al., 2017). Additionally, keeping cells chilled on ice should slow down cellular activities and could mitigate the stress following single-cell dissociation or flow cytometry-based sorting. To test the effects of these culture conditions on single-cell survival after flow cytometry based sorting, Three different hPSC lines were sorted into Geltrex-

A

Dissociation reagent	Enzymatic (Y/N)	Single cell dissociation
L7	N	37°C, 7 minutes
GCDR	N	37°C, 7 minutes
Versene	N	37°C, 5 minutes
Accutase	Y	37°C, 5 minutes
0.75X TrypLE-Select	Y	37°C, 3 minutes
1X TrypLE-Select	Y	37°C, 3 minutes

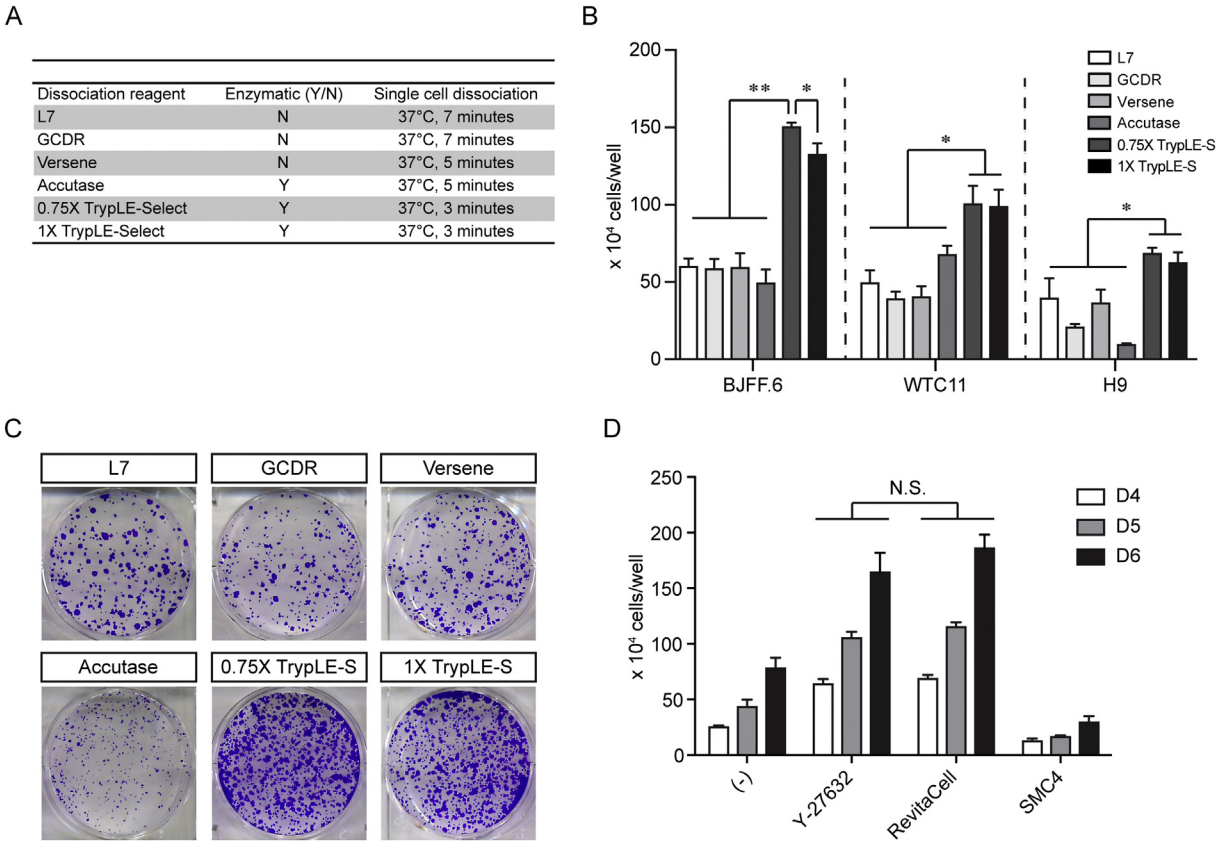


Fig. 1. TrypLE-Select supports faster recovery of hPSCs growth following single-cell dissociation. (A) Comparison of different hPSC passaging reagents for single-cell dissociation. hPSCs dissociated with TrypLE-Select showed the best recovery from single-cell dissociation in (B) cell number and in (C) the number and size of hPSC colonies (crystal violet staining) without addition of ROCKi ($n = 3$). (D) Improved cell survival and growth was observed in E8-Flex: Matrigel supplemented with ROCK inhibitor (Y-27632 or RevitaCell), but not SMC4 after single-cell passaging (n = 3). Error bars denote the mean ± SEM. (*) $p < 0.05$, (**) $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

coated plates with defined culture media (E8-Flex: RevitaCell). Keeping cells chilled during the cell dissociation and flow sorting process did not increase clonal survival of BJFF.6 or H9 cells, but a decrease in clonability was observed in WTC11 cells (Fig. 2D). While a subtle increase (5% increase) in clonal efficiency was observed using 50% conditioned medium in BJFF.6 cells, there was no significant clonal survival difference between controls and fibronectin-supplemented or conditioned medium (Fig. 2D). However, in contrast to normoxia (20% O_2), physiological oxygen (2.5% O_2) reduced hPSC clonal growth in BJFF.6, WTC11, and H9 lines. (Fig. 2D). Together, these findings demonstrate that the combination of RevitaCell with Geltrex or VN significantly increased clonal efficiency following our single cell sorting workflow.

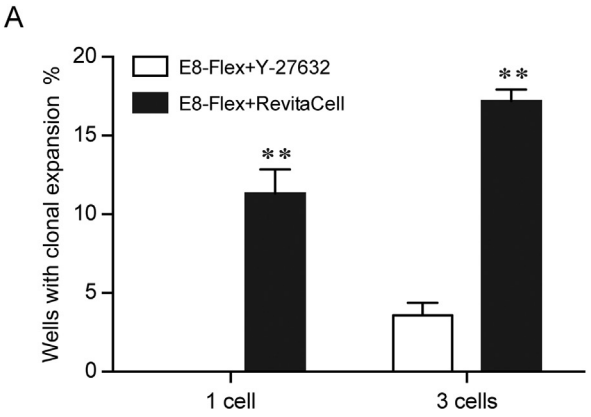
3.3. Easy adaption of single-cell cloning workflow

The advantages of the RevitaCell and Geltrex/VN were combined to develop a high-throughput method for obtaining clonally derived hPSCs following a genome-engineering workflow (Fig. 3A). Colony formation could be seen as early as 3 days post-sorting, and colonies could be harvested for the downstream analysis as early as 11 or 12 days post-sorting (Movie S1). To test whether our single-cell cloning workflow can support robust clonal efficiency using different culture systems, we compared the workflow using E8-Flex with four other media. RevitaCell supplement was used in E8-Flex, Stem-Flex and DEF-CS culture systems. For mTeSR1 and TeSR-E8, CloneR was used per manufacturer's instructions. While the addition of ROCKi was not recommended by the manufacturer for the DEF-CS culture system, we found that clonal efficiency was almost 4-fold higher when RevitaCell was added (Fig. S2A). RevitaCell was therefore used in DEF-CS culture medium in our subsequent comparison for single-cell clonal efficiency. We assessed the clonal efficiency for 4 different hPSC lines. Cloning rates up to 55% were achieved with Stem-Flex:Geltrex, and the cloning efficiency was consistently higher using E8-Flex or Stem-Flex (Fig. 3B).

To further compare the clonal efficiency of different culture systems, three iPS cell lines with low clonability (< 25%) using E8-Flex:Geltrex were chosen. The Stem-Flex:Geltrex culture system showed better overall clonal efficiency (Fig. 3C). It should be noted that the dramatic difference on clonal efficiency was observed in F10336.3 and HT112F when using E8-Flex, mTeSR1, TeSR-E8 or DEF-CS systems, underscoring the importance of culture medium on single cell cloning. Together, these data demonstrate that single-cell clones can be isolated using our single-cell workflow, albeit at different efficiencies, in all tested culture systems, and that consistent and high clonal efficiency can be achieved in the Stem-Flex culture system.

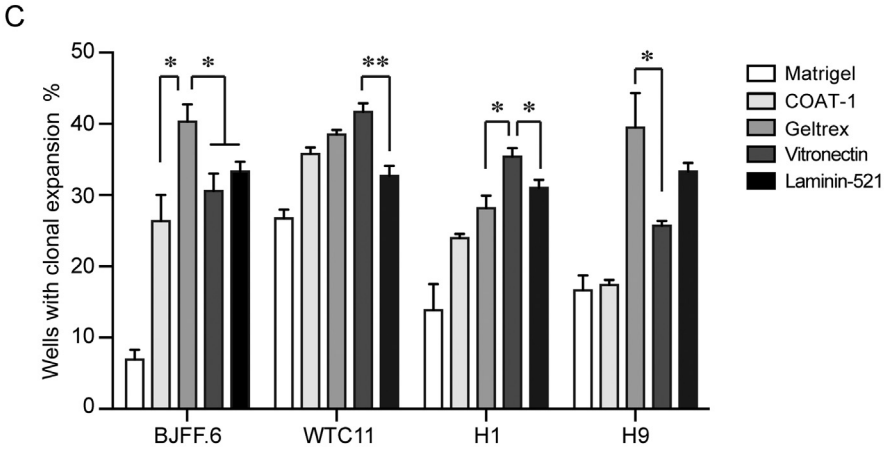
3.4. Single cell cloning protocol does not increase genetic abnormalities

Over 100 genome engineering projects have been completed using a variety of hPSC lines in our center (Table S1 and S2). Clonal efficiency for each project was conducted at day 11–12 following single cell sorting and projects were grouped by basal culture medium (E8-Flex and Stem-Flex) and type of modifications (Fig. 4A). Karyotype analysis was routinely performed in selected final edited clones to assess the chromosomal integrity. A representative karyogram of a selected clone demonstrates that hPSCs retained a normal karyotype (Fig. 4B). Moreover, hPSCs maintain expression of pluripotency markers, SSEA4, OCT4, SOX2 and TRA-1-60 (Fig. 4C) after single-cell cloning and expansion processes in E8- or Stem-Flex culture medium. Final edited



B

Coating matrix	Xeno-free (Y/N)	Dilute solution	Coating procedure
Matrigel	N	DMEM/F-12	RT, 1 hour
COAT-1	Y	DPBS(+/+)	37°C, >20 minutes
Geltrex	N	DMEM/F-12	37°C, 1 hour
Vitronectin	Y	DPBS (-/-)	RT, 1 hour



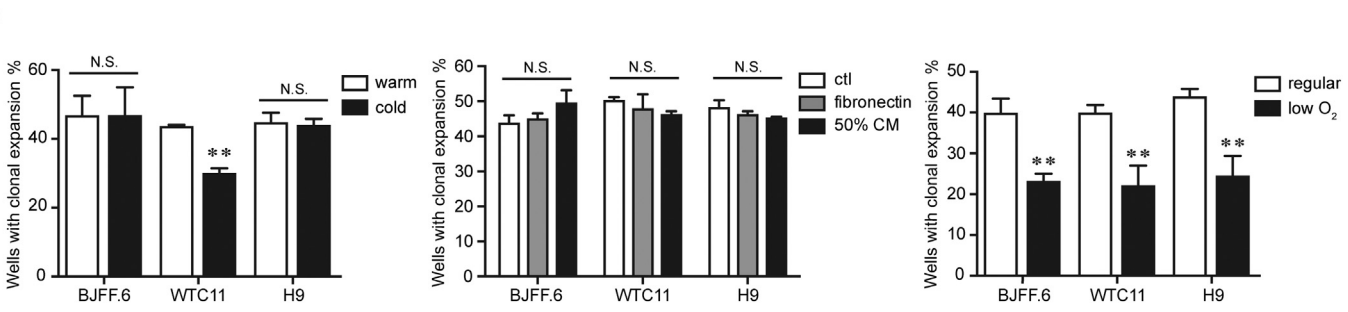


Fig. 2. RevitaCell and Geltrex/VN support cell survival after single cell sorting. (A) RevitaCell-supplemented medium (E8-Flex) exhibited increased single cell cloning efficiency on surface coated with Matrigel compared to traditional ROCKi controls (n = 3). (B) Comparison of different coating matrices for hPSC culture. (C) Single-Cell cloning efficiencies of different hPSC lines on different coating matrices (n = 3). (D) Different culture conditions (cold, fibronectin and conditioned medium) did not improve single cell cloning efficiency, low oxygen tension (2.5%) decreased single clonal recovery relative to controls (n = 3). Error bars denote the mean ± SEM. Scale bar, 100 μm. (*) $p < 0.05$, (**) $p < 0.01$.

clones were routinely subjected to spontaneous differentiation by embryoid body formation and subsequently plating on Matrigel with serum-containing medium. After 4 weeks, hPSCs showed successful differentiation into all three germ layers as assessed by immunofluorescence staining for specific markers for three somatic lineages (Fig. 4D). Taken together, the robustness of this single-cell cloning method can support screening, hPSC maintenance and expansion following genome-engineering processes.

4. Discussion

The recent development of site-specific nucleases and specifically CRISPR-Cas9 to provide precise genome manipulation in hPSCs has created new opportunities for studying human genetics and diseases. However, genome editing in hPSCs remains difficult because of their intrinsically poor capacity to grow in single or very low cell densities. While several defined matrices and additives have been shown to improve hPSCs single-cell survival and expansion (Emre et al., 2010; Pijuan-Galito et al., 2016; Rodin et al., 2014; Valamehr et al., 2012; Watanabe et al., 2007), none of the currently available methods fulfill the need for a simple, robust, and cost-efficient method for single-cell expansion and cloning. In addition, traditional clonal isolation of edited hPSCs involves limiting dilution and manual picking processes, which often leads to mosaicism and are inefficient and labor intensive (Li et al., 2016; Yumlu et al., 2017). Therefore, a standardized method for high-throughput hPSC clonal expansion following genome engineering is needed.

While several studies have successfully demonstrated a workflow for genome editing using hPSCs, these protocols rely heavily on FACS enrichment, drug selection and manual colony picking (Byrne et al., 2014; Santos et al., 2016; Yumlu et al., 2017). Additionally, some improvements for single-cell cloning have been reported, but with only modest improvements in efficiency (between 5 and 20%) (Pijuan-Galito et al., 2016; Valamehr et al., 2012). Here, we present a simple and

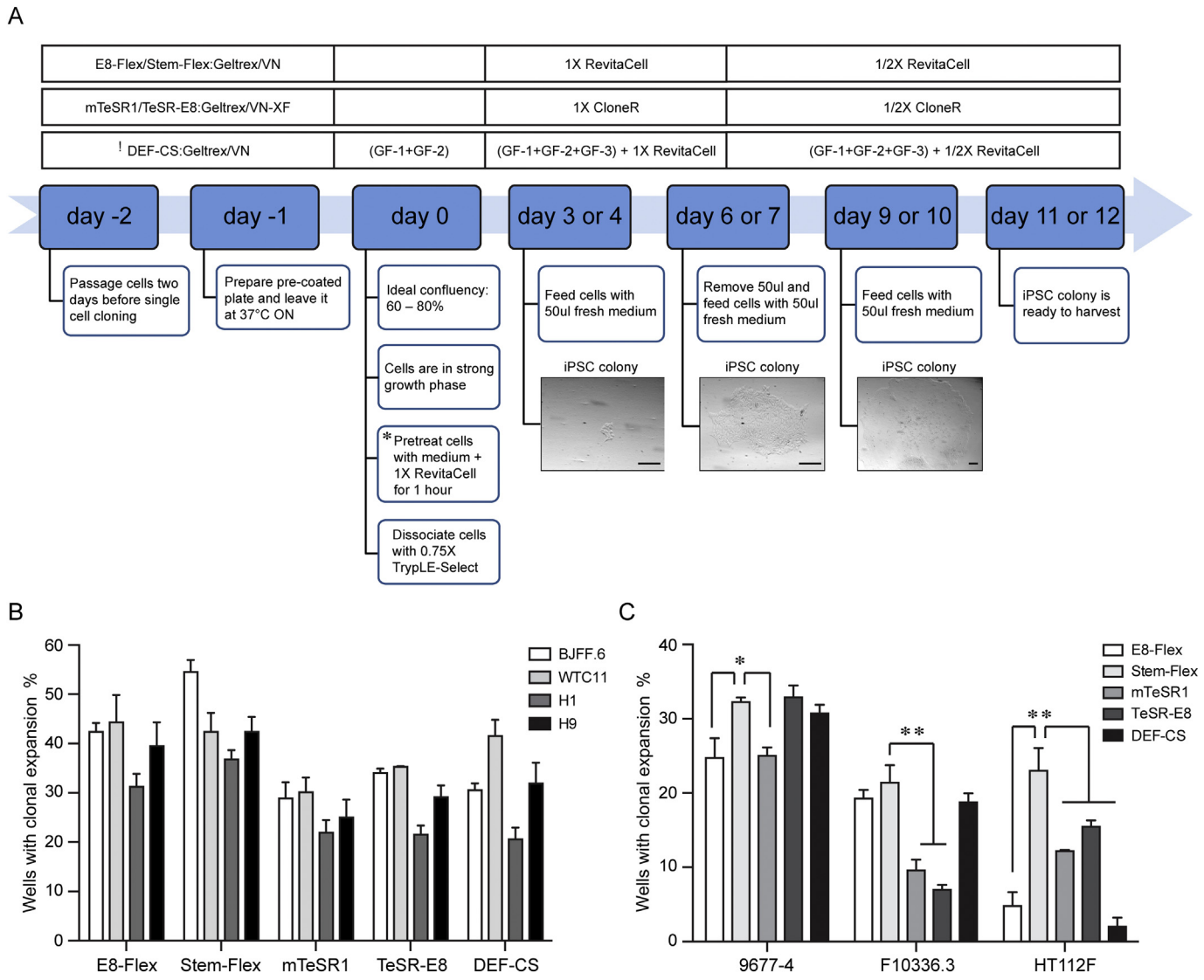


Fig. 3. E8-Flex and Stem-Flex demonstrate robust clonal expansion following single-cell sorting workflow. (A) A schematic workflow of single-cell sorting methodologies for high efficient single cell clonal expansion. (!) Fresh DEF-CS medium was prepared with additives (GF-1, GF-2 and GF-3) on the day of intended use. (*) Pretreatment is not required for the use of CloneR. (B) Unbiased comparison of different commercial hPSC culture systems for single-cell cloning efficiency (n = 3). (C) Stem-Flex shows consistently higher clonal efficiency in difficult iPSCs (n = 3). Error bars denote the mean ± SEM. Scale bar, 50 μm. (*) $p < 0.05$, (**) $p < 0.01$.

robust protocol to enhance hPSC single-cell survival and cloning with efficiencies as high as 60% that also yields genomically stable hPSC clones.

We evaluated different passaging reagents, cell matrices, additives and media to develop a robust protocol for selection and feeder free single-cell cloning following genome editing. We found that 0.75× TrypLE-S showed the best recovery rate for hPSCs following single cell dissociation, consistent to previous findings (Nishishita et al., 2015). Prior reports demonstrate that TrypLE-S is gentler on the cell surface (Ellerstrom et al., 2007). Moreover, ROCK inhibitors, Y-27632 and RevitaCell showed significant improvement on single-cell survival. In contrast, the SMC4 inhibitor cocktail did not improve single-cell survival, revealing ROCK inhibition is beneficial for single-cell survival.

To establish a robust platform for single-cell cloning, we flow-sorted hPSCs as single cells into Matrigel-coated plates. While both Y-27632 and RevitaCell supported single-cell survival when plating pools of single-cell clones, single-cell derived clones were only observed in medium supplemented with RevitaCell. Additionally, we found that the coating matrix used also influenced the clonal efficiency. This is the first time that hPSCs have been shown to have different responses to the cell matrices for single-cell cloning. Surprisingly, the most commonly used matrix, Matrigel, showed the worst performance on single-cell cloning. In contrast, Geltrex, VN and Laminin-521 demonstrated much more consistent and higher cloning efficiencies.

While many hPSC culture systems have been shown to support single-cell culture, expansion and maintenance of pluripotency, standard culture system may perform differently during single-cell cloning. The reported clonal efficiencies for each medium are E8-Flex: < 5%; Stem-Flex: 20 to 25% (SCIENTIFIC, 2017); mTeSR1 and TeSR-E8: ~20% (TECHNOLOGIES, 2017); DEF-CS: 25 to 30% (TakaRa, 2017). In this study, we first established the single-cell cloning workflow based on E8-Flex culture system, and then applied it to different culture systems with minor modifications to fit the workflow. E8-Flex and Stem-Flex showed overall higher cloning efficiency compared to other systems with Stem-Flex out-performing even E8-Flex in more difficult hPSC lines. While similar results were observed in Stem-Flex in response to single cell dissociation (Fig. S2B vs. Fig. 1B), Stem-Flex showed improved cell survival in SMC4 compared to E8-Flex (Fig. S2C

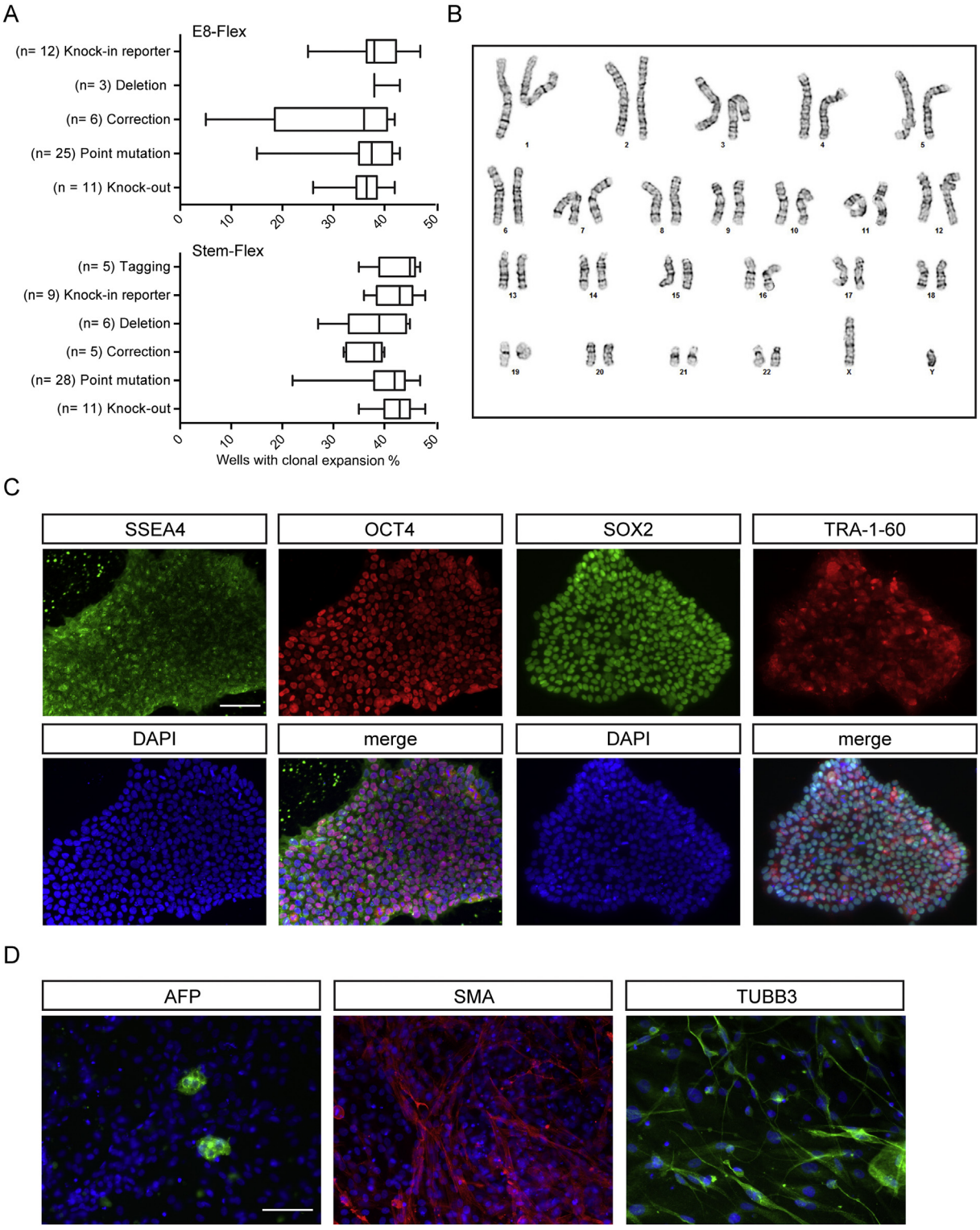


Fig. 4. Genome edited clones maintains normal hPSC properties. (A) Summary of single clonal efficiency in different genome engineering projects. (B) Representative karyogram of a final edited clone showing a normal karyotype. (C) Immunofluorescence staining for pluripotency markers, SSEA4, OCT4, SOX2 and TRA-1-60. (D) Immunofluorescence staining for markers of three germ layers, endoderm with AFP (green), mesoderm with SMA (red) and ectoderm with TUJ1 (green), after 4 weeks of differentiation. Nuclei were counterstained with DAPI. Scale bar, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vs. Fig. 1D). More importantly, overall clonal efficiency was higher following our workflow (E8-Flex: 30 to 50%; Stem-Flex: 37 to 58%; mTeSR1: 22 to 30%; TeSR-E8: 22 to 35% and DEF-CS: 20 to 45%), regardless of the culture system. In contrast to the typical hPSC morphology, cells in DEF-CS culture medium showed less packed, but much more scattered monolayer morphology (Fig. S2D). It should be noted that CloneR supplement made a significant improvement in single-cell cloning following flow sorting for both mTeSR1 and TeSR-E8 culture systems as cloning efficiencies without CloneR in these two medium was close to zero (data not shown). Additionally, we found more

consistent results following single-cell cloning by gradually reducing CloneR concentrations as cells expanded rather than performing complete media changes as per manufacturer's instructions (data not shown). While the use of Laminin-521 as cellular substrata has shown to improve single-cell growth of hPSCs under xeno-free and chemically defined conditions (Rodin et al., 2014), the current use of laminin for high-throughput hPSC maintenance and expansion is limited due to the high cost. In this study, we did not observe a significant increase in clonability across different hPSCs using Laminin-521 (Fig. 2C, 3B and S2E) compared with Geltrex or VN.

In this report, we summarize over 100 genome engineering projects including parental cells, culture medium, modification, type of donor, and clonabiity following the single-cell cloning method (Table S1 and S2). Interestingly, we found that type of modification or donor was not associated with single cell cloning efficiency (Fig. 4A), whereas some particular patient iPSC lines show poor clonability. Additionally, most knockout genes or deletion mutations have no significant impact on hPSC single cell cloning efficiency indicating that the defect on gene function could be cell-type dependent. The presence of genetic variations in iPSCs could be the actual factor for adverse effects on single cell growth, moreover hampering the process of genome engineering. Stressful single-cell dissociation under suboptimal conditions may lead to selection of abnormal cells or to an in vitro-adapted cell line (Draper et al., 2004; Imreh et al., 2006; Mitalipova et al., 2005). Interestingly, there doesn't appear to be a correlation between reprogramming method and clonability. Moreover, passage number is unlikely to affect clonability as we try to maintain hPSCs at low passage numbers and it in general takes < 8 passages post nucleofection to identify a single cell derived clone. While the causes for genetic changes of hPSCs are poorly understood, we have not observed any karyotypic abnormalities post single-cell enzymatic dissociation in any of the 41 hPSC genome editing projects and total 92 modified clones that have been karyotyped. Single-cell propagation and characterization of randomly selected edited clones further support that this workflow is reliable and robust.

5. Conclusions

We have successfully leveraged different culture methods to develop a simple workflow that offers a reliable and cost-efficient solution to the current need for hPSC single-cell cloning. This method can be combined with the CRISPR-Cas9 technology to scarlessly generate engineered hPSCs and further facilitate stem cell research.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.08.003.

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