TECH NOTE

Superior Single-Cell Cloning of iPS Cells with the Cellartis DEF-CS 500 Culture System

Higher single-cell cloning efficiency
The Cellartis DEF-CS 500 Culture System better supports establishment of hiPS cell clones than competitors' culture systems >>

Robust expansion of clones
The Cellartis DEF-CS 500 Culture System promotes robust proliferation of clonal hiPS cell colonies >>

Pluripotency maintained after single-cell cloning
Clones established and grown in DEF-CS maintain high expression levels of TRA-1-60 and SSEA-4, while clones established and grown using competitors' systems lose expression of these pluripotency markers >>

Introduction

Recent progress in gene editing technology has made it possible to introduce targeted genetic alterations in cells and organisms. These methods are being applied in human induced pluripotent stem (hiPS) cells, for functional studies of genetic variation in disease models and for the larger field of regenerative medicine; however, a major barrier remains at the level of selecting single cells with the desired mutations. Currently, it is difficult to treat isolated clonal cells in a manner that allows them to survive the gene editing procedure and thrive. This single-cell cloning bottleneck occurs because delicate hiPS cells typically grow in colony- and/or feeder-dependent conditions, and re-plating as single cells removes survival and growth signals, reducing viability. Promoting survival and proliferation at the single-cell level is critical for expansion of any clonal colonies containing the mutation of interest from the gene editing process.

Unfortunately, gene editing protocols often subject stem cells to harsh conditions that compromise their survival (*e.g.*, electroporation), a problem that is compounded by the innate challenges of single-cell culture. A culture system that supports single-cell cloning and expansion of hiPS cells could overcome the current barrier of poor outcomes for single cells. This type of improved culture system should be feeder-free and defined, as the use of feeder cells results in a more complicated workflow, more labor, inaccurate quantification, and an increased risk of heterogeneous cultures.

Additionally, the ideal system should sustain a feeder-free hiPS cell *monolayer* culture while going through the gene editing technique of choice, and then further ensure survival and proliferation once single cells are plated and expanded for mutant allele identification. Monolayer culture systems and colony systems differ in the growth patterns of the hiPS cells they support; the former supports a sheet of cells and the latter multiple colonies.

The Cellartis DEF-CS 500 Culture System, recognized for its suitability for genome engineering (Valton, J., *et al.* 2014) and single-cell cloning (Feng, Q., *et al.* 2014), promotes reliable growth of hiPS cells in a feeder-free and defined environment. Cells are grown as a homogeneous monolayer and are enzymatically passaged as single cells that maintain pluripotency with a stable karyotype for more than 20 passages (Asplund, A., *et al.* 2016). The same reagents are used for monolayer and single-cell culture, reducing variability. In the following experiments, we demonstrate that the Cellartis DEF-CS 500 Culture System is ideal for single-cell culture. We

also report a comparison between this system and two commercially available, feeder-free culture systems on their performance with single-cell cloning and expansion of cloned hiPS cells. Cloning efficiency measurements and pluripotency analysis indicate that the Cellartis DEF-CS 500 Culture System is the most efficient system for single-cell cloning, supporting expansion of clones while maintaining pluripotency.

Cellartis DEF-CS 500 Culture System Enables Establishment of Robust Colonies after Single-Cell Seeding

For gene editing experiments, edited cells can be passaged by serial dilution into single-cell culture conditions (or FACS sorted, one cell per well). Supporting survival and growth of "emerging clones" at this stage is critical for success; any colony loss could mean a loss of the desired mutant. Optimal performance of hiPS cell culture systems occurs when there is a high enough seeding density for the cells to support their growth through cell-cell contact signals and a sufficient concentration of secreted soluble factors. However, system performances may diverge as the seeding density decreases.

In order to determine the performances of the Cellartis system and the systems from Vendor 1 and Vendor 2 over a range of seeding densities, cells from Cellartis Human iPS Cell Line 18 (ChiPSC18, a delicate stem cell line) were diluted serially and seeded at concentrations ranging from ~0.5–450 cells/well into 96-well plates. After 9–11 days, wells with surviving cells were counted to determine the supportive capability of each system. While the Cellartis DEF-CS 500 Culture System performed as well as or better than competitors' systems in the range of ~2–50 cells/well (the light yellow section of Figure 1, below), it showed a unique capability to support an initial seeding density of ≤1 cell/well (the dark yellow section). At a seeding density of one cell per well, the DEF-CS system was the only system that showed robust survival and proliferation of clones, indicating the suitability of DEF-CS for experiments that require single-cell survival.

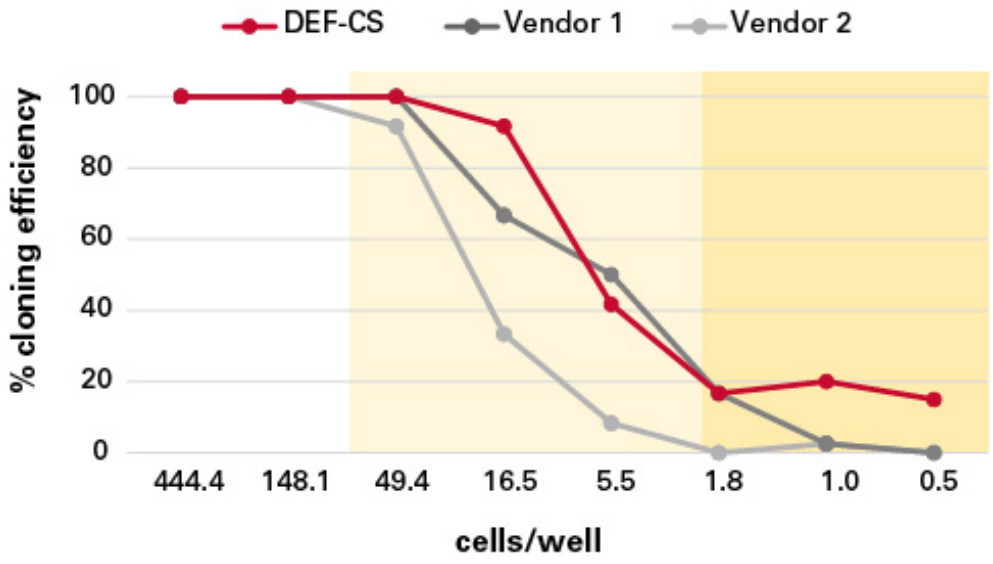

Figure 1. The Cellartis DEF-CS 500 Culture System successfully supports the growth of clones. ChiPSC18 cells were plated at concentrations ranging from ~0.5–450 cells/well in 96-well plates. Wells in each condition were plated at the same initial seeding density, and n = 40 for the 1 cell/well and 0.5 cell/well conditions. Cell attachment and growth were observed by phase contrast microscopy. Single-cell cloning efficiency was calculated as described in the Methods section. When the seeding density was decreased to 50 cells/well and below, the supportive capabilities of the culture systems started to diverge. At a seeding density of ≤1 cell/well, the performances of Vendors 1 and 2 dropped, each supporting only one colony out of 40, while the DEF-CS culture system supported 14 colonies out of 40.

Since the competitors' systems were unable to support viable colonies in the previous experiment using a delicate stem cell line, they were additionally tested with more robust cell lines: ChiPSC22 (known for its robustness) and ChiPSC12 (with more average features). In Figure 2, cells from ChiPSC12, ChiPSC18, or ChiPSC22 were grown in the DEF-CS system, Vendor 1's system, or Vendor 2's system (three cell lines x three culture systems = nine conditions total). After single-cell cloning in 96-well plates (as described in the Methods section), the growth of the emerging clones was observed by phase contrast microscopy, and the number of wells containing healthy-looking colonies was counted.

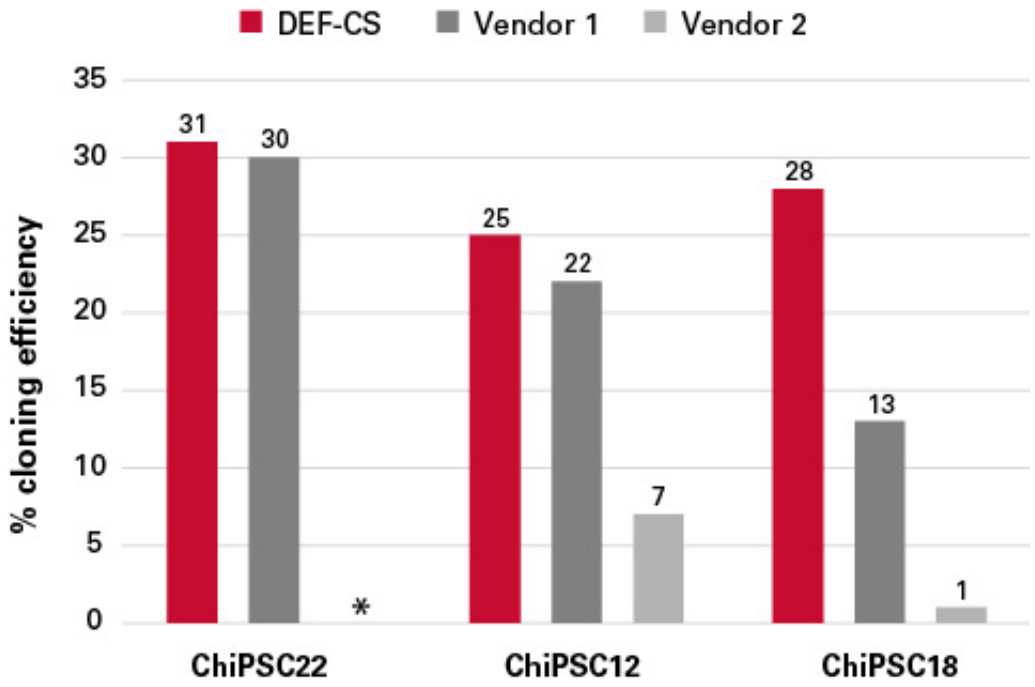

Figure 2. The Cellartis DEF-CS 500 Culture System supports single-cell cloning using additional hiPS cell lines. To further assess the single-cell cloning efficiency starting from 1 cell/well for the different systems, clones were isolated from three cell lines via enzymatic passaging (or equivalent), seeded at a density of 1 cell/well, and allowed to expand. Cell attachment and growth were observed by phase contrast microscopy, the number of successful colonies was counted, and single-cell cloning efficiency was calculated as described in the Methods section. Data was combined from two experiments, where n is the number of wells seeded with a single cell. For ChiPSC22, n = 96; for ChiPSC12, n = 156; and for ChiPSC18, n = 156. In the case of Vendor 2's system using ChiPSC22, the asterisk indicates that no single clones were obtained.

Cellartis DEF-CS 500 Culture System Maintains Pluripotency of Cloned hiPS Cells

For successful single-cell cloning, not only do the cells need to give rise to emerging clones (through healthy proliferation of the seeded single cells within the original wells), but the emerging clones also need to be pluripotent, robust, and expandable (able to maintain proliferative capacity after passaging into larger wells, or to be an "expandable colony"). The stability of hiPS cells as they are repeatedly dissociated, plated, and expanded is critical for successful gene editing; cells must maintain their original pluripotent identity, indicating their potential to be differentiated into cell types from all three germ layers. A solid indicator of stemness is the presence of pluripotency markers TRA-1-60 and SSEA-4.

Emerging clones from experiments 1 and 2 were passaged according to each culture system's manual and the established hiPS cell cultures were analyzed for protein expression of TRA-1-60 and SSEA-4. For cells grown in DEF-CS, 29 clones were analyzed (26% of the emerging clones). For cells grown in Vendor 1's system, 14 clones were analyzed (19% of the emerging clones), and for Vendor 2's system, 6 clones were analyzed (55%

of the emerging clones). Flow cytometry analysis indicated that the clones established in the DEF-CS system maintained the highest proportion of TRA-1-60-positive, SSEA-4-positive, and double-positive cells, while the clones in the competitors' systems partially or completely lost the expression of these pluripotency markers. In Figure 3, the results from up to five representative colonies established and grown in the different culture systems are compared; each set of three bars shows data from one representative expandable colony derived from each viable emerging clone.

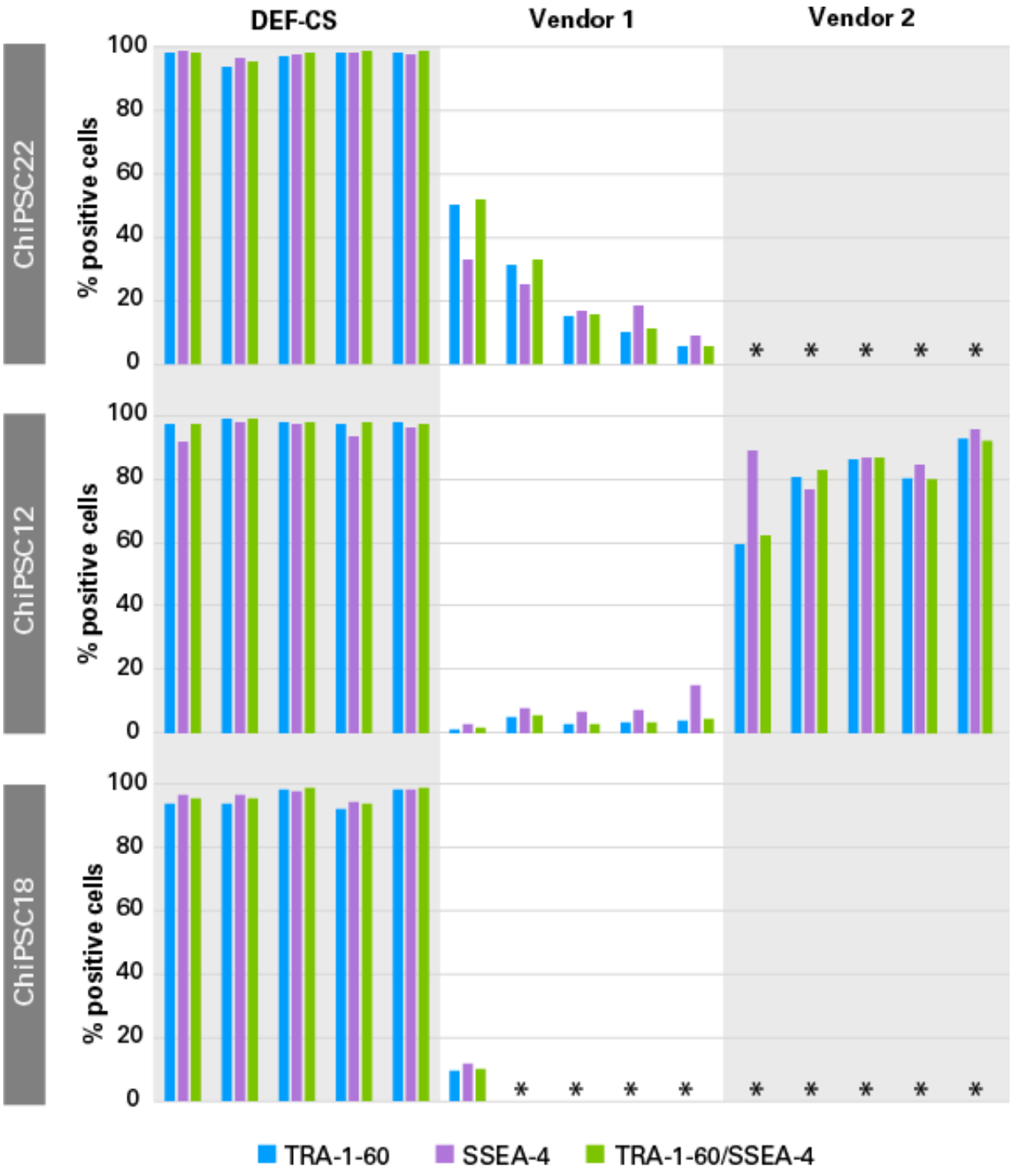

Figure 3. The Cellartis DEF-CS 500 Culture System maintains the highest expression of pluripotency markers

TRA-1-60 and SSEA-4. After single-cell cloning and passaging in each of the nine conditions (three cell lines x three culture systems), hiPS cells were analyzed for TRA-1-60 and SSEA-4 protein expression by flow cytometry (see Methods section for details). The percentages of TRA-1-60-positive, SSEA-4-positive, and double-positive cells are indicated for each clone. An asterisk indicates that the emerging clone did not survive, so pluripotency data could not be obtained.

In order for single-cell cloning to be truly successful, a high efficiency in the number of seeded single cells that become emerging clones must be combined with a high capability of the emerging clones to be converted into expandable colonies—that is, established, undifferentiated hiPS cell lines. Emerging colonies must be able to be passaged separately, and after passage, >90% of the cells in each expandable colony must stain positive for TRA-1-60 and SSEA-4, representing high-quality hiPS cells. In the following analysis, data from ChiPSC12, ChiPSC22, and ChiPSC18 were averaged across the cell lines. For cells grown in DEF-CS (29 clones analyzed), 97% met the threshold of >90% TRA-1-60+/SSEA-4+ cells. For cells grown in Vendor 1's system (14 clones analyzed), only 7% met this threshold, and for cells grown in Vendor 2's system (6 clones analyzed), 33% met the threshold. Figure 4 shows the total percentage of seeded single cells that could be converted into undifferentiated lines. DEF-CS stands out as highly effective for the conversion of emerging clones into high-quality cell lines.

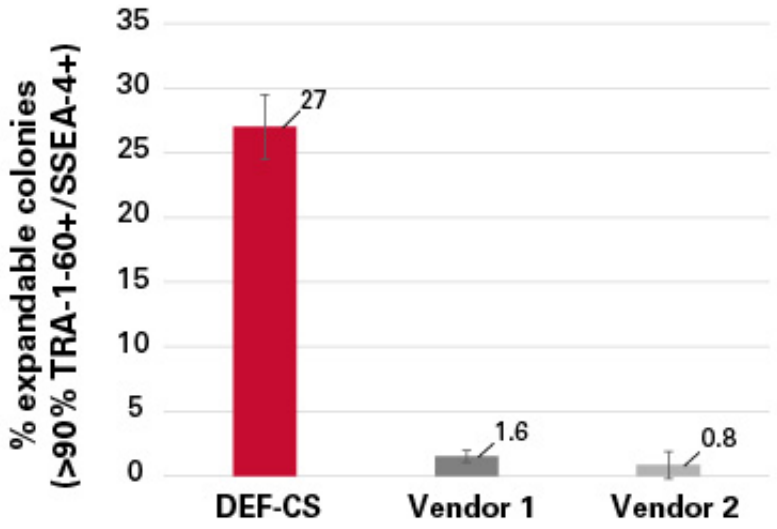

Figure 4. The Cellartis DEF-CS 500 Culture System enriches for undifferentiated hiPS cell lines from single cells. Based on the single-cell cloning efficiency data from Figure 2, and the average percentage of analyzed cells that met the threshold of >90% TRA-1-60+/SSEA-4+ cells from Figure 3, the percentage of expandable colonies per seeded single cell was calculated.

Cellartis DEF-CS 500 Culture System Promotes Robust Clonal Expansion

The ultimate goal for single-cell culture is to generate stable, healthy clonal cell populations that can be used for a variety of experiments. The DEF-CS system reaches this goal; it provides the ideal environment for single-cell survival and colony growth by fostering growing colonies so they are fit for subsequent expansion, and by maintaining a pluripotent status throughout the process. In the time-lapse images below, a seeded single cell was grown using DEF-CS and followed over eight days to track its emergence into a healthy colony.

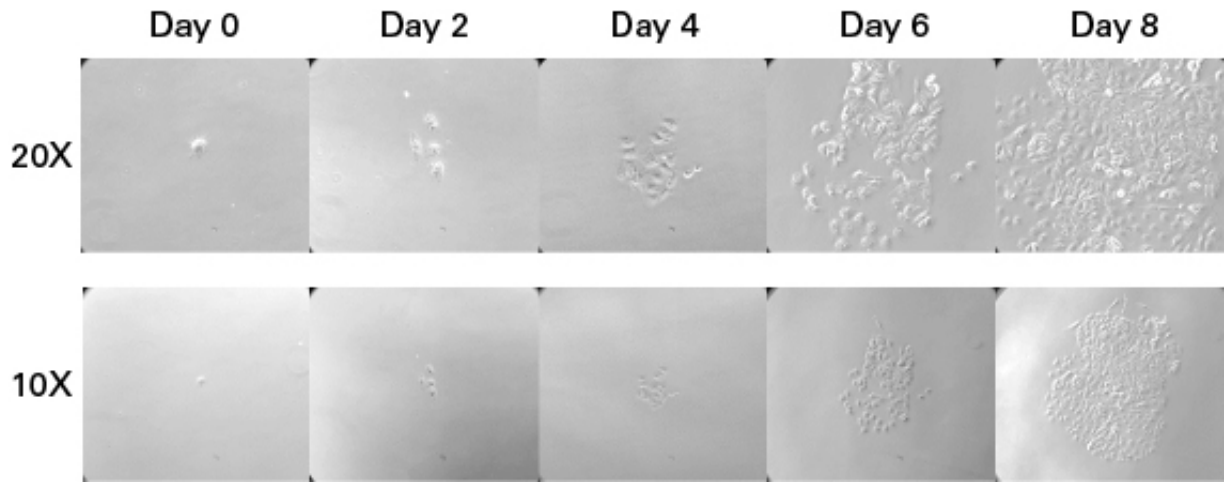

Figure 5. The Cellartis DEF-CS 500 Culture System supports emerging hiPS cell colonies. ChiPSC22 cells were diluted serially and plated as single cells in individual wells of a 24-well plate. One well was selected for analysis, and phase contrast images were taken of cell proliferation over 48-hour intervals for a total of eight days. Images show the formation of a healthy colony originating from the original seeded single cell.

Results demonstrated that the Cellartis DEF-CS 500 Culture System showed robust expansion ability in all three iPS cell lines, while the other systems failed in supporting the establishment and expansion of single cells (Figures 1, 2, and 3) and/or in maintaining pluripotency levels (Figures 3 and 4).

Conclusions

Gene editing of human induced pluripotent stem cells requires a culture system that can support single-cell culture, robust proliferation, expansion, and maintenance of pluripotency. The type of culture system should be considered: standard stem cell culture systems may perform differently with single-cell culture than those designed for monolayer culture. In this study, the performance of the Cellartis DEF-CS Culture System, a monolayer culture system, was compared with those of two commonly used colony culture systems from different vendors, using multiple cell lines. The data demonstrated that DEF-CS allowed efficient single-cell cloning and expansion of hiPS cells, and the established clones maintained their dual expression of TRA-1-60 and SSEA-4. Indeed, the other systems were not able to promote robust single-cell survival, proliferation, or expansion to the same degree as DEF-CS, and the clones in those systems completely or partially lost expression of the aforementioned pluripotency markers. The DEF-CS system proved superior for single-cell cloning, making it an ideal choice for advancing experiments that require sensitive handling of edited hiPS cells.

Methods

Cell culture

Cellartis Human iPS Cell Lines 12, 18, and 22 were cultured in the Cellartis DEF-CS 500 Culture System and competitors' feeder-free culture systems (Vendors 1 and 2) for three weeks according to the manufacturers' protocols. Then, cells were enzymatically passaged (or equivalent) and seeded onto coated 96-well plates at ~0.5–450 cells/well for the experiment represented by Figure 1, and at 1 cell/well for the remaining figures. For the experiments represented by Figures 2, 3, and 4, expandable clones were created by passaging colonies (1:1) from the original 96-well plates into 48-well plates. Medium was changed every other day until analysis was performed between days 9 and 11.

Cloning efficiency and flow cytometry

After seeding, phase contrast microscopy was used to verify wells containing single cells. Wells containing

multiple cells were eliminated from the calculation of single-cell cloning efficiency. Ten days after seeding, cell growth in 96-well plates was again observed by microscopy, the number of wells containing healthy-looking colonies was counted, and cloning efficiency was calculated. Single-cell cloning efficiency was expressed as a %; it was calculated by dividing the number of healthy-looking wells (emerging clones) by n (# of seeded wells) and multiplying by 100. For the experiments represented by Figures 3 and 4, healthy-looking colonies were passaged and further expanded. These clones were stained with TRA-1-60 and SSEA-4 antibodies conjugated to Alexa Fluor 488 and phycoerythrin, respectively. The cells were analyzed by flow cytometry, and the percentages of TRA-1-60-positive, SSEA-4-positive, and double-positive cells were quantified.

References:

Asplund, A., *et al.* (2016) *Stem Cell Rev and Rep.* **12**(1):90–104.
Feng, Q., *et al.* (2014) *Stem Cell Reports* **3**(5):817–831.
Valton, J., *et al.* (2014) *Methods* **69**(2):151–170.