A Small Molecule Cocktail Promotes Survival Of Human Pluripotent and Differentiated Cells

Yu Chen, Carlos A. Tristan, Lu Chen, Vukasin M. Jovanovic, Claire Malley, Pei-Hsuan Chu, Seungmi Ryu, Tao Deng, Pinar Ormanoglu, Dingyin Tao, Jaroslav Slamecka, Sam Michael, Christopher P. Austin, Anton Simeonov, Ilyas Singeç

National Center for Advancing Translational Sciences (NCATS), Stem Cell Translation Laboratory (SCTL), NIH, Rockville, MD 20850

Abstract

Access to human induced pluripotent stem cells (iPSCs) has created novel paradigms for drug discovery and regenerative medicine. However, poor survival of iPSCs during routine passaging, cell differentiation, and cryopreservation poses major challenges to the establishment of standard operating procedures to produce and store iPSCs on a large scale, and to the development of efficient genome editing protocols based on optimized single-cell cloning procedures. The ROCK inhibitor Y-27632 has been widely used to improve cell survival, but significant amounts of cell death remain evident in many iPSC applications. Here, we developed a four-component small-molecule cocktail named "CEPT" that dramatically improves iPSC viability. Testing 15,333 compounds in quantitative high-throughput screening (qHTS), we first identified 113 hits that improved iPSC survival. Advancing 29 hits to combination screening based on their diverse modes of action, we discovered compound C and compound E as a synergistic pair (C+E) that improved iPSC survival during routine passaging by approximately 50% as compared to Y-27632. Despite the dramatic effect of C+E during routine passaging, only modest improvement was obtained when iPSCs were seeded at a low density (25 cells/cm^2) or in a 1 cell/well condition. Therefore, we designed another combination screening assay to search for additional compounds that, when applied together with C+E, can further enhance iPSC survival at ultra-low cell density conditions. Screening 7,599 compounds using the new assay, we found that C+E together with two additional compounds (compounds P and T) dramatically improved iPSC survival at ultra-low cell density, increasing single-cell cloning efficiency up to ~55% as compared to the ~10% with Y-27632. We then extensively tested CEPT and demonstrated that this cocktail was highly efficient in improving cell survival during routine cell passaging, embryoid body formation, genome editing using CRISPR/Cas9, the establishment of new iPSC lines, and cryopreservation/thawing various differentiated cells. Hence, the versatility of CEPT provides a powerful chemical platform for establishing efficient protocols and may become a widely used approach in cryobiology, drug development, and regenerative medicine.

Materials and Methods

- Fully automated screening: touch and go!
- Throughput: up to 1 million wells/day
- Miniaturization: 1536-well plates capable of 2-8 μL/well
- Reliability: plate to plate variation minimal
- On-line compound storage and access
- High-speed plate imaging

Fig. 1 Quantitative High-Throughput And Matrix Screening.
Acoustic liquid dispensing allows combinatorial matrix screening by mixing multiple compounds in miniaturized 1536-well plates. NCATS has access to libraries with over 500,000 small molecule compounds and quantitative high-throughput (qHTS) screening performed as titration series with full dose-response curves for every library compound (Inglese et al., 2006, PNAS 103:11473-8). This approach results in significantly lower false-negative and false-positive hits.

Results

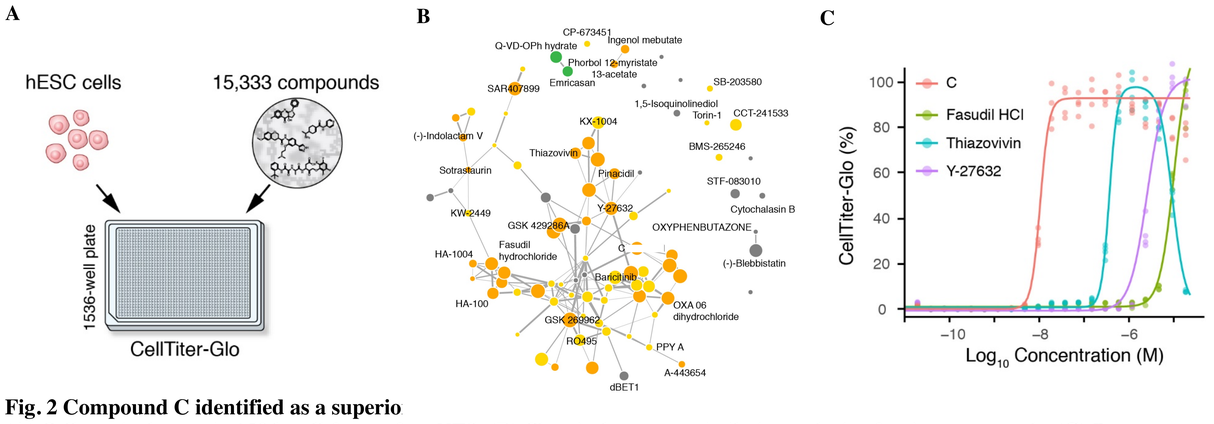

Fig. 2 Compound C identified as a superio
(**A**) Cell survival assay in 1536-well format for qHTS. (**B**) Chemical structure similarity analysis of active compounds. (**C**) Dose-response curves of selected ROCK inhibitors, including C, Fasudil, Thiazovivin and Y-27632. Note the toxicity of Thiazovivin at higher concentrations.

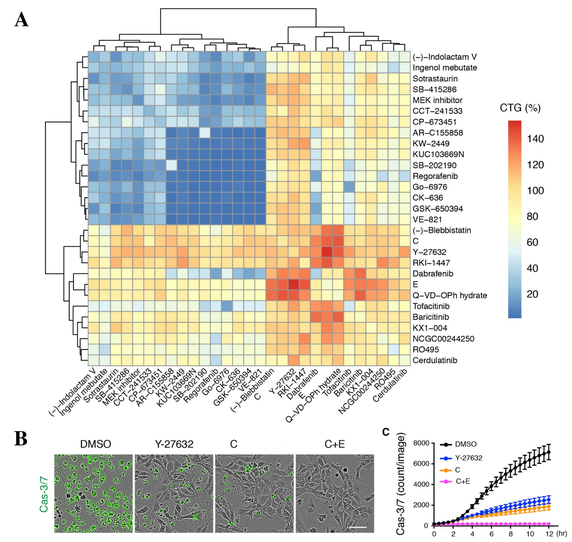

Fig. 3 Combinatorial matrix screen identifies compounds with synergistic activities.
(**A**) Heat-map summarizing the maximum CellTiter Glo (CTG) readings of all-versus-all dose matrices for 29 compounds (812 drug-drug combinations tested in total). CTG readings were normalized to the value obtained with 10 μM Y-27632 representing 100% (control). (**B, C**) C+E improve cell survival and reduce the number of apoptotic cells. hESCs (WA09) cells were dissociated with Accutase and plated on VTN-N in E8 medium. Caspase-3/7 green detection reagent was used to monitor caspase activation.

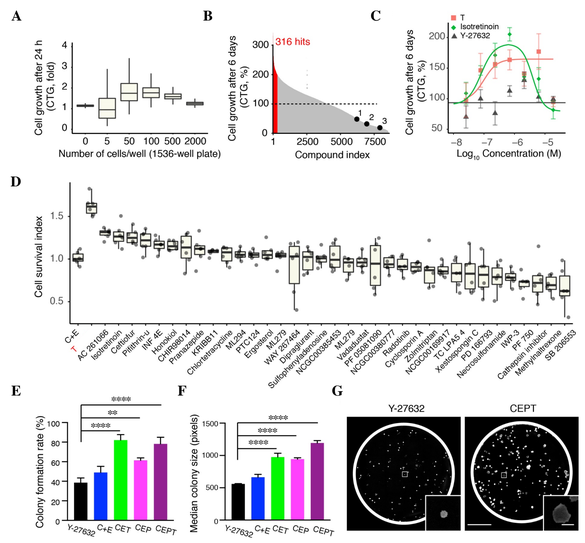

Fig. 4 CEPT promotes clonal growth and expansion of genetically stable hPSCs.
(**A**) Assay development for hESCs (WA09) in 1536-well format to determine optimal survival and growth at different cell densities. (**B**) Summary of qHTS performed at ultra-low cell density (10 cells/well). All compounds (tested at 10 μM in triplicates) were used in combination with C+E, and ranked based on their median CTG readings. Data were normalized to the average CTG reading obtained with C+E. (**C**) Example dose-response curves of T, Isotretinoin, and Y-27632. (**D**) Follow-up screen highlights T among top 36 hits identified from previous ultra-low cell density screen. hESCs (WA09) were plated on LN521 at 25 cells/cm^2 in StemFlex medium with the indicated compounds in addition to C+E. After a 3-day incubation with compounds, cell confluency was quantified using calcein green AM to label live cells on day 6. Data are presented as box plot (n = 6 for each group). Cell survival index represents cell confluency normalized to the C+E group. (**E, F** and **G**) Systematic comparison of small molecule combinations and their effect on colony number and colony size of hESCs plated on VTN-N at 25 cells/cm^2 in StemFlex medium. Quantification was performed 6 days after plating.

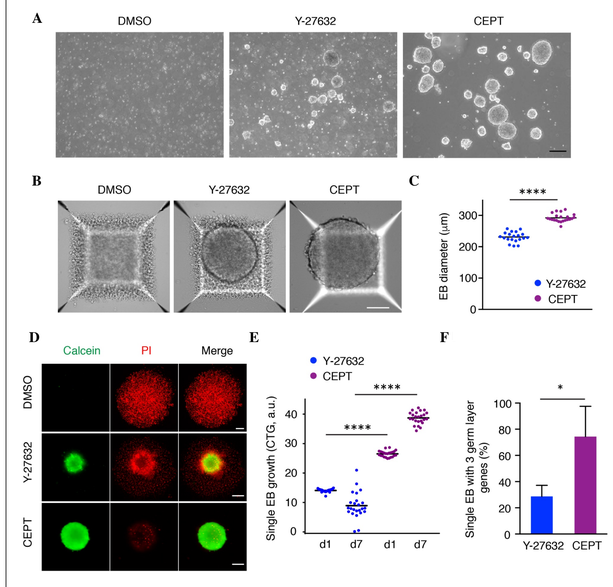

Fig. 5 CEPT improves EB formation and differentiation.
(**A**) EB formation in the presence of DMSO, Y-27632 and CEPT. (**B**, **C**) Single EBs generated in Aggrewell. (**D**) Single EB formation in 96-well ULA plates. Live and dead cells were stained with calcein green AM and propidium iodide (PI). (**E**) Cell survival of single EBs at day 1 and day 7. (**F**) CEPT supports differentiation of single EBs into the three germ layers.

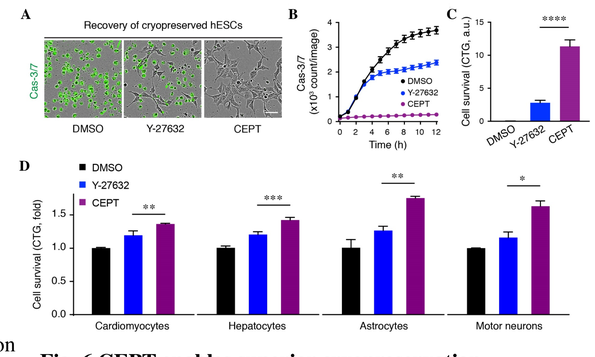

Fig. 6 CEPT enables superior cryopreservation.
Cryopreserved hESCs, iPSC-derived cardiomyocytes, hepatocytes, astrocytes, and motor neurons were thawed. Caspase-3/7 green detection reagent and the CTG assay were was used to monitor apoptosis and quantify cell survival.

Summary

1. We developed a small molecule cocktail named "CEPT" by using qHTS in 1536-well format and combination screening.
2. CEPT is highly efficient in improving cell survival during routine cell passaging, embryoid body formation, single-cell cloning, cryopreservation, genome editing (data not shown), and the establishment of new iPSC lines (data not shown).
3. The versatility of CEPT provides a powerful chemical platform for establishing efficient and clinically-relevant protocols and may become a widely used approach in drug development and regenerative medicine.

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