Influence of Physical and Chemical Treatments on Cell Survival and Acquisition of Pluripotency

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Abstract

Pluripotent human embryonic stem (ES) cells represent a promising tool for research and regenerative medicine. Their use as standard research material is hindered by several ethical problems regarding the use of embryos and by their limited sources. Reprogramming techniques allow to obtain large quantities of patient-specific pluripotent cells from fully differentiated and functional somatic cells, overcoming both ethical and immune-rejection issues, and presenting new unlimited sources of material for research and therapies. Recently, new data reported the reprogramming of murine somatic cells into a state of pluripotency through the induction of external strong stimuli, such as the exposure to an acidic solution and/or physical stress. The aim of this study was to reproduce the accomplished results on different mouse cell types, and also to optimize the described procedure to accomplish a reprogramming event using human cells. Several different types of murine somatic cells were directly isolated and exposed to three consecutive physical trituration steps with increasingly smaller lumen pipettes, followed by 30 minutes exposure to an acidic solution. The same procedure was adapted to both mouse and human embryoid bodies (EBs) grown in the lab. Cell survival and resistance was monitored during the experimental steps. The results obtained showed incapacity of the described technique to induce pluripotency in all the studied different types of cells. The challenging protocol and prolonged exposure of the cells to harsh conditions resulted in constant cell death or damage during the exposure to external stimuli, and consequent deficient cell culture. The possible reprogramming of somatic cells into pluripotency through this simple and fast method would represent a remarkable improvement in the use of stem cells-based therapies.

Keywords: Pluripotency, Differentiation, Somatic Cells, Embryoid Bodies, Reprogramming, External Stimuli

Introduction

ES cells are pluripotent cells isolated from the inner cell mass (ICM) of the pre-implantation stage embryo and can be expanded *in vitro* under pluripotency-supportive conditions[1,2]. These cells are characterized by two remarkable features: their proliferative capacity, allowing unlimited self-renewal, and their pluripotency, being capable to give rise to every cell type of a fully developed adult body[3]. Human ES cells, since they were firstly isolated from humans[4], represent a valuable promise for cell-based therapies, drug discovery, disease modeling and pharmaceutical applications. Through the generation of specialized cells, ES cells can be a promising source for cell replacement therapies, restoring damaged tissues[5]. The use of ES cells for research and therapies faces several ethical issues regarding the use of embryos[6]. Moreover, the limited quantities of stem cells available and the immune rejection following a transplant represent common issues that impair the development of stem-cell based therapies.

Reprogramming techniques largely changed the use of stem cells, enabling the generation of undifferentiated patient-specific ES-like cells. The induction of a pluripotent state through exposure to extrinsic pluripotency-related transcription factors[7,8] enables the generation of patient-specific induced pluripotent stem (iPS) cells. This technique overcomes ethical and immune-rejection issues, is not dependent on any embryo-related material, and enables the generation of an unlimited amount of undifferentiated cells. This reprogramming method is particularly simple, since iPS cells can be generated in any laboratory[9]. However, the use of retrovirus to expose the cells to the extrinsic factors may lead to several safety-related issues, impairing the use of these cells for therapies. New reprogramming methodologies, as well as iPS cells-based method improvements using fewer transcription factors, are of upmost importance.

Recently, a new reprogramming technique denominated stimulus triggered acquisition of pluripotency (STAP) claimed the possibility of reprogramming fully differentiated and functional murine somatic cells into a state of pluripotency

following induction of a strong external stimulus, such as exposure to an acidic solution for 30 minutes[10]. The resulting undifferentiated cells had all the pluripotency-related characteristics of mouse ES cells, showing capacity to construct an entire embryonic structure in a tetraploid complementation assay. Interestingly, it was even suggested that other stresses, especially physical stress by membrane trituration, may play an important role in the reprogramming phenomenon.

The main goal of the present work was to try to accomplish the same reprogramming event using human differentiated cells. The successful acquisition of pluripotency in human cells following this simple and quick procedure would present a great value to the use of pluripotent stem cells in general medicine. A completely adapted protocol following the generation of differentiated cells (through EBs' formation) was followed, and cells from the human UGENT11-2 and reporter H1 cell lines, as well as mouse ES cells, were tested. Moreover, it was attempted to accomplish the same results claimed by Obokata by adapting the defined protocol to several murine cells directly isolated (Granulosa and cumulus cells (GCs) and mouse tail tips (TTs) cells) or expanded *in vitro* (mouse embryonic fibroblasts (MEFs)). Due to the technically challenging protocol that resulted in cell death or damaged in most of the experimental works performed, it was decided to monitor cell progression after the main steps to evaluate the influence of each perturbation to the described observations. During the whole time that the present work was conducted, not a single laboratory around the world was able to reproduce the reprogramming event, placing Obokata's work under investigation, and remaining the uncertainty if the reprogramming technique could work.

Materials and Methods

Mouse embryonic fibroblasts seeding and growth. CD1 MEF cells were thawed at passage 3 and cultured in MEF media containing DMEM 1X (Invitrogen cat. 41965-039), Fetal Bovine Serum (10% (v/v), Invitrogen cat. 10270-106), L-Glutamine (1% (v/v), Invitrogen cat. 25030-024) and penicillin/streptomycin (1% (v/v), Invitrogen cat. 15140-122), in T75 flasks (VWR cat. 734-0046). As feeder layer, MEFs were inactivated (using Mitomycin C (SIGMA-ALDRICH cat. M4287)) and seeded in T25 flasks (VWR cat. 734-0044) (pre-coated with 0.1% gelatin (SIGMA-ALDRICH cat. G1890)).

Stem cells culture and expansion. Human UGENT11-2 ES cell line was on passage 50 when culture and expansion started. Cells were expanded until passage 53 (EBs were grown for EB1), passage 56 (EB2) and passage 59 (EB3). Cells were cultured in human ES cell media containing KnockOut DMEM (Invitrogen cat. 10829-018), KnockOut Serum Replacement (20% (v/v), Invitrogen cat. 10828-028), L-Glutamine (2.5% (v/v)), MEM non-essential amino acids (1% (v/v), Invitrogen cat. 11140-035), penicillin/streptomycin (1% (v/v)), β-mercaptoethanol (0.2% (v/v), Invitrogen cat. 31350-010) and basic FGF (0.008% (v/v), Peprotech cat. 100-18B). For cell passaging, cells were detached from MEF feeder cells using 3ml/T25 flask of collagenase type IV solution (100mg collagenase type IV (Invitrogen cat. 17140-019) in 100ml KnockOut DMEM) and colonies were cut using sterile glass beads (approximately 5-6 beads/flask). Human H1 reporter cell line was cultured and passaged using the same methods described above, and cells were expanded until passage 107, when EB's differentiation for EB4 started. Both cell lines were expanded in T25 flasks, using MEFs as feeder cells. Cells were refreshed every two days and split according to cell density observed (usually split every one week). Mouse ES cells were expanded from passage 38 until 40, when EBs' culture (EB5) was initiated. These cells were cultured in 2i/LIF media containing base N2B27 medium (DMEM/F12 (Invitrogen cat. 31331-028) and Neurobasal media (Invitrogen cat. 21103-049) in equal quantities, 1% (v/v) N2 supplement (Invitrogen cat. 17504-044), 2% (v/v) B27 (Invitrogen cat. 17504-044), 1% (v/v) MEM non-essential amino acids, 1% (v/v) L-glutamine, 1% (v/v) penicillin/streptomycin, 0.2% (v/v) β-mercaptoethanol and 0.5% (m/v) BSA (Calbiochem cat. 12657-5)) supplemented with mouse LIF (1000U - 1µl/ml, SIGMA-ALDRICH cat. L5158-5UG) and the two inhibitors PD0325901 1µM (1µl/ml, Cayman cat. 13034) and CHIR99021 0.3µM (0.6µl/ml, Axon Medchem cat. 1386) on gelatin coated T25 flasks. Cells were passaged using conventional trypsin-EDTA method (0.05% trypsin-EDTA, Invitrogen cat. 25300-054).

Embryoid bodies differentiation. Previously described human stem cell lines were re-suspended in differentiation media containing KnockOut DMEM, Fetal Bovine Serum (20% (v/v)), L-Glutamine (1% (v/v)), MEM non-essential amino acids (1% (v/v)), Penicillin/streptomycin (1% (v/v)), β-mercaptoethanol (0.2% (v/v)). Differentiation culture lasted 14 days. Cells were cultured in ultra-low attachment 24 wells plate (SIGMA-ALDRICH cat. CLS 3473-24EA) and refreshed every 2 days from day 0. For EB1 and EB2 experiments, EBs were grown in 6 well of the 24 well plate. For EB3 and EB4, 9 wells of a 24 well plate were used. For EB5, mouse ES cells were differentiated into EBs by culturing them in the previously described N2B27 media, without the addition of LIF and the two inhibitors. Cells were cultured in a 24 wells plate. The first refreshing should be performed by collecting all the cells in a 15ml Falcon tube (VWR cat. 734-0450), letting the cells settle at room temperature for 10-15 minutes and finally removing the old media and adding fresh differentiation media. In the following refreshing days, old medium was removed directly from the wells, adding the same volume of fresh differentiation media. Each well had a total volume of 1ml of medium.

Physical and chemical stresses: experiments with EBs. The protocol was technically based on the information published by Obokata et al (2014). Five main experiments using EBs growth were performed: three with human UGENT11-2 cell line (EB1, EB2 and EB3), one with human H1 reporter cell line (EB4), and a final experiment with mouse ES cells (EB5). EBs were recovered in a 15ml Falcon tube (in EB5 were collected in a 50ml Falcon tube (VWR cat. 734-0453)) and were

centrifuged at 750 rpm for 5 minutes (Eppendorf cat. 5804R). Supernatant was aspirated and cells were re-suspended in 3ml 0.05% trypsin-EDTA in EB1 and EB2 experiments and alternatively in 0.25% trypsin-EDTA (Invitrogen cat. 25200-056) in EB3, EB4 and EB5 experiments. Following incubation at 37ºC for 5 minutes (8 minutes in EB2), and agitating the tube every minute (except EB1), trypsin-EDTA was neutralized by adding 6ml of differentiation media (6ml of N2B27 in EB5 experiment) and the cell suspension was centrifuged at 1200 rpm for 5 minutes. Cells were re-suspended in 2ml (3ml in EB5) of neutral HBSS (Invitrogen cat. 14170-112) at a concentration of $2x10^5$-$3.3x10^6$ cells/ml and the cell suspension was transferred (except in EB1) to a smaller 5ml tube (VWR cat. 734-0436) to avoid attachment of the pipette tips to the wall of the 15ml tube. A concentration of $1x10^6$ cells/ml was suggested by Obokata et al (2014). Due to limited quantity of cells available, EB1 and EB2 did not reach such concentration. Physical stress started with a first pipetting step, using an aspirator tip (1.5mm, Hilgenberg pasteur pipettes) attached to the tip of a 5ml sterile Falcon graduated pipette (VWR cat. 734-0350). Cell suspension was triturated in and out for 5 minutes, with special attention for the cell suspension not to touch the attached 5ml tip, and pre-coating the aspirator pipette with neutral HBSS. A second pipetting step was performed using a Yellow Stripper Tip (135µm, Origio cat. MXL3-135). Cell suspension was pipetted for 10 minutes, followed by a third pipetting step using a tip with approximately 60µm diameter pre-prepared in the lab. Cell suspension was pipetted for 15 minutes. After being centrifuged at 1200 rpm for 5 minutes, cells were re-suspended (EB1, EB2, EB3 and EB4 in 1ml, EB5 in 4ml) in acidic HBSS at a concentration of $3x10^5$-$2x10^6$ cells/ml (neutral HBSS was titrated with 1M HCl (SIGMA-ALDRICH H9892) until pH of 5.4). A concentration of $2x10^6$ cells/ml was suggested by Obokata et al (2014). Due to limited quantity of cells available, only EB5 reached that concentration. Cells were incubated at 37ºC for 25 minutes and centrifuged at 1200 rpm for 5 minutes. Cells were re-suspended (EB1 and EB2 in 1ml, EB3 and EB4 in 2ml, and EB5 in 12ml) in sphere media (DMEM/F-12, 1% (v/v) penicillin/streptomycin and 2% (v/v) B27), at a concentration of 1-$3.5x10^5$ cells/cm^2. A culture concentration of $1x10^5$-$1x10^6$ cells/cm^2 was suggested by Obokata et al (2014). In the experiments involving human cells (EB1-4), the media was supplemented with heparin (0.2%, Stem Cell Technologies cat. 07980), EGF (20ng/ml, Peprotech cat. 100-15) and bFGF (20ng/ml), whereas in the experiment involving mouse cells (EB5 and following experiments with somatic cells), the media was supplemented with heparin, EGF and mouse LIF (1000U, 1µl/ml). Cells were cultured in ultra-low attachment 24 wells plate, using the convention of 1ml/well.

Mouse tail tips experiments (TTs1 and TTs2). B6D2/F1 hybrid strain of mice was purchased from Charles River Laboratories (Brussels, Belgium). All animal experiments were approved by the Animal Ethics Committee of the Ghent University Hospital, Belgium (ECD No. 12/61). Two experiments using mouse tail tips were performed. In the first experiment (TTs1), two tail tips were used. In the second experiment (TTs2), the amount of tail tips used was increased to six. The protocol followed the same steps for both experiments. Tail tips were cut from freshly killed mice. These tips were kept in 1X PBS (prepared from a dilution of 10X PBS (Invitrogen cat. AM9624)) until they were used. Tail tips were taken out from the buffer, placed in an organ culture dish (VWR cat. 734-0961) and covered with collagenase type IV solution. Tissue was scrapped and minced until a gelatinous tissue was obtained, to release the cells to the solution, resulting in a more opaque liquid. To facilitate this procedure, hair was first removed and tail was cut in the middle. The cell suspension (gelatinous tissue) was transferred to a 15ml Falcon tube and agitated at 37ºC and 90rpm in the warm water bath. A certain volume of neutral HBSS (double the collagenase volume) was added in to neutralize collagenase, and the first pipetting step was started. Hairs, skin and the rest of the tissue were maintained in the cell suspension during this first pipetting step in order to allow the release of cells still attached to the tissue. Attention must be paid in order not to clog the tip with the tissue debris. Cell suspension was then filtered through a 100µm cell strainer (Falcon cat. 352360), washing once with 5ml DPBS (-/-) (Invitrogen cat. 14190-094). Following a 5 minutes centrifugation at 1200 rpm, cells were re-suspended in 3ml of neutral HBSS at a concentration of $1x10^6$ cells/ml. Cell suspension was transferred to a 5ml tube, and the protocol followed the exact same steps already described, starting from the second pipetting step. Cells were suspended in acidic HBSS at a concentration of 1.5-$1.6x10^6$ cells/ml, and cultured at a concentration of 2-$2.2x10^5$ cells/cm^2.

Mouse granulosa cells experiments (GCs1 and GCs2). The cumulus and granulosa cells were collected from B6D2/F1 mice. 6-14 weeks-old female mice were superovulated by intraperitoneal injection of 7.5IU equine chorionic gonadotrophin (eCG, Folligon, Intervet, Oss, the Netherlands) followed by 7.5IU human chorionic gonadotrophin (hCG, Chorulon, Intervet) at an interval of 46-48 hours. Females were kept with males after the second injection. Zygotes were recovered, from the swollen ampulla, 21hrs post-hCG injection. The cumulus zygote complexes were briefly incubated in 200IU/ml hyaluronidase (type VIII) to free the cumulus cells. The GCs were used for this project. Two experiments using GCs were performed. In the first (GCs1) experiment, GCs from two mice were used, whereas in the second (GCs2) experiment, GCs from six mice were subjected to the protocol. Cells were centrifuged at 1200 rpm for 5 minutes, and re-suspended in 2ml of neutral HBSS, at a concentration of 5-$5.5x10^5$ cells/ml. Following transfer of the cell suspension to a 5ml tube, the protocol followed the exact same steps already described, starting from the first pipetting step. Cell suspension in acid had a concentration of $4.2x10^5$ cells/ml, and cells were cultured at a concentration of 1-$1.6x10^5$ cells/cm^2.

Mouse Embryonic Fibroblasts experiments (MEFs1 and MEFs2). Two experiments (MEFs1 and MEFs2) were performed with MEFs commonly grown to be used as feeder cells for human ES cells culture. These MEFs were grown in T75 flasks as described, being one flask used for the first experiment and three flasks used for the second trial. The protocol for either the experiments was exactly the same. MEFs, grown in MEF medium were washed once with DPBS (-/-) and incubated for 5 minutes (37ºC) in the presence of 3ml/flask of 0.25% trypsin-EDTA. Following incubation, trypsin was neutralized by 6ml of MEF medium (double the volume of trypsin) and cell suspension was transferred to a 15ml Falcon tube (in the second experiment, cell suspensions were divided into two 15ml Falcon tubes). Cells

were centrifuged at 1200 rpm for 5 minutes and re-suspended in 3ml of neutral HBSS at a concentration of $0.8x10^6$ cells/ml (MEFs1) and $1.3x10^6$ cells/ml (MEFs2). The protocol followed the exact same steps already described, starting from the first pipetting step. Cells from MEFs1 showed low density, and were suspended in 1ml of acidic HBSS at low concentration, whereas cells from MEFs2 were suspended at a concentration of $1.4x10^6$ cells/ml acid. Cells were cultured at a concentration of $1\text{-}5x10^5$ cells/cm^2. A negative control was performed, in which MEFs were directly cultured in sphere media at a concentration of $5x10^5$ cells/cm^2.

Cell's Culture. Human cells were cultured in sphere media supplemented with bFGF, heparin and EGF, whereas mouse cells were cultured in sphere media supplemented with mouse LIF, heparin and EGF. Cells were cultured for 7 days and at day 1 extra media (1ml) was added to each well. From day 2 until day 7, cells were refreshed every day by removing 1ml of old media from the wells with a 1ml graduated pipette (VWR cat. 612-3707) and adding 1ml of fresh media. Cells from the EB5 experiment, cultured in 12 wells, showed high cell density at day 1 of culture. All the cells were collected, centrifuged at 750 rpm for 5 minutes, re-suspended in 24ml of media and cultured in the whole 24 wells plate. Cell density was high in the following days. Excess cells were removed every day from day 2 to day 7, by mixing and removing 1ml of media (including cells), and adding 1ml of fresh media. In all the experiments, each well was pipetted constantly and gently for 5 minutes using a 1 ml sterile Falcon pipette. 1000U of mouse LIF was added every day, from day 2 to 7, to the experiments involving mouse cells.

Immunostaining analysis. Immunostaining samples were taken after 7 days of culture, and fixed in coverslips (VWR Microscope cover glass cat. ECN 631-1578). For this purpose, coverslips were pre-coated with a 17.5% collagen type I solution, composed by 1/4 of a 70% collagen solution (collagen type I (Corning cat. 354249) diluted on deionized water (SIGMA-ALDRICH cat. 38796-1L)) and 3/4 of a 60% ethanol solution (absolute ethanol (VWR cat. 20816-298) diluted on deionized water). Coverslips were inserted in the wells of a 4-wells plate (VWR cat. 734-2176), covered with 100-150µl pre-defined collagen solution and left to dry overnight at room temperature. Next day, a sample containing cells to analyze (around 200µl) was placed on top of the pre-coated coverslip and left to dry in the incubator (37ºC) for 3-4 hours, in order to attach cells to the coverslip. Coverslips were washed once with 1X PBS and placed in 4% paraformaldehyde (SIGMA cat. F1635) solution, pre-warmed to room temperature for 20 minutes. Samples were washed twice with 1X PBS for 5 minutes and covered by a permeable solution (1X PBS with 0.1% Triton X100 (Sigma cat. T8787)) for 5 minutes. Following two more washes in 1X PBS (5 minutes), coverslips were covered by a blocking solution (1X PBS with 0.05% Tween (Sigma P2287) and 1% BSA) for 1 hour. Coverslips were then placed in drops of 50µl of primary antibody solution containing primary antibodies for *Oct4* and *Nanog* genes (see next sub-section), and left in the 4ºC fridge overnight, properly covered. Next day, coverslips were washed twice with 1X PBS for 5 minutes and placed in secondary antibody solution, and left in the dark for 1 hour at room temperature. This was followed by a double wash in 1X PBS, after which coverslips were placed in glass slides (Marienfeld cat. 1000000 76x26x1mm) on a drop of DAPI (Vector laboratories - Vectashield mounting medium with DAPI-H1200) and stored in the dark at 4ºC until posterior analysis. For EBs' staining, EBs from mouse ES cells, cultured for 14 days, were washed once with 1X PBS, fixed with 4% paraformaldehyde (at room temperature) for 20 minutes and dehydrated with methanol (10 minutes in 25% methanol, 10 minutes in 50% methanol, 10 minutes in 75% methanol, 10 minutes in 90% methanol and 10 minutes in 100% methanol). All the solutions were prepared by diluting 100% methanol (SIGMA-ALDRICH cat. 34860-1L) with *PBT* solution (1X PBS with 0.1% Tween). EBs were then stored at -20ºC in 100% methanol until further use. After rehydration (same set of methanol solutions in opposite order), EBs were washed for 10 minutes twice with *PBT* and washed for 15 minutes once in *TBST* (1% PBS with 0.1% Triton X-100). EBs were then transferred to a blocking solution (*TBST* with 0.5% BSA) and kept overnight at 4ºC. Next day, EBs were placed in the primary antibodies' solution (for *Oct4* and *Nanog* genes) and kept for 2 days at 4ºC in a rotating plate. EBs were washed with *TBST* five times for 6 minutes each, rotating, after which they were transferred to secondary antibodies solution and kept overnight, at 4ºC, rotating, and in the dark. Glass slides were prepared with a small droplet of DAPI on top of them. Finally, EBs were washed again for five times (6 minutes each) with *TBST* and transferred to the drops of DAPI previously placed in the slides (around 4 to 5 EBs per drop of DAPI) and a coverslip was placed on top of the drop to press and fix the sample in the slide. Samples were kept in the dark at 4ºC until further use.

Antibodies solutions. Two pluripotency genes were analyzed: *Oct4* and *Nanog*. The primary antibodies' solutions, used both for cell suspension and antibodies analysis, contained the primary antibodies anti-goat *Oct4* (Santa Cruz Biotechnology) diluted 1:200 and anti-rabbit *Nanog* (R&D Systems) diluted 1:200. Dilutions were made with blocking solution, which varies if cell's suspensions or EBs were being analyzed, as shown before. Secondary antibodies' solutions contained the secondary antibodies *Oct4* donkey anti-goat (stained with FiTC: green, Bioconnect) diluted 1:100 and *Nanog* donkey anti-rabbit (stained with cy3: red, Bioconnect) diluted 1:500. Again, dilutions were made in blocking solution, which varied if cell's suspensions or EBs were being stained.

qPCR analysis. Samples to be analyzed by quantitative polymerase chain reaction were collected and re-suspended in 1ml TRIzol (Invitrogen cat. 15596-026) and frozen at -80ºC until further use. RNA extraction was performed using the RNeasy Mini Kit (Qiagen cat. 74106), followed by cDNA generation using the iSCRIPT Advanced cDNA Synthesis Kit (Biorad cat. 1708843). Quantification was performed using the Qubit ssDNA Assay Kit (Invitrogen cat. Q10212) and gene expression analysis was done using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The examination included the pluripotency associated markers for *Nanog*, *Gapdh*, *SSEA1*, *Rex1* and *Gbx2* (for the mouse samples) and markers for *OCT4*, *NANOG*, *SSEA1*, *GAPDH* and *B2M* (for the human samples). A mastermix, comprising gene-specific primers and either iTAQ Universal SYBR Green Supermix (Biorad cat. 172-5122) or iTAQ Universal Probes Supermix (Biorad cat. 172-5133) was prepared. All the samples were analyzed in triplicates with a total final reaction volume of 25µl, including 10ng cDNA template

(previously generated) and 20µl of mastermix. Real time quantitative polymerase chain reaction was performed using the following thermic cycles: 2 minutes at 95ºC, 45 cycles at 95ºC for 15 seconds and 1 minute at 60ºC. The normalization of the CT values was done against housekeeping genes (*GAPDH* and *B2M*) and the ΔΔCT method was used to calculate the fold change (comparison with control samples for each sample: mouse ES cells for the mouse samples and human H1 ES cells for the human samples).

Results and Discussion

Cell survival during the experiments

Experiments with embryoid bodies. The first two experiments conducted with EBs from human UGENT11-2 cell line (EB1 and EB2) showed inefficient cell dissociation and disruption of the EBs' structures, thus resulting in lower cell density to start the physical stress (only EB2 reached $1x10^6$ cells). Low cell density was obtained following each step of the protocol (data not shown), resulting in deficient post-stresses cell culture. Moreover, the incomplete EBs' structure disruption resulted in big mass bulks that, in addition to retain most part of the cells, interfered with the physical stress imposed to the cells. Contrastingly, the use of 0.25% rather than 0.05% trypsin-EDTA in the following experiment (EB3) resulted in significantly more efficient EBs' disruption and cell's release, thus resulting in later exposure to acidic HBSS at a proper concentration ($1.5x10^6$ cells/ml). The same procedure was used for the following experiments: EBs from human reporter *OCT4-GFP*$^+$ H1 cell line (EB4) and EBs from mouse ES cells (EB5), both resulting in proper dissociation of the EBs into single cells. Cell density remained at proper levels (data not shown) after each step of the protocol performed in these last three experiments (for EB5, cell density remained at extremely high values). Acid treatment was shown to be responsible for a slight decrease in EB3 experiment's cell density, whereas EB4' cell density was slightly reduced after the second pipetting step.

***Experiments with murine somatic cells*.** The first experiment (GCs1) with GCs made use of cells isolated from 2 mice, whereas the second (GCs2) used cells from 6 mice. The amount of cells isolated from this later experiment was interestingly similar to the first one. Generally, cell density was reduced through the experiment, being particularly sensible to the acid treatment, and cell density after GCs2 was lower than after GCs1 (data not shown). Globally, GCs were shown to be present in sufficient but random quantities in both experiments, since the experiment using 6 mice yielded a similar amount of cells as compared to the use of 2 mice, suggesting it to be difficult to isolate GCs in both high and controlled quantities. The huge cell loss verified though the experiment resulted in cell culture at a low concentration ($1\text{-}1.6x10^5$ cells/cm^2), as compared to the optimal range of ($1x10^5\text{-}10^6$ cells/cm^2) suggested by Obokata et al (2014). Therefore, for proper further experiments, a significantly higher amount of starting material would be needed, resulting in both economical and ethical implications, associated with the necessity of a high amount of mice.

A first experiment with mouse tail tips (TTs1) used tail tips from 2 mice, whereas the second experiment (TTs2) used 6 tail tips. Cell density was drastically reduced after acid treatment (data not shown), suggesting particular fragility of these cells to acid exposure. During the second experiment, cell density was tracked after every pipetting step, identifying the second and third trituration steps as responsible for some cell loss. Nevertheless, exposure to acidic HBSS confirmed the low resistance of these cells to this treatment, resulting in high cell loss. The significantly different size of the first pipette (1.5mm) as compared to the following ones (135 and 60µm) may have contributed to the observed cell response. Introduction of an intermediary pipetting step, used as a cross-over to the low lumen pipettes, could help to prepare the cells for the severe pipetting. Alternatively, increasing the diameter of the second and third pipettes could prevent the cell loss observed.

Both the first (MEFs1, using 1 T75 flask) and the second (MEFs2, using 3 T75 flaks) experiments with MEFs resulted in high cell loss after the first pipetting step. Cell density was maintained during the following physical stress, and suffered a significant decrease after the acid exposure. The second experiment showed globally higher amounts of cells, and cells were cultured at a concentration five times greater ($5x10^5$ cells/cm^2). To minimize cell loss, a third experiment was conducted, exposing MEFs to physical stress only. This experiment yielded results similar to the ones from MEFs2. Acid treatment on cells can be considered as a severe method that leaves few chances of acquiring a proper functional cell. By disrupting membrane proteins' structure due to pH changes, breaking their supportive ionic bonds, the presence of an acidic environment leads the cells both to open potentially dangerous channels in their membrane and lose their physical integrity[11-13]. It was expected that an acidic solution would potentially kill the cells. Such evidence can explain both the cell loss verified after some of the acidic treatments realized and the incompetence of the surviving cells to be

maintained and proliferate in culture. Different kinds of cells may have different resistance to the exposure to equally different acidic solutions and/or compounds[14]. Interestingly, acid can kill cancer cells[15]. On the other hand, gastric mucosa, the cells that line the stomach, are daily exposed to pH around 2 and still maintain their integrity. It is therefore difficult to predict the response of different cell types to the acidic solution, as performed in these experiments. Different cells had different resistance to acid treatment, although most part of them resulted in significant cell death. Further studies to evaluate the resistance of the different studied cell types to different acidic solutions and pH levels could elucidate about an optimal acid exposure, optimizing a protocol to be applied to each cell type.

Cell culture following treatment

Experiments with embryoid bodies. Following 7 days in the post-stress culture, EB1 and EB2 experiments resulted in deficient cell culture with total cell absence and single cells/small colonies (with no proliferation capacity) in the EB1 (data not shown) and EB2 (Figure 1-A) experiments, respectively.

Both experiments conducted with human cells (EB3 and EB4) showed high proliferation during the 7 days of culture, resulting in big colonies (Figure 1-B, C) with differentiation tendencies, resembling typical EBs' structures. The exposure to acid at a proper concentration ($1.5x10^6$ cells/ml) resulted in cells that maintained their integrity, and could be maintained and proliferate in culture. As stated[10], an initial cell loss in culture would be expected, followed by a maintenance of cell's density and formation of small sized and round shaped cells, thus arguing against a possible reprogramming event in these experiments. The results obtained suggested incapacity of the defined sphere media to support growth and maintenance of undifferentiated pluripotent cells. Despite being stated that these cells should acquire an ES cell-like morphology[10] without differentiation tendencies, morphology observations suggested that there was no acquisition of pluripotency by these cells. It must be kept in mind that during EBs' differentiation, incomplete differentiation may result in some cells residing in a pluripotent state, contributing to the high proliferation observed. However, a negative control (Figure 2-Negative Control: EBs) proved that EBs did not show pluripotency after 14 days in culture, thus eliminating this possibility. The significant proliferation capacity observed is characteristic of pluripotent ES cells population, since some specialized cells, upon differentiation, do not replicate themselves. Nerve cells, as well as muscle cells, for example, reside in G_0 phase and thus do not replicate[16], similarly to other cells (like bone cells) which enter temporarily in the G_0 phase. Therefore, due to the possible presence of such cell types in the fully differentiated EBs, the proliferation capacity should have been highly reduced as compared to this same ability in undifferentiated cells (which do not stop replicating). The high proliferation capacity is, therefore, an intriguing result. It was demonstrated that mouse ES cells, upon direct culture in sphere media without any stress, showed significant cell death and complete absence of proliferative capacity, resulting in reduced cell density following 7 days of culture (Figure 1-K). The maintenance of the ground-state of pluripotency[17] is dependent on the use of two inhibitors along with LIF, supporting the idea that the defined sphere media could not maintain or help to induce a pluripotent state. Nevertheless, this media should at least support cell survival and possibly induce mouse ES cells, directly cultured in it, to adopt a differentiated status as result of uncontrolled proliferation and loss of their pluripotency. It is intriguing why this media was said to efficiently promote puripotency. Associated with the low efficiency that characterizes reprogramming techniques[7,10,18], the low amount of starting material used in these experiments (only 9 wells of a 24 wells plate with EBs) contributed in less chance of finding possibly reprogramed cell aggregates. Further studies, using at least 2 plates full of EBs, could yield more favorable results. The similar resultes obtained with EB3 and EB4 demonstrated the coherence obtained with the method when cells with similar origin were used (both human ES cell lines). Interestingly, cells resulting from the EB5 experiment, despite showing completely uncontrolled proliferation (Figure 1-D), showed perfect mouse ES-like morphology as single cells (data not shown) after being daily refreshed and the cells excess removed. This may suggest that these cells, or at least a few cells, did not reside in a differentiated state. However, the mouse ES-like morphology observed could be suggestive of incomplete differentiation during EBs' culture, resulting in permanence of ES cells during the whole protocol and posterior culture. Mouse ES cells survived the whole protocol (data not shown), and acquired differentiated-like morphology during the 7 days of culture (Figure 1-L). Therefore, the fact that all the cells in EB5 experiment showed ES-like morphology suggests that the incomplete EBs' differentiation is improbable. Moreover, immunostaining analysis performed on day 14 EBs showed absence of pluripotency (Figure 1-E) confirming that the high

proliferation observed in these last three experiments was not a result of residual ES cells, and thus the high proliferation observed could be a result of the presence of efficiently reprogrammed cells. Since the reprogramming method in study[10] was stated to work with murine cells, and EBs grown from mouse ES cells should represent the non-pluripotent cells residing in a less differentiated status used in the whole work, EB5 logically represented the experiment from which the best results would be expected. The complete absence of pluripotency in this sample left low expectations for the following studies.

The conjunction of all these results pointed the high proliferation observed as an intriguing observation, most probably explained by uncontrolled cell growth and differentiation rather than presence of pluripotency.

Experiments with murine somatic cells. Cell culture following GCs1 experiment resulted in total cell death. Cells resulting from the GCs2 experiment showed some proliferation capacity in the first days in culture. This tendency was lost in the last days, resulting in a lower amount of cells after the 7 days of culture. These cells were dissociated from each other, staying in culture as single cells or small clumps (Figure 1-E). Obokata et al (2014) claimed that cells, after being cultured, would suffer high loss in the first days, followed by cell density stabilization (and further acquisition of pluripotency). However, these cells showed exactly the opposite: high initial proliferation and consequent loss of that capacity. This result suggested that these cells did not experienced any reprogramming event. It remains unclear why the cells resulting from GCs2 resisted to the culture conditions, contrastingly to the cells from GCs1, since they were cultured at a most favorable concentration. Additionally, it must be taken under consideration the time-consuming protocol that was performed. All the perturbations, along with all the centrifugations and observations of cell density between each steps easily reached 2 hours, and thus cells suffered with this long exposure to the external environment out of the incubator, contributing to the cell loss or damage, resulting in deficient cell culture.

Cells from TTs1 were cultured at a concentration of $2.2x10^5$ cells/cm^2 and showed some initial proliferation capacity, although it was lost and cell density was maintained until day 7 (Figure 1-F). TTs2 experiment resulted in cell culture at a concentration of $2x10^5$ cells/cm^2, similar to the first trial. However, no proliferation capacity was observed resulting in almost complete cell loss after 7 days in culture (Figure 1-G). Significant contamination was observed in culture, resulting from hair, skin, and possibly bone, which remained in the suspension during the experiment. The contamination observed interfered with both the cell culture and the physical pipetting steps, blocking the passage of the cells through the tips and representing extra physical stress imposed to them. A cell strainer with smaller pores (50µm) could reduce the chances of having debris in suspension, although possibly reducing the amount of cells.

Although being present in really few number, cells resulting from MEFs1 experiment had significant

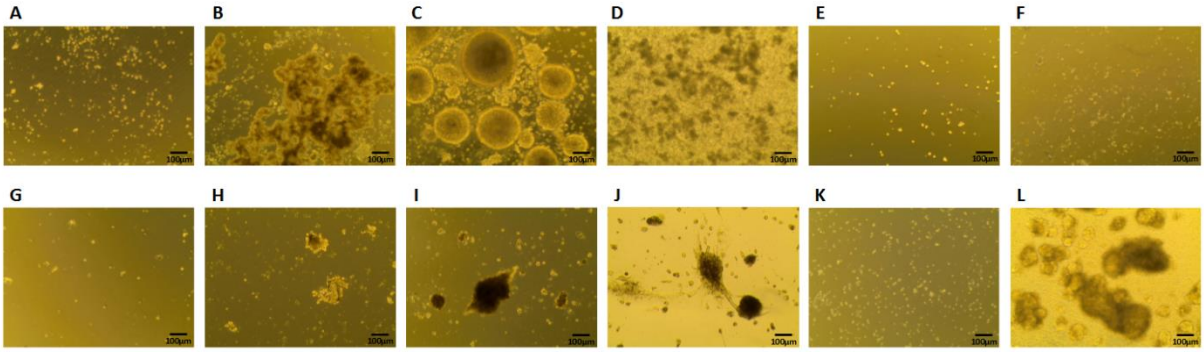

Figure 1: Cells from different experiments tend to adopt very different morphologies and survival rates in culture. An increase in survival and proliferation capacities was observed in the experiments EB2 (A) and EB3 (B). The proliferation capacity observed was even higher in the experiment EB4 conducted with cells from the H1 cell line (C). However, differentiation tendencies are also present in both experiments. The last experiment using EBs (EB5) shows uncontrolled proliferation rate (D), although the dark color observed may be due to high density and aggregation rather than differentiation, since cells adopt ES-like morphology when refreshed. Both GCs2 (E), TTs1 (F) and TTs2 (G) experiments result in dispersed single cells. Although not adapting differentiation tendencies like the previous ones, the absence of final proliferation enough for culture formation suggests that the cells eventually died. Both experiments with MEF cells resulted in apparent differentiation. While MEFs1 experiment (H) shows only small cell aggregates, some cells from MEFs2 (I) and MEFs with physical stress only (J) adapted an elongated morphology resembling true MEFs, thus suggesting maintenance of the epigenetic characteristics and absence of pluripotency. Moreover, mouse ES cells directly cultured on the sphere media (K) lose their viability and are drastically reduced in number, resulting in isolated cells, whereas these same mouse ES cells when exposed to all the perturbations can be efficiently cultured, resulting in cell survival and acquisition of a differentiated-like status.

proliferation capacity, leading to high cell growth and density, even after only 3 days in culture. Following 7 days in culture, cells acquired a morphology typical of completely differentiated cells (Figure 1-H). Several clumps dark in color were observed, contrasting to the small size isolated cells tendency verified with STAP cells. Cell's culture from MEFs2 experiment resulted in much higher cell density and equal differentiation tendency. Interestingly, after 7 days in culture, MEFs-like morphology was observed in some colonies (Figure 1-I), characterized by elongated structures. This observation suggested that some of these cells within these aggregates maintained their epigenetic characteristics and did not acquire any pluripotency, returning to their normal functionality when properly stable in culture. The same results were observed in MEF cells exposed only to physical stress (Figure 1-J).

Immunostaining Results

Experiments with embryoid bodies. The first three experiments conducted with EBs (EB1-3) resulted in complete absence of pluripotency (data not shown). The EB4 experiment (Figure 2-EB4) showed almost perfect, although very faint, overlapping of *OCT4* and *NANOG* genes expression, matching with DAPI staining. The perfect overlapping between both genes and DAPI, marking perfectly defined cells, increased the probability of effective pluripotency expression. The particularly low efficiency usually associated with reprogramming techniques could explain the few number of cells expressing pluripotency.

EB5 experiment also resulted in cells with apparent overlapping expression of both pluripotency genes, *Oct4* and *Nanog*, perfectly matching with DAPI staining (Figure 2-EB5). Interestingly, the cells in which *Oct4* and *Nanog* expression levels were higher were the ones poorly expressing DAPI, whereas the cells not expressing pluripotency genes highly expressed DAPI. This observation could be an evidence of unspecific binding spots that for some reason also stained for DAPI. However, the perfect overlapping and strong expression of both pluripotency genes in several perfectly defined cells was an argument strong enough to consider a possible acquisition of pluripotency.

Immunostaining analysis performed on mouse ES cells confirmed the efficiency of both the staining method and the antibodies used (Figure 2-Positive Control), used as positive control for pluripotency expression. Also, as stated before, 14 days culture EBs showed absence of pluripotency (Figure 2-Negative Control: EBs) before the cells were exposed to the protocol, confirming that a possible pluripotency expression would be acquired. Cells from the EB4 and EB5 experiments were frozen for qPCR.

Experiments with murine somatic cells. Both experiments with GCs (stated as easier to reprogram[19]) and TTs resulted in complete absence of pluripotency, characterized by unspecific blurry and dispersed *Oct4* and *Nanog* expression (data not shown). These results were expected due to the cell culture observations previously described.

Cells resulting from MEFs1 experiment showed apparent positive results. The expression patterns of

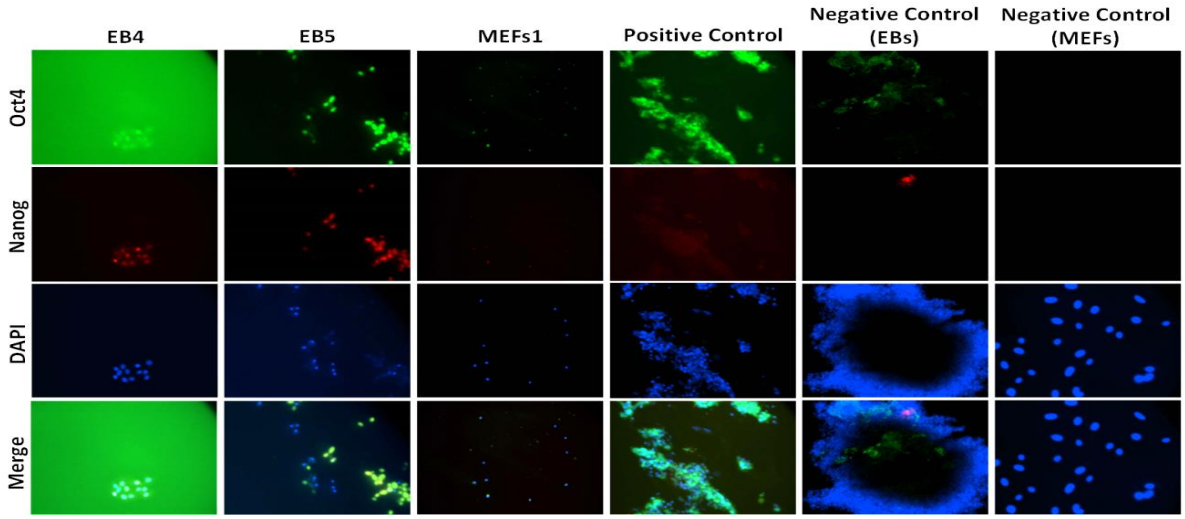

Figure 2: Immunostaining analysis showing possibly positive results. Cells from the EB4 experiment some almost perfect overlapping in a group of cells, although Oct4 expression was difficult to focus and identify. Contrastingly, some cells from EB5 experiment show perfect simultaneous expression of both Oct4 and Nanog, despite the fact that the apparently pluripotent cells weakly express DAPI. MEFs1 experiment resulted in some cells with equally interesting results, showing perfect overlapping of both Oct4, Nanog and DAPI, although the expression of the pluripotency genes is weak. Yet, a positive control performed with mouse ES cells confirms the efficiency of both the staining method and the antibodies in use, whereas two negative controls confirm the absence of pluripotency in EBs grown for 14 days and in MEFs prior to the experiment.

both *Oct4* and *Nanog* matched, although not perfectly with DAPI (Figure 2-MEFs1). Despite showing differentiation tendencies during culture, the simultaneous expression of both pluripotency genes argued in favor of the acquisition of pluripotency. As stated before, the efficient pluripotency expression observed in mouse ES cells confirms the efficiency of the antibodies in use and the adopted staining protocol (Figure 2-Positive Control). Also, a negative control showed clear absence of pluripotency in MEF cells prior to the protocol (Figure 2-Negative Control: MEFs). Intrigued by these observations, a second experiment (MEFs2) and an extra one (physical stress only) were performed. Both showed absence of pluripotency (data not shown), thus refuting the pluripotency possibly acquired in GCs1. Following the interesting results obtained in the MEFs1 experiment, cells from the MEFs2 experiment were frozen.

qPCR Results

This analysis included a comparison of the collected samples with two control pluripotent lines (mouse ES cell line for the mouse samples and human H1 ES cell line for the human sample), observing fold change expression of pluripotency genes.

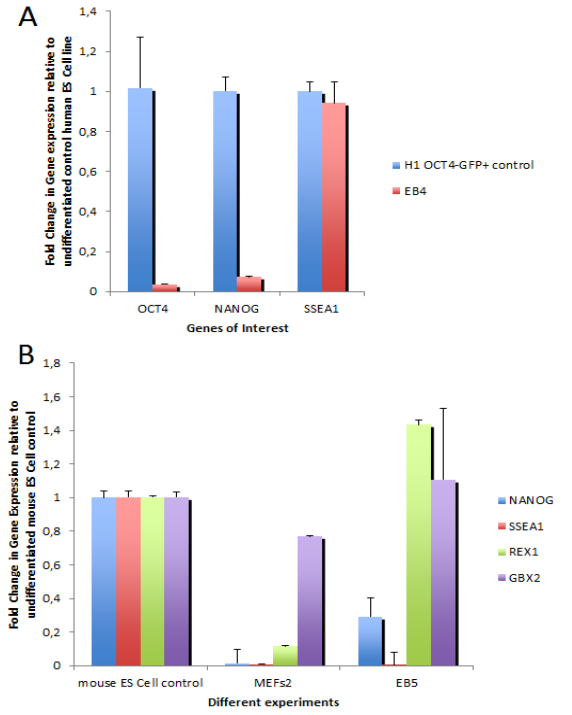

Figure 3: Human cells from the EB4 experiment show clear absence of pluripotency (A), showing low expression of both pluripotency genes. From the experiments conducted with murine cells (B), although cells from the MEFs2 experiment show clear absence of pluripotency, cells from the EB5 experiment show some interesting GBx2 and Rex1 expression levels. However, the low expression level of both Nanog and SSEA1 clarifies about the differentiated status of these cells.

Regarding the sample from EB4 experiment, qPCR results showed clear absence of pluripotency, with almost imperceptible *OCT4* and *NANOG* genes expression, showing only SSEA1 expression (Figure 3-A). Regarding mouse experiments, the results obtained (Figure 3-B) suggested the absence of pluripotency in the MEFs cells exposed the protocol, described by almost complete absence of *Nanog*, *SSEA1* and *Rex1* genes expression in MEFs2, as compared to the control. A negative control showed that MEF cells directly cultured in sphere did not acquire pluripotency (data not shown).

Contrastingly, EB5 experiment cells showed some interesting results. *Gbx2*, a mouse homeobox gene, which is expressed in mouse ES cells, having its expression decreased in differentiated cells[20], showed similar expression levels in the EB5 sample as compared to the control. *Rex1*, gene for a zinc finger protein expressed mainly in undifferentiated cells[21] showed an expression level comparable to the control. Despite showing slight *Nanog* expression, these cells did not acquire pluripotency. The surface marker gene *SSEA1* expression level was particularly low. Since the expression level of this gene is known to decrease upon differentiation[22], this consisted in an evidence of differentiation of these cells.

Conclusions and Perspectives

The ultimate goal to be achieved in the present work was the efficient reprogramming of human somatic cells, assuming that the same reprogramming with mouse cells would be possible. However, the results obtained showed divergence from a possible pluripotency acquisition. It was shown to be very difficult to conduct the whole protocol and reach culture phase, forcing the analysis to be equally focused on the monitoring of the cells' survival during the experiments and later on possible acquisition of pluripotency.

The results obtained showed the incapacity of differentiated cells to acquire pluripotency following acid exposure, in contrast to what was previously claimed[10], even using physical perturbations that were stated to help the reprogramming and even starting with EBs, presumably in a less differentiated state than fully differentiated cells.

Several drawbacks were observed during the present work. The limited amount of initial material surely contributed to reduce the chances of finding efficiently reprogrammed cells. Associated with the possibly inefficient dissociation of EBs into single cells, this

factor was critical. Despite optimal concentration of cells to be used in each step was suggested by Obokata et al (2014), including suspension in HBSS, suspension in acid and posterior culture, the high cell death and the limited quantity of material resulted in particularly low cell density that rarely reached the optimal values. Future works using considerable higher amounts of starting cells could increase the chances of a reprogramming event. The long exposure of the cells to external conditions, out of the incubator turned them more susceptible to the stresses imposed. Following the controversy in which this work was involved, and the suggestions of physical treatment along with acid exposure as essential for the reprogramming event, experiments using acid treatment only were discarded. The initial 30 minutes protocol was increased to at least 2 hours. Posterior studies using acid exposure only should be performed. Expedite methods for the isolation of murine somatic cells should be studied. Significant contamination was observed in the experiments conducted with tail tip cells, presenting extra stress to the cells, hindering them of being efficiently exposed to the physical pipetting, and interfering with their posterior culture.

The existence of STAP stem cells would be remarkable. The exceptionally easy method is just unbeatable in the stem cells field, having the answer to both ethical concerns, immune-rejection and virus activation (characteristic of iPS cells). However, if cells could be reprogrammed and lose their functionality through external stresses, this would theoretically mean that a common cell from a common living being, which is daily exposed to strong environmental conditions, could spontaneously lose its functionality anytime, and this possibility cannot be discarded. This common sense-related evidence surely argues against the functionality of this reprogramming technique.

It is intriguing why a physical and/or chemical stress could lead to the acquisition of pluripotency. Following the proof of mix or switch of cells and the inclusion of fraudulent data by Obokata in her publications, the possible reprogramming triggered by an external stimulus remains a mystery. However, at this points, the evidences of the results obtained suggest that the reprogramming method, in the way it was suggested[10] and in the way it was adapted to the present work, is incapable of inducing pluripotency.

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