Cryopreservation of Human Embryonic Stem Cells by Cell Alive System (CAS)

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Abstract

Human embryonic stem cells (hESCs) are pluripotent cells with unlimited proliferative potential and have capability to differentiate into all kinds of lineage cells of three germ layers. Up to now, there are three FDA-approval clinical trials undergoing in the US using hESC-derived cells. Due to the nature of hESC growing characteristics, these cells always grow as clumps to maintain their stemness. Therefore, there is always an obstacle in cultivation of hESCs：low survival and recovery rate after cryopreservation.

The Cell Alive System (CAS) is a technology that generates a special magnetic field around a subject material by using pulsed magnetic field, low-frequency wave and several types of weak energy. By Combine the technology with refrigerating process, water molecules within cells or tissues are frozen instantaneously from its super-cooled state. Different from the conventional cryopreservation, the system make cells or tissues frozen in vitrification method and minimize the damage due to crystallisation. Vitrification also can be achieved by applying open pulling straw (OPS) method with non-equilibrium solution. But OPS method is time-consuming, operator-dependent and limitation on small volume. Most scrupled is possible contamination due to the direct exposure to liquid nitrogen

Therefore we expect improvement on the recovery rate of hESCs by CAS. In our study, comparing with conventional slow cooling method, hESCs (TW1) frozen by CAS show obviously higher attachment rate after thawing. We also inspect whether the cells frozen by CAS remain pluripotency. Surface marker assay by flow cytometry and immunofluoroscence suggest the cells express markers of hESCs such as SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. The results of embryoid body (EB) forming assay also reveal the cells can exactly differentiate into different cells of three germ layers in vitro.

Purpose

hESCs and induced pluripotent cells (iPS) are stable cell lines retaining capacity to differentiate into all cells from three germ layer cells. Both of them are conspicuous and expected a lot in clinical medicine and drug discovery. However, these cells frozen by conventional slow cooling method are low recovery rate and high differentiated rate after thawing. Hence a excellent method of cropreservation is ergen to develop.

CAS is a technology invented for returning food to original taste and freshness. Different with conventional slow cooling rate method, CAS add a special magnetic field around materials during cooling process and make materials freeze in vitrification way. Such a remarkable technology may also useful for cryopreservation of bio-materials. Indeed CAS applied in cryopreservation of intact teeth and periodontal ligament cells shows great results.

hESCs grow as clumps and hard to attach or survive if trypsinized to single cells. That's why the low recovery rate of hESCs. The success of CAS on food tissues and cell tissues inspire us. So in this study we applied CAS on cryopreservation of hESCs:TW1 cells and inspect whether the cells maintain stemness after thawing.

Materials and Methods

TW1 cells were used in this study. The components of freezing medium is culture medium with 25% KnockOut Serum Replacement (Gibco, Cat. no.10828) and 5%-10% DMSO. TW1 cells were treated with collagenase for 1-2 hours until the borders of colonies round up. Then collect cells, re-suspend with freezing medium and aliquot into cryovials. TW1 cells frozen by Mr. Frosty were transferred to liquid nitrogen on next day. TW1 cells frozen by CAS were transferred to liquid nitrogen immediately after the freezing procedure. In the process of thawing, TW1 cells were re-suspended with culture medium (w/ or w/o Y27632). Anakaline phosphatase (AP) staining was performed to assay the attachment rates of TW1 cells.

Results and Discussion

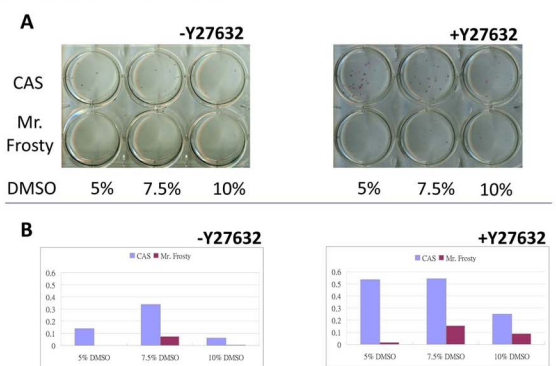

Fig. 1 Comparison of TW1 cells attachment rates after thawing which cryopreservation by Mr. Frosty and CAS.
(A) AP staining of hESC TW1 cells 7 days after thawing. Left : cells re-suspend with culture medium (w/oY27632) ; right : cells re-suspend with culture medium (w/ Y27632)
(B) Statics of attachment rates (%). Left : cells re-suspend with culture medium. (w/oY27632) ; right : cells re-suspend with culture medium (w/ Y27632)

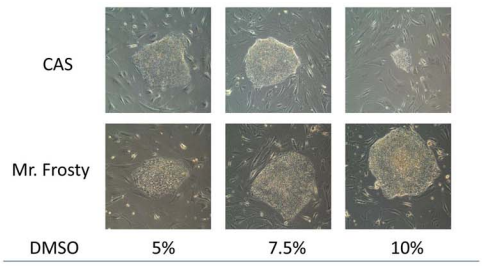

Fig.2 The morphology of TW1 cells after thawing
After freezing by Mr. Frosty or CAS, TW1 still can grow in undifferentiated morphology. The pictures are colonies five days after thawing.

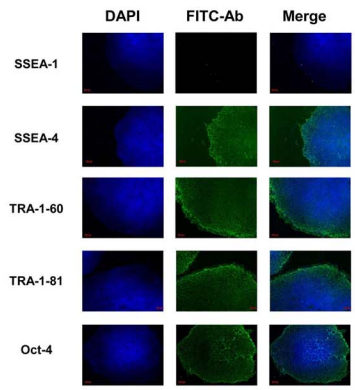

Fig.3 Detection of pluripotent markers by immunofluorescence.
We analyze surface markers of TW1 cells frozen by CAS(7.5% DMSO) and Y27632 added while thawing. The results show high expression of pluripotent markers in surface and nuclear.

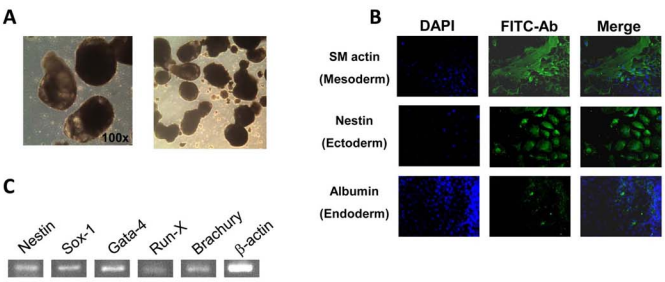

Fig.5 In vitro differentiation of hESC TW1 by EB formation.
(A) EB formation after 7 days suspension culture on ultra-low plate
(B) Immunofluorescence staining of three germ layer markers
(C)RT-PCR reveals the cells express marker s of three germ layers

In this study, hESCs TW1 cells frozen by CAS show improved attachment rate than by Mr. Frosty no matter with or without Y27632 in thawing process. 3th passage after thawing, the cells expressed pluripotent markers such as SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and Oct-4. From the results of EB formation assay, the cells frozen by CAS still can differentiate into cells of three germ layer cells.

Conclusion

The study reveals that CAS is a powerful system on cryopreservation of hESCs (TW1). From IF and EB formation assay, pulsed magnetic field and low-frequency wave etc. extra energy didn't affect differentiated capacity of TW1 cells. We will keep evaluating the characteristics of hESCs for higher passage to confirm the method is feasible.

Reference

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