POSITIVE EFFECT OF HUMAN ESC CONDITIONED MEDIUM ON SOMATIC CELL REPROGRAMMING

Katarzyna Tilgner [1,2], Lyle Armstrong [1,2] and Majlinda Lako [1,2]
[1] Centro de Investigacion Principe Felipe, Valencia, Spain
[2] Institute of Human Genetics, Newcastle University, United Kingdom

ABSTRACT

Induced pluripotent stem cells (iPSC), created by reprogramming of somatic cells into embryonic-like state are the latest developments in stem-cell research. These cells have a great potential in research and medicine, however the full exploitation of these cells is hampered by several issues such as derivation efficiency, heterogeneity and safety. Here we describe a simple method to enhance generation of fully reprogrammed human iPSCs from dermal fibroblasts. We have shown that the addition of culture medium that has been previously conditionned by human embryonic stem cells (hESC-CM) at the final stage of reprogramming procedure (≥3rd week) improves the efficiency of somatic cell reprogramming, possibly by promoting the transition of pre-iPSC colonies to a fully reprogrammed state. This approach may offer an alternative to the epigenetic modulators and chemicals method in the generation of safer and more efficient iPSCs.

Introduction Several pieces of research including the cloning of Dolly the sheep (Wilmut et al., 1997), put to rest for once and for all a long believed dogma that the cellular patterning of cells during the mammalian embryogenesis is irreversible. This break-through was accomplished due to iPSC technology, a method by which any somatic cell type may be reprogrammed in vitro to a pluripotent-like state by the introduction of specific transcription factors and subsequent culture under ESC-like conditions. Cells generated in such way, referred to as induced pluripotent stem cells (iPSCs), have been proven to be similar to embryonic stem cells in terms of morphology, gene expression and differentiation abilities. Such characteristics have raised hopes for the generation of patient specific iPSCs for biomedical research and clinical applications. Though, before any personalized stem cell-based therapies can be considered, a number of limitations needs to be addressed. The risk of potential mutagenesis due to the genomic insertion of exogenous reprogramming factors has been partially overcomed in recent years by replacing integrating retro- and lentiviruses with transiently transgene-expressing carriers (such as inducible/excisable vectors, adenoviruses), however the slow kinetics coupled with very low efficiency (≤0.01%) (Takahashi et al., 2007, Yu et al., 2007) and heterogeneous nature of emerging iPSC colonies are still significant handicaps for iPSC technology. As part of the undergoing effort to solve these problems we found that hESC-culture medium conditioned with hESC (hESC-CM) improves the efficiency of somatic cell reprogramming possibly by promoting the transition of pre-iPSC colonies to a fully reprogrammed state.

MATERIALS AND METHODS

Lentiviral transduction and reprogramming culture: Lentiviral vectors for human OCT4, NANOG, LIN28 and SOX2 were obtained from Stemgent (iPSC Generation Human TF Lentivirus Set; Cat. No.00-005). Lentiviral transductions of neontal human fibroblasts (NHDF, Lonza) were carried out (at an M.O.I of 5) with cells in attachment (1x105 cells/2ml/well of 6-well plate seeded the day before transduction) in fibroblasts medium in the presence of polybrene (0.6 μg/ml final concentration, Sigma). Following the overnight incubation with lentiviral transduction mixture, human somatic cells were dissasociated with trypsin and transferred to 6-well plate seeded with Mitomicin C inactivated MEFs (from 1 well of 6-well plate transduced cells to 3 wells of 6-well MEF feeder plate). Twenty-four hours later the fibroblasts medium was replaced with hESC culture media in which cells were maintained for one week. After this time the transduced cells were either cultured (1) continuously in human ESC culture medium conditioned with inactivated MEF (F-CM) or (2) starting from day 21- in human ESC medium conditioned with inactivated MEF (F-CM) supplemented 1:1 with hESC culture medium collected from cultivated hESCs (referred here as human ESC medium conditioned with hESC (ESC-CM). ESC-CM medium was collected from H9 cells 3-5 days after plating and prior use was filtered through 0.2 μm filter to avoid cross-contamination. In both strategies media were refreshed every day until colonies with a similar morphology to hESCs were observed, which was usually between 28-35 days after transduction. Once iPSC colonies were identi-

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Correspondence to: Majlinda Lako, Centro de Investigacion Principe Felipe
Avda. Autopista del saler, 16-3 Junto Oceanografico 46012 Valencia Spain,
tel:+34 9632 89681 ext. 1211, mlako@cipf.es

fied, they were manually picked and serially expanded on Mitomycin C inactivated MEF feeder plates in conventional hESC growth conditions for several passages.

Immunocytochemistry: ALP staining was performed according to the manufacturer's instructions using the Alkaline Phosphatase Detection Kit (Milipore). For pluripotency markers staining, cells were fixed in 4% paraformaldehyde for 15 min at room temperature (RT) and washed with PBS. Additionally for internal marker staining the cells were incubated in permabilisation solution (0.2% Triton X-100 (Sigma-Aldrich) in PBS) for 10 min at RT. They were then incubated for 30 min in blocking buffer (5% goat serum and 1% BSA in PBS). Afterwards the primary antibodies (mouse anti -OCT4 1:100 (Millipore); mouse anti-TRA-1-60 1:100 (Millipore) and mouse anti-SSEA-4 1:100 (BD Pharmingen), were applied in blocking buffer for 1 hour at RT. Then the cells were washed with PBS and incubated with secondary antibodies (anti-mouse IgG FITC conjugated 1:500 (Sigma)) in blocking buffer for 45 min at RT in dark. Nuclei were detected by DAPI (Sigma-Aldrich) staining. Images were captured using a Xiovert software and a Zeiss microscope.

RESULTS

Generation of induced pluripotent stem cells by defined factors is greatly improved by medium conditioned with hESC (ESC-CM)

To induce reprogramming, human dermal fibroblast cells (NHDF) were co-transfected with a set of viruses coding genes known to be critical for maintenance of pluripotency in embryonic stem cells (OCT4, SOX2, NANOG and LIN28). Transduced cells were then cultured under conditions specified in materials and methods (see also Figure 1A) and the reprogramming progress was monitored on a daily basis by epifluorescence microscopy. As presented in Figure 1B morphological changes were observed as early as day 4 post-transduction. At this time some fibroblast appeared spherical in their morphology and formed initially loose followed by densely packed clusters. These early iPSC colonies, displaying a granulated non-human ES cell-like morphology, became dominant in early reprogramming days as they proliferated actively, however at the second week post-transduction the majority of them disappeared. Since it has been demonstrated by others that it is at ~10-12 post-transduction when the switch from the viral transgene expression to endogenenous gene activation takes place (Stadtfeld et al., 2008), it is very likely that most of our early iPSC colonies did not pass this crucial step for further reprogramming and possibly reverted back into fibroblast or simply died. Colonies which displayed the appropriate hESCs morphology (Figure 1B) emerged much later (≥day 28) and interestingly their number differed significantly between different culture conditions (Figure 1C). When OSLN transduced cells were maintained from day 7 up to the end (35 days) in F-CM, only a few colonies appeared (average of 3±1 colonies per 1x105 cells initially used). However, when transduced cells from day 21 were treated with F-CM in combination with hESC-CM (at 1:1 ratio), a significant increase in the number of colonies that closely resembled the hESC morphology was observed (20±2 colonies per 1x105cells) (Figure 1C). In both cases the colonies with good morphology, i.e. tightly packed, flat and with defined edges, were manually picked out for expansion and identity analyses. The immunocytochemistry assay (Figure 1D), showed that selected clones, regardless of the culture strategy used, exhibited strong Alkaline Phosphatase (AP) activity and were positive for the pluripotency marker-specific cell surface (TRA-1-60, TRA-1-81, SSEA-4) and nuclear (OCT4, SOX2) markers. Further molecular examination for the expression of pluripotency and differentiation markers, bisulphite genomic sequencing analyses of certain promoters together with more sophisticated in vitro and in vivo analyses will further dissect how closely newly generated iPSCs resemble hESCs and whether the hESC-CM supplement has affected the quality of reprogrammed iPSC clones.

Discussion Conventionally human ESCs are maintained in culture in undifferentiated state either on inactivated feeder layer in serum-free medium or as feeder-free in the presence of conditioned medium produced by inactivated feeders. Several studies on conditioned medium have shown that secreted factors produced by fibroblasts are important for hESC cultivation (Lim and Bodnar, 2002; Xie et al., 2004; Chin et al., 2007). It is therefore not surprising that conditioned medium has been utilised the majority of current reprogramming procedures, including ours. By culturing OSLN transduced fibroblast in conditioned media we indeed observed a large number of potential iPSC colonies emerging at early days of reprogramming, however only a very small number of them made into the final stage ultimately giving rise to fully reprogrammed iPSCs. This is why we decided to further modify the reprogramming procedure by supplementing the conditioned media collected from feeders (F-CM) with conditioned media collected from hESC cultures (hESC-CM). We speculated that hESCs may secrete to the media some factors which may support their maintenance (positive loop) and accordingly may help to gain pluripotent state by cells undergoing reprogramming. In our study, treatment of OSLN transduced cells with hESC conditioned medium (starting from day 21) reproducibly enhanced the reprogramming efficiency at least 10 times, possible by promoting the transition of pre-IPSC colonies to a full reprogrammed state. This observation encourages more proteomic analyses on hESC-conditioned media. Such screens may lead to the identification of molecules that will become powerful tools in providing us with new insights into the reprogramming process and may ultimately lead to an efficient iPSC generation protocol.

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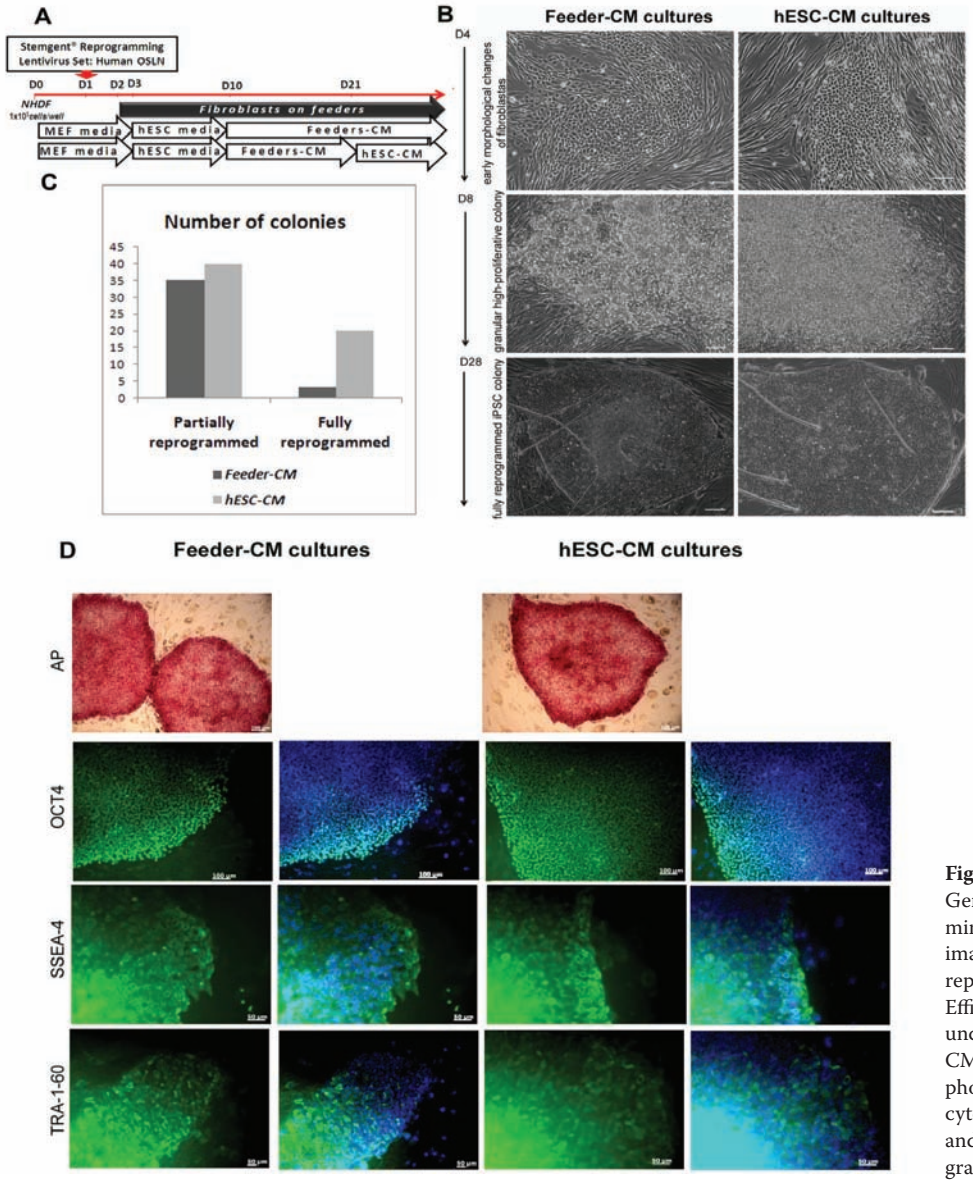

Figure 1
General outline of the reprogramming procedure (A); Microscope images of early, partially- and fully reprogrammed iPSC colonies (B); Efficiency of colony formation under F-CM and F-CM –hESC-CM (1:1) conditions (C); Alkaline phophatase staining and immunocytochemistry of OCT4, SSEA-4 and TRA-1-60 on fully reprogrammed colonies (D).