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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-