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CELL SURVIVAL AND PRESERVATION OF SIRNA-MEDIATED PROTEIN KNOCK-DOWN UPON SERUM-FREE CRYOPRESERVATION (-80°C)

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The small G proteins of the Rho family (Rho GTPases) are key operators in the signaling arising from integrins and cell-matrix focal adhesions. They function as binary molecular switches that cycle between an inactive GDP-bound and an active GTP-bound form that in turn activates downstream effectors and a variety of signaling pathways. The best known Rho GTPases, RhoA, Rac1 and Cdc42 control the organization of the cytoskeleton and the focal adhesions-mediated transduction of exogenous and endogenous mechanical signals. Their localization at the cross-road between signaling, mechanical forces and the cytoskeleton along with reported effects of microgravity on cell shape and cytoskeletal arrangement led us to hypothesize that Rho GTPases might be involved in the reception and reaction of cells to gravity. To explore this hypothesis we created stable cell lines, SV40-transformed fibroblasts (WI-26), expressing a constitutively active form (QL) of RhoA, Rac1 or Cdc42 (manuscript in preparation). The reverse situation, i.e. suppression of the GTPases expression was obtained by transfecting small interfering RNA (siRNA) targeting each of these GTPases (Deroanne et al., 2003, 2005).

Experiments using these cells have been selected for the 2nd batch of experiments to be conducted in the Biolab facility on-board of the International Space Station. The deep impact of seric factors on the activity of Rho GTPases together with practical constraints related to the storage of frozen cells, their thawing and dilution on the ISS imposed a cryopreservation of the cells in serum-free conditions at -80°C. Here we describe the cryopreservative potential of various serum-free mediums that allow the persistence of the knock-down of the GTPases by siRNA after freeze-thawing of the cells.

WI-26 cells were transfected by siRNA targeting RhoA, Rac1 and Cdc42 using calcium phosphate precipitate. Naïve cells, cells treated with calcium phosphate alone or a with a scramble siRNA were used as control. After 16 hours the precipitate was washed-out and the cells kept for 48 hours in culture before cryopreservation procedure. Cells were detached from culture dish with trypsin, pelleted by centrifugation and suspended at 10^6 cells/ml in Dulbecco's modified Eagle Medium (DMEM). Aliquots of cell suspension were pelleted, suspended in the indicated cryopreservative mediums, left at -20°C for 40 minutes and stored at -80°C. After the indicated periods of time, cells were thawed, diluted (1/20) in serum-free DMEM complemented or not by 1% bovine serum albumin (BSA), plated on collagen-coated wells and transferred at 37°C. After 4 hours the wells were washed twice with phosphate buffered saline and the number of attached cells was measured by the content of DNA assayed by fluorimetry. The attachment of cells frozen in control cryopreservative medium (Medium A: fetal calf serum (FCS)/ dimethyl sulfoxide (DMSO) (95:5)) was taken as 100%.

In preliminary experiments, wild-type WI-26 cells were frozen in DMEM alone or supplemented with dextran, hydroxyethyl starch (HES), trehalose or DMSO as cryoprotective agents. After 11 days of storage at –80°C in DMEM alone about 25% of cells attached to the support. The addition of dextran (2 and 8%) and HES (2.5 and 5 %) resulted in a lower survival than DMEM alone, while trehalose (200 mM and 1 M) did not affect the cryoprotective effect of DMEM. As expected DMSO added some cryoprotective effect when present at 5%, and to a lesser extent at 10% (not illustrated).

In a second set of experiments, the cryoprotective potency of four mediums were tested on WI-26 (wild-type, expressing Rho GTPase QL or transfected with siRNA). The selected mediums were: DMEM/DMSO (95/5, = Medium B), Medium B containing 5% BSA (w:v, = Medium C), DMEM/ProFreeze (Cambrex)/DMSO (50/45/5, = Medium D) and Medium D containing 5% BSA (w:v, = Medium E). After 50 days at –80°C, cells were thawed and diluted (1/20) in DMEM containing 1% BSA before plating. Similar data (illustrated for WI-26 expressing Rac1 QL in Fig. 1) were obtained with the wild-type WI-26 and the various derived cells. They indicate that the rank of cryopreservative potency is Medium A = Medium C = Medium E > Medium D > Medium B.

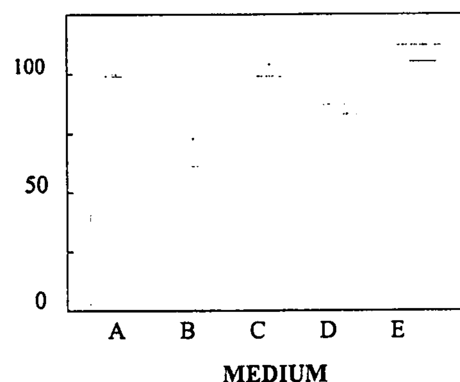

Fig. 1: *Cryopreservation of WI-26 cells in various mediums. WI-26 cells expressing constitutively active Rac1 were frozen at –80°C in various cryopreservative mediums. After 50 days they were thawed, plated onto collagen in serum-free DMEM, 1% BSA for 4 hours and the relative number of attached cells was measured. A: FCS/DMSO (95:5, = control medium); B: DMEM/DMSO (95:5); C: DMEM/DMSO (95:5) + BSA (5%, w:v); D: DMEM/ProFreeze/DMSO (50:45:5); E: DMEM/ProFreeze/ DMSO (50:45:5) + BSA (5%, w:v).*

The cryoprotection offered by Medium C and E was further confronted to that of control medium for a variety of cell types (see Fig. 2). Data show that Medium E offered cryoprotection similar to or slightly better than control medium for the various tested cells except NIH3T3. Medium C was significantly less efficient.

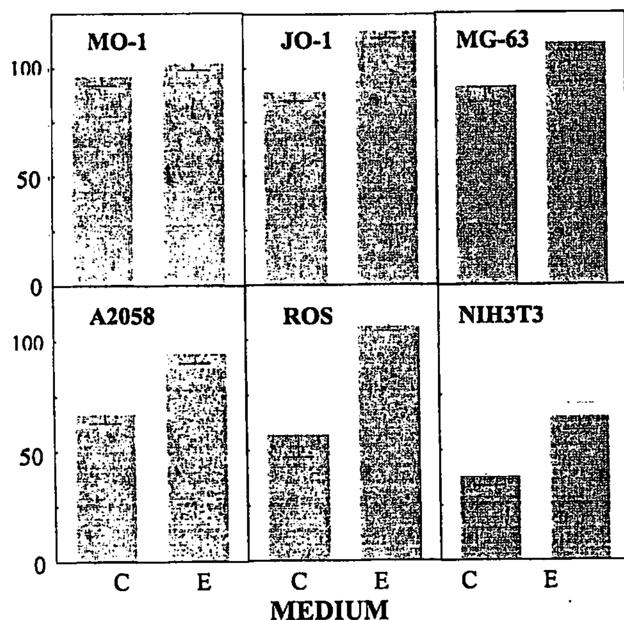

Fig. 2. *Survival of several cell types after cryopreservation at –80°C for 50 days in Medium C and E. Data are expressed as the percentage (mean ± S.D) of attached cells as compared to control medium taken as 100%. MO-1, JO-1: human primary fibroblasts; MG-63: human osteosarcoma cells; A2058: human melanoma cells; ROS; rat osteosarcoma cells; NIH3T3: mouse transformed fibroblasts.*

Finally, the impact of freeze-thawing on the knock-down of the GTPases expression by siRNA was tested. WI-26 cells were transfected by siRNA as previously described (Deroanne et al., 2005) before freezing. After 50 days of storage, cells were thawed, harvested in DMEM containing 1% BSA, plated for 24 hours and then lyzed in Laemmli buffer. The expression of the GTPases was measured by Western blotting using antibodies specific for the three GTPases or against ERK1/2 as a control of protein loading. Transfection with the cognate siRNA reduced the expression of RhoA and Cdc42 by about 90 % and that of Rac1 by about 70 % , i.e. to levels identical to those observed in the cells before freezing, regardless of the cryopreservative medium used (illustrated for Rac1 and Cdc42 in cells frozen in medium C, Fig. 3).

In conclusion these data demonstrate the feasibility to store cells frozen in serum-free cryopreservative medium at –80°C on Earth and to plate them after dilution with serum-free medium on ISS. Moreover, they show that siRNA-directed knock-down of proteins is retained in these conditions.

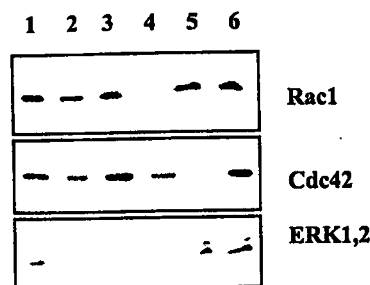

Fig. 3: *Knock-down of Rho GTPases expression by siRNA is effective and retained after storage at –80°C. Cells were left untreated (1), mock-transfected (2) or transfected with siRNA against RhoA (3), Rac1 (4) or Cdc42 (5) or a scramble siRNA (6). After two days the cells were frozen in medium C for 50 days, thawed, plated and harvested after 24 hours. Total cell extracts were run on SDS-polyacrylamide gels and blotted to membranes probed with anti-Rac1, anti-Cdc42 or anti-ERK1,2 antibodies.*

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