Efficient and Safe Single-Cell Cloning of hPSCs using the CEPT Cocktail

Summary

Single-cell cloning and gene editing patient-specific human pluripotent stem cells (hPSCs), particularly through CRISPR/Cas9 techniques, has enormous potential to advance the field of regenerative medicine. The main challenges to the hPSC gene editing field are 1) low cloning efficiencies, 2) severe observed cellular stress, 3) risk of inducing genetic mutations, 4) identification of correctly edited cell populations, and 5) establishment of a healthy, self-renewing clonal cell line.

The CEPT cocktail is a chemically defined small molecule combination of Chroman 1, Emricasan, and Trans-ISRIB (CET), along and a polyamine solution (CEPT), proven to improve cell viability and genomic stability of hPSCs in single-cell and low-density cultures through potent ROCK pathway inhibition, promotion of cell adhesion, maintenance of cellular structure and membrane integrity, and reduction in DNA fracture.

Cytoprotection from the CEPT cocktail provides superior cell survival under stressful conditions, including single-cell passaging, cloning, and gene editing protocols. This research spotlight highlights a 2022 Nature Protocols publication by Tristan et al., outlining a reproducible and scalable workflow for establishing clonal cell lines and a standardized gene editing procedure. The process combines the unique cellular protection from the CEPT cocktail with a streamlined and gentle process for single-cell dispensing into clonal wells through a low-pressure microfluidic platform.

The Spotlight

Research area: hPSC culture, clonal cell line generation, reporter and isogenic cell line establishment, gene editing, hPSC single-cell dissociation, disease modeling, regenerative medicine, cell-based therapies

Cell type(s): hPSCs, hiPSCs, hESCs

Experiment purpose: Efficient hPSC single-cell cloning protocol that provides a low-cost, chemically defined method to generate genetically stable clones, overcomes hPSC's sensitivity to dissociation, and allows significant improvement in gene editing workflows.

Product Highlights

CET Small Molecules

CET small molecules including Chroman 1, Emricasan, and Trans-ISRIB have been found to:

- Promote improved recovery after cryopreservation
- Enhance survival and expansion of dissociated hPSCs
- Provide comprehensive cytoprotection of cells

Experiment overview

CEPT cocktail prevents cellular stress and DNA damage

- The cytoprotective properties of CEPT treatment are fast-acting to mitigate cell stress.
- CEPT treatment maintains proper cell morphology and results in better cell attachment to the culture substrate than treatment with Y27632 or CloneR®.

CEPT improves single-cell cloning efficiency and maintains pluripotency

- Using both FACS-based cell sorting and image-based single-cell printing methods, CEPT treatment increased cloning efficiency when compared with Y27632.
- Clonal cell lines derived using a microfluidics-based platform and CEPT cocktail express pluripotency-associated markers and normal cell growth.

Optimized standardized protocol for generation of hPSC clonal lines

- The protocol reviews thawing and maintaining hPSCs using the CEPT cocktail, as well as a fast and practical approach to single-cell dissociation of non-clonal hPSC cultures to generate a clonal cell line from a single hPSC.
- This protocol presents a standardized solution to overcome current challenges in hPSC technology.

Results

hPSCs are damaged by passaging and dissociation.
Cellular stress and DNA double-strand breaks are more common than previously suspected, even in routine cell passaging. Impairment of cell function and structure, including blebbing, is seen immediately upon dissociation and lasts for several hours.

hPSCs experience a hyperactive ROCK pathway during passaging, inducing apoptosis and anoikis. This yields low cell viability, particularly after single-cell dissociation and/or seeding cells at low densities after passaging. The inherent low viability of hPSCs after single-cell dissociation directly leads to highly inefficient and variable cell cloning assays, making standardization of gene editing protocols extremely difficult.

ROCK (Rho-associated kinase) inhibition can significantly improve cell viability during dissociation by protecting cells from dissociation-induced apoptosis. The demonstration that the ROCK inhibitor Y27632 could greatly improve hPSC cloning efficiency by Watanabe et al. in 2007 was a critical discovery and is still the most widely used method to improve hPSC survival. Recent optimization of hPSC cytoprotection by Chen et al. in 2021 showed that the small molecule Chroman 1 is a much more potent and selective ROCK inhibitor than Y27632. Further optimization studies by Chen et al. revealed that a small molecule cocktail including Chroman 1, Emricasan, and Trans-ISRIB (CET), in addition to a polyamine solution (CEPT), was a superior supplementation strategy to prevent cell dissociation-related stress, yielding improved cell viability and long-term healthy pluripotent cultures over treatment with a ROCK inhibitor alone (Figure 1).

Microfluidic cell sorting in combination with CEPT cocktail enables efficient high-throughput single-cell cloning.
The action of low-pressure cell dispensing can improve viability by minimizing the mechanical forces and cellular stress seen in traditional FACS- or imaging-based cell sorting techniques. The use of a gentle microfluidic cell dispensing platform along with CEPT cocktail supplementation is more efficient than any combination of Y27632, CloneR, and sorting methods tested (Figure 2).

Using CEPT with a microfluidic dispensing platform supports a user-friendly and reproducible high-throughput protocol that can greatly improve gene editing workflows for hPSC researchers.

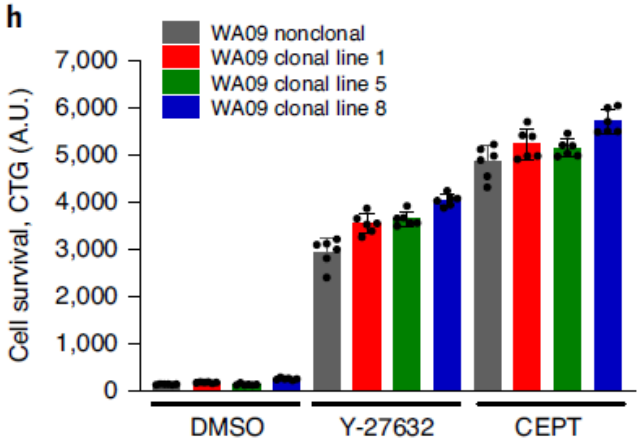

Figure 1. Single-cell survival improves. Data from a luminescent cell viability assay shows improved cell survival after single-cell dissociation of parent and clonal cell lines in the presence of either the ROCK inhibitor Y27632 or the CEPT cocktail. Interestingly, sensitivity to enzymatic cell dissociation is comparable between parent lines and clonal lines generated using CEPT supplementation. Data published from Tristan et al. Nature Methods (Fig. 3, h.).

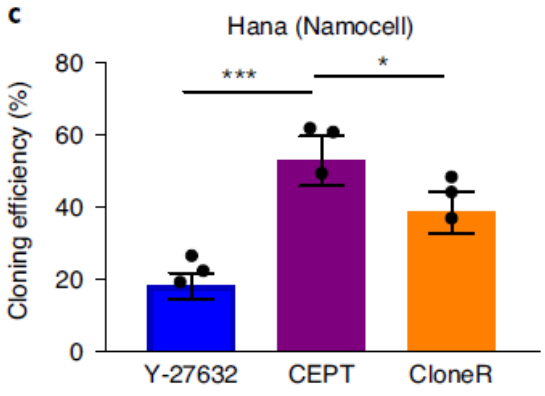

Figure 2. Single-cell cloning efficiency improves. Quantification of hPSC cloning efficiency shows highest efficiency with CEPT supplementation, as compared to treatment with either Y27632 or commercially available CloneR reagent. CEPT-supplemented cloning efficiency is further improved in combination with the gentle microfluidic sorting system, represented by the use of the Hana instrument. Data published from Tristan et al. Nature Methods (Fig. 3, c.).

CEPT supplementation promotes the establishment of healthy single-cell clones.
Clonal cell lines generated using media supplemented with CEPT cocktail and plated with the microfluidic platform were shown to have similar proliferation rates and sensitivity to single-cell dissociation as the parental line (Figure 3). The new clonal lines remain undifferentiated in culture, express expected pluripotency markers, and demonstrate pluripotency through directed differentiation methods. Clonal cell lines generated with CEPT supplementation maintain normal karyotypes, with no detected chromosomal abnormalities or p53 mutations at genomic cancer hotspots.

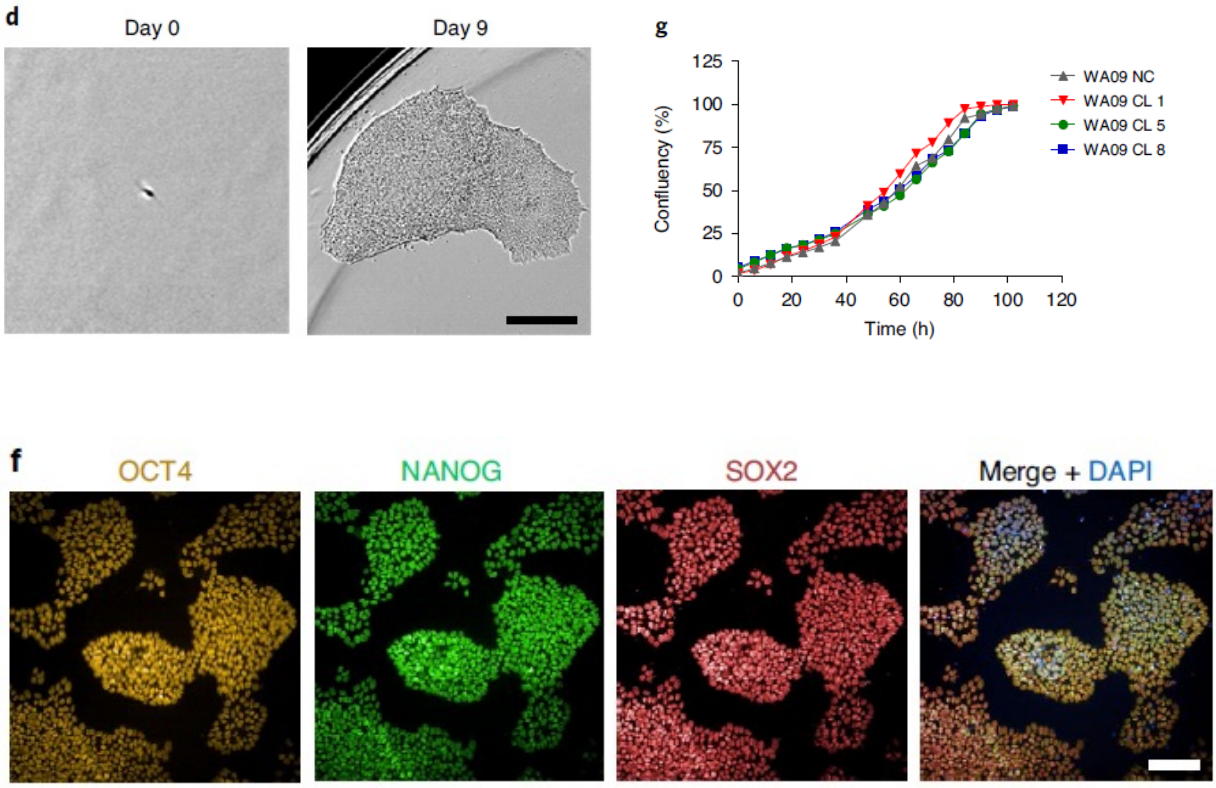

Figure 3. Single-cell cloning of WA09 hPSC using CEPT supplementation and microfluidic cell sorting platform. Phase-contrast images show a single cell after dissociation and the resulting clonal colony (d). The non-clonal WA09 parent line and resulting clonal lines show comparable growth rates (g). ICC images of clonal cell line exhibiting proper pluripotency markers in culture (f). Data published from Tristan et al. Nature Methods (Fig. 3, d, f, g).

Protocol Tips & Highlights

Using chemically defined media and substrates in the culture workflow enables seamless transition of clonal hPSC lines to cGMP applications. Before cloning, ensure the non-clonal parent line is high-quality and experiencing minimal stress in culture by exposing the cultures to CEPT at the time of passage for a minimum of 7 passages prior to experimentation.

Cloning efficiency with the CEPT cocktail can vary between 20% and 80% efficiency, and is largely dependent on variability in cell lines, as some cell lines are inherently more sensitive to single-cell dissociation and cloning procedures. Low cloning efficiencies may be due to slow proliferation rates, increased activation of cell signaling pathways that create a more extreme stress response during cell dissociation, or other variables.

With this protocol, single cells are observed to be attached to the culture substrate within 10 to 15 minutes of plating, and the first successful clones can be seen within the first 2 to 3 days, depending on the parent cell line.

This protocol allows for the establishment of single-cell clonal colonies in 1 to 2 weeks from most parent lines. The cytoprotection provided by CEPT cocktail supplementation generates up to 6-fold higher cloning efficiencies over the ROCK inhibitor Y27632, and 1.5-fold higher efficiencies over the commercially available CloneR reagent.

Conclusion

Single-cell cloning is the most rigorous way to evaluate a stem cell line's true "stemness", and improvements in this technique can allow researchers to establish next-generation clonal lines with improved overall quality, homogeneity, and genomic stability.

The combination of CEPT cocktail supplementation to overcome cellular stress and maintain hPSC structure and function after single-cell dissociation with a gentle microfluidic dispensing technique provides the optimal physiochemical conditions for efficient single-cell cloning of hPSCs and therefore allows standardization and scalability of hPSC gene editing workflows. This protocol ultimately improves the efficiency and utilization of these cells in a wide range of regenerative medicine and disease modeling applications.

References

1. Tristan et al. 2022. Efficient and safe single-cell cloning of human pluripotent stem cells using the CEPT cocktail. Nature Protocols.

2. Chen, et al. 2021. A Versatile Polypharmacology Platform Promotes Cytoprotection and Viability of Human Pluripotent and Differentiated Cells. Nature Methods.

3. Tristan et al. 2021. Robotic high-throughput biomanufacturing and functional differentiation of human pluripotent stem cells. Stem Cell Reports.

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