Journal of Research and Development

Extended Abstract

Euro Biotechnology 2018: Stopping biological time: science and art of biostabilization- Igor L Katkov- US Belgorod National State Research University

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Abstract

Introduction: Human pluripotent stem cells (hPSCs) hold good potential for cell therapy and regenerative medicine and as helpful tools to demonstrate in vitro embryotoxicity. Cryopreservation (CP), storage, and shipment of hPSCs are key elements for eventual clinical applications that will need large numbers of quality-controlled pluripotent stem cells. While effective CP protocols for regular cell lines and even murine embryonic stem cells (mESCs) are well established, this is not the case for hPSCs due to low recovery rates of viable and pluripotent cells following freezing, and the slow hESC growth rate, so the time from thawing to getting cultures suitable for experimentation can be week. This problem is not simply an inconvenience due to extend culture periods exert increased selective pressure on the cell population improving the likelihood of phenotypic variation and/or alterations in potency.

General Materials & Methods: Derivation of hESC-iPSCs - Cells were cultured in Knockout Dulbecco's modified Eagle's medium (KODMEM, Invitrogen, no. 10829-018) supplemented with 1 mM L-glutamine with 20% Knockout Serum Replacement medium (KOSR, Invitrogen), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (NEAA, Invitrogen), 50 U/mL penicillin, 50 μg/mL streptomycin (Invitrogen), 0.1 mM beta-mercaptoethanol (Invitrogen), and 8 ng/mL basic fibroblast growth factor (bFGF, Sigma no. F0291-25UG). hESCs and hiPSCs were grown on Matrigel (growth factor-reduced, BD Bioscience) coated 6-well plates (Corning, Inc. no. 3506) on a feeder layer of primary MEFs from E13.5 CD-1 mice. H9 and hiPSC lines were passaged following enzymatic digestion with collagenase IV (Invitrogen, no. 17104-019) approximately every 7 days. Cells were routinely tested for mycoplasma (MycoAlert; Cambrex, Walkersville, MD).

Generation of H9-Derived Fibroblasts - Undifferentiated H9 cells were subjected to a regular spontaneous discrimination protocol via EB formation. Briefly, collagenase IV-treated hESC colonies are distributed by mechanical pipette trituration into cell aggregates of 500 to 800 cells.

Generation of Human iPS Cells - We applied standard Yamanaka protocol with minor modifications. Original pMXs retroviral vectors encoding for hOct4, hSox2, hKlf4, and hc-Myc were collected from Addgene and pseudotyped with VSV-G envelop protein (a gift from Gerald Pao, Salk Institute). Semiconfluent hdFs were converted overnight with the supernatant containing all four viruses.

Immunochistochemistry - The cells are rinsed with PBS, microphotographed in bright field (BF) on a CKX 41 inverted microscope, and settled with 4% paraformaldehyde (PFA) diluted in PBS for 10 min at room temperature (RT).

Particular M & M and Results: Toxicity of Four Different Cryoprotectants. We earlier hypothesized that DMSO can be intrinsically toxic for hPSCs and have to be substituted with a nontoxic CPA. In a pilot study we identified that ethylene glycol (1,2-ethane diol, EG) given equal protection in cryopreservation of 293 cells so we considered this diol as a best candidate for substitution of DMSO.

We also tested propylene glycol (1,2-propane diol, PG) and glycerol (GLY). The cells were exposed in 10% (w/w) of the CPAs for 30 min at 37°C. Recovery of the CalceinPM+/7AAD-cell is comparatively with in control (QUANTA allows precise measurement of the cell concentration that was converted into total cell yield).

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After that cells are detached and plated as per the routine procedure, and on day 2, before the first change of the medium, the unattached cells were also harvested.

The average attachment efficiency of the untreated control was in a range 65–77%, and practically 95% of the attached iPS cells were Nanogpositive after 2 days of incubation. Cryopreservation of Dissociated iPS Cells - As described above, cell detachment from surfaces containing ECM molecules can cause massive cell death and differentiation. However, from a cryobiological perspective, CP of single cell suspensions is preferable to clusters. We address this issue by adding a Rho-kinase (ROCK) inhibitor Y-27632 (RI in the text) that helps cells withstand detachment and dissociation. Cryopreservation Attached Cells in Plates - Since the standard freezing protocol requires detachment, we hypothesized that the recovery could be improved if cells were not disturbed from their "natural" environments. We thus froze cells in 4-well plates.

Discussion: While many aspects of these developments were used in previously documented protocols; however, the reported results differ widely among labs and cell lines. Our clear distinction and innovation was to use the cumulated data in concert as a whole. Another problem is that a wide variety of the methods of assessment of recovery used in the abovementioned publications makes direct quantitative comparison of different results very difficult if not sometimes impossible.

We have decided not to use any colony assay due to large variability of the size and amount of the colonies that can be plated into a particular well, thus, making the "number of colonies" as an unreliable parameter for quantification. We instead, used the number (% to the total amount of harvested cells) of GFP-positive cells that excluded a negative viability marker 7AAD as the characteristic of the attached cells.

We as well accurately (using QUANTA Coulter counter option) calculated the amount of nonattached and attached cells because it was also impossible to plate the same amount of cells per a well even if the cells were dissociated prior to freezing and plating, and especially if they were frozen in clumps.

That combination of the assessment of the cells that reattached to the surface of the well versus floating non-attached cells and the percentage of Oct4- positive viable (7AAD-) reattached cells will give us a fair assessment of the overall recovery, as in, the yield of viable pluripotent SCs after CP (VY).

Conclusions: Both dissociation of iPSCs with Accutase in the appearance of a ROCK inhibitor and ethylene glycol AND programmable freezing in adherent stage (ComfortFreeze) have drastically improved (up to 6-fold) the yield of pluripotent cryopreserved stem cells is comparatively standard freezing in clumps without ROCK inhibitor. Cryopreservation of adherent cells in plates has also showed a higher efficacy and lower time for restoration of the colonies to the unfrozen level than dissociation with Accutasdeclining traditional sericulture would be redeemed.

This work is partly presented at 21st European Biotechnology Congress on October 11-12, 2018 held at Moscow, Russia