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Commentary

GM-CSF and MEF-conditioned media support feeder-free reprogramming of mouse granulocytes to iPS cells

Jaber Firas [a,b,1], Xiaodong Liu [a,b,1], Christian M Nefzger [a,b,*], Jose M Polo [a,b,**]

[a] Department of Anatomy and Developmental Biology, Monash University, Clayton 3800, Victoria, Australia
[b] Australian Regenerative Medicine Institute, Monash University, Clayton 3800, Victoria, Australia

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ABSTRACT

Induced pluripotent stem cells (iPSCs) are characterised by their ability to differentiate into any cell type of the body. Accordingly, iPSCs possess immense potential for disease modelling, pharmaceutical screening and autologous cell therapies. The most common source of iPSCs derivation is skin fibroblasts. However, from a clinical point of view, skin fibroblasts may not be ideal, as invasive procedures such as skin biopsies are required for their extraction. Moreover, fibroblasts are highly heterogeneous with a poorly defined developmental pathway, which makes studying reprogramming mechanistics difficult. Granulocytes, on the other hand, are easily obtainable, their developmental pathway has been extensively studied and fluorescence activated cell sorting allows for the isolation of these cells at high purity; thus iPSCs derivation from granulocytes could provide an alternative to fibroblast-derived iPSCs. Previous studies succeeded in producing iPSC colonies from mouse granulocytes but with the use of a mitotically inactivated feeder layer, restricting their use for studying reprogramming mechanistics. As granulocytes display poor survival under culture conditions, we investigated the influence of haematopoietic cytokines to stabilise this cell type *in vitro* and allow for reprogramming in the absence of a feeder layer. Our results show that treatment with MEF-conditioned media and/or initial exposure to GM-CSF allows for reprogramming of granulocytes under feeder-free conditions. This work can serve as a basis for future work aimed at dissecting the reprogramming mechanism as well as obtaining large numbers of iPSCs from a clinically relevant cell source.

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1. Introduction

Induced pluripotent stem cells (iPSCs) are characterised by their ability to self-renew and differentiate into any cell type of the body, mirroring the potential of embryonic stem cells (ESCs) (Takahashi and Yamanaka, 2006). iPSCs circumvent many of the ethical and legal issues associated with ESC research and hold vast therapeutic potential for disease modelling, pharmaceutical screening and autologous cell therapy applications (Ebert et al., 2009; Juopperi et al., 2012; Nguyen et al., 2011; Moretti et al., 2010; Itzhaki et al., 2011; Hanna et al., 2007; Wernig et al., 2008; Nelson et al., 2009; Miura et al., 2009; Nishimura et al., 2013; Vizcardo et al., 2013). They were originally generated through the forced expression of four pluripotency associated transcription factors: Oct4, Sox2, Klf4 and c-Myc (OSKM) in mouse fibroblasts (Takahashi and Yamanaka, 2006). Subsequently, different groups were able to derive iPSCs from fibroblasts from other species such as rat (Liao et al., 2009; Li et al., 2009), monkey (Liu et al., 2008) and human (Lowry et al., 2008; Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007). Furthermore, iPSCs can be derived from other somatic cell sources such as stomach cells (Aoi et al., 2008), hepatocytes (Aoi et al., 2008), keratinocytes (Aasen et al., 2008; Maherali et al., 2008), melanocytes (Utikal et al., 2009), pancreatic beta cells (Stadtfeld et al., 2008), neural progenitors (Eminli et al., 2008; Kim et al., 2008; Shi et al., 2008; Silva et al., 2008) and nucleated blood cells (Eminli et al., 2009; Hanna et al., 2008; Loh et al., 2009). However, the best candidate cell source for human iPSCs remains elusive. Human iPSCs are generally derived from skin fibroblasts (Park et al., 2008) which usually require invasive collection methods involving surgical biopsies. Also, the exposure of the skin to ultraviolet light from the sun has been shown to cause somatic mutations (Tyburczy et al., 2014; Abyzov et al., 2012; Jonason et al., 1996; Ling et al., 2001; Ikehata et al., 2003). This raises the possibility that the skin-derived iPSCs might carry an increased mutational load. Blood, on the other hand, is an easily accessible tissue that can be obtained in a minimally invasive way. Furthermore, the markers associated with the haematopoietic system have been extensively studied which allow for the isolation

* Corresponding author at: Department of Anatomy and Developmental Biology, Monash University, Clayton 3800, Victoria, Australia. Tel.: +61 3 9905 0096.
** Corresponding author at: Department of Anatomy and Developmental Biology, Monash University, Clayton 3800, Victoria, Australia. Tel.: +61 3 9905 0005.
E-mail addresses: christian.nefzger@monash.edu (C.M. Nefzger), jose.polo@monash.edu (J.M. Polo).
[1] These authors contributed equally.

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of specific populations with a distinct phenotype and function at high purity (Chao et al., 2008). Examples of other easily accessible cell types are renal epithelial cells in urine (Zhou et al., 2012) and hair follicle associated cells (Wang et al., 2013) which have been successfully reprogrammed to iPSCs. However, previous studies have also shown that different starting cell types are able to reprogram at different rates, and can transiently retain a transcriptional and epigenetic memory of their cell of origin (Polo et al., 2010; Kim et al., 2010, 2011). Such differences could conceivably be utilised in a clinical setting. Thus, further understanding of the underlying mechanisms of reprogramming from different starting cell types is needed.

Granulocytes are highly abundant and constitute 40–60% of all white blood cells. They include neutrophils, eosinophils, basophils and mast cells, with neutrophils being the most abundant (Duffin et al., 2010). Furthermore, fluorescence activated cell sorting (FACS) allows for the isolation of these cells at high purity. This makes them a possible viable cell source for derivation of iPSCs in the clinical arena, as well as a source to investigate the underlying mechanisms of reprogramming from a relatively homogenous starting population. Reprogramming of granulocytes to iPSCs has been reported previously using lentiviral vectors and reprogrammable mouse strains (Eminli et al., 2009; Polo et al., 2010; Stadtfeld et al., 2010). In humans it has been shown that mononuclear blood cells (agranulocytes) can be reprogrammed using a cytokines cocktail, to enhance their initial survival. However, those studies reprogrammed these cells in the presence of a layer of mitotically inactivated mouse embryonic fibroblasts (otherwise known as a MEF feeder layer). The presence of such a layer imposes restrictions as it hinders the use of granulocytes for studying reprogramming mechanistics and in case of human cells is unwanted in a clinical setting. A previous study has shown that the mean *in vitro* half-life of mouse peripheral blood neutrophils and bone marrow neutrophils is 6 and 12 h respectively (Boxio et al., 2004), which might be extended by the use of cytokines. Hence, we investigated the influence of different haematopoietic growth factors to stabilise this cell type *in vitro* and allow for more efficient reprogramming in a feeder-free system. We addressed this question by using a transgenic system that enables homogeneous doxycycline-inducible OSKM expression in somatic cells (Carey et al., 2010).

Our results showed that early transient treatment with GM-CSF has a significant effect in enhancing the generation of iPSC cells from granulocytes. Furthermore, we have found that treatment of granulocytes with MEF-conditioned media could serve as an alternative to the use of feeders during reprogramming.

2. Materials and methods

2.1. Mouse model

We used reprogrammable mice (Carey et al., 2010) that carry a polycistronic cassette encoding OSKM targeted to the *Col1a1* locus, under control of a tetracycline-dependent promoter (*tetOP*). These mice also express a reverse tetracycline-dependent transactivator (M2rtTA) from the ubiquitous *Rosa26* locus. When doxycycline is added, M2-rtTA binds to *tetOP* and induces OSKM expression.

2.2. Bone marrow extraction

Reprogrammable mice homozygous for M2-rtTA and OSKM, 6–8 weeks of age, were culled by cervical dislocation. The femurs and tibiae of the hind limbs and the humeri of the forelimbs were carefully dissected and cleaned from adherent tissue. The tip of each bone was removed, followed by the insertion of a syringe needle (23-gauge) into one end of the bone and by flushing it into 1% Bovine Serum Albumin/Dulbecco's Phosphate-Buffered Saline (BSA/DPBS; Gibco). The flushed cells were passed through a 70-μm nylon mesh filter (BD Biosciences), then incubated for 5 min with lysis buffer (Zhang et al., 2008) at room temperature to lyse the red blood cells. Cells were then washed and prepared for flow cytometry.

2.3. FACS purification of granulocytes

Harvested bone marrow cells were incubated with conjugated antibodies against Sca1 (Pacific Blue-conjugated, E13-161.7; Biolegend), Mac1 (R-Phycoerythin-conjugated, M1/70; Biolegend), B220, Ter-119, CD5 (Fluorescein isothiocyanate-conjugated, RA3-6B2, TER-119, 53-7.3; BD Biosciences), Gr1 (Allophycocyanin-Cy7-conjugated, RB6-8C5; Biolegend), SSEA1 (biotinylated, eBioMC-480; eBioscience) and cKit (Allophycocyanin-conjugated, 2B8; BD Biosciences) for 20 min at 4 °C. The cells were then washed with DPBS, and incubated with R-Phycoerythin-Cy7-streptavidin (eBioscience) for 20 min at 4 °C. Then, they were washed with DPBS, resuspended in propidium iodide (2 ng/μl)–1% BSA/DPBS solution and passed through a 40 μm cell strainer (BD Falcon) to warrant single cell suspensions. Cells with the following marker profile $CD5^-B220^-Ter119^-Sca1^-cKit^-Ssea1^-Gr1^+Mac1^+$ were defined as granulocytes and isolated with an Influx cell sorter instrument (BD Biosciences).

2.4. Generation of MEF-conditioned media

MEF-conditioned ES media were generated from irradiated MEFs. Initially, irradiated MEFs were plated at ~20,000 cells/cm^2 in MEF media. After 4 h, the media were exchanged with the ES media (Knock-out DMEM, 15% FCS, 1% glutamax, 1% NEAA, 1% Penicillin/Streptomycin, 0.1% B-mercapto, and 0.1% Lif (Millipore)) and MEF-conditioned media were collected in 24 h intervals for up to five days. Upon collection, MEF-conditioned media were filtered with a 0.22 μm stericup (Millipore).

2.5. Granulocytes reprogramming

Granulocytes were cultured in ES media under normoxic conditions and at 5% CO_2. Cells were plated at a seeding density of ~50,000 cells/cm^2 on either a feeder layer of irradiated MEFs (~20,000 cells/cm^2), or grown in feeder-free ES media or in feeder-free MEF-conditioned media, as indicated. Media were changed every 2–3 days. In the first week, with every media change, non-adherent cells were collected by pelletting at 1500 rpm for 3 min. Pellets were resuspended in fresh media and then added back to their wells of origin. Doxycycline treatment (2 μg/ml) was for 16 days. Subsequently, cells were cultured in doxycycline-free media for 3–5 days.

The following cytokines were used for different durations depending on the experiment: granulocyte colony-stimulating factor (G-CSF; 10 ng/ml; PeproTech), granulocyte-macrophage CSF (GM-CSF; 5 ng/ml; PeproTech), interleukin-3 (IL3; 10 ng/ml; PeproTech), Fms-like tyrosine kinase 3 (Flt3; 10 ng/ml; PeproTech) and Stem Cell Factor (SCF; 10 ng/ml; PeproTech).

2.6. FACS analysis

Cultures at days 6, 9, 12 and 16 of reprogramming were washed with DPBS then harvested by incubation in 0.25% trypsin/1 mM EDTA (Invitrogen) for 3 min at 37 °C. In addition on day 6, non-adherent cells in the culture media were collected by centrifugation and combined with enzymatically liberated cells. Cells were then incubated with SSEA1 (biotinylated, eBioMC-480;

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