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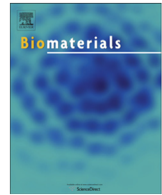

A defined xeno-free and feeder-free culture system for the derivation, expansion and direct differentiation of transgene-free patient-specific induced pluripotent stem cells

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ABSTRACT

A defined xeno-free system for patient-specific iPSC derivation and differentiation is required for translation to clinical applications. However, standard somatic cell reprogramming protocols rely on using MEFs and xenogeneic medium, imposing a significant obstacle to clinical translation. Here, we describe a well-defined culture system based on xeno-free media and LN521 substrate which supported i) efficient reprogramming of normal or diseased skin fibroblasts from human of different ages into hiPSCs with a 15–30 fold increase in efficiency over conventional viral vector-based method; ii) long-term self-renewal of hiPSCs; and iii) direct hiPSC lineage-specific differentiation. Using an excisable polycistronic vector and optimized culture conditions, we achieved up to 0.15%–0.3% reprogramming efficiencies. Subsequently, transgene-free hiPSCs were obtained by Cre-mediated excision of the reprogramming factors. The derived iPSCs maintained long-term self-renewal, normal karyotype and pluripotency, as demonstrated by the expression of stem cell markers and ability to form derivatives of three germ layers both *in vitro* and *in vivo*. Importantly, we demonstrated that Parkinson's patient transgene-free iPSCs derived using the same system could be directed towards differentiation into dopaminergic neurons under xeno-free culture conditions. Our approach provides a safe and robust platform for the generation of patient-specific iPSCs and derivatives for clinical and translational applications.

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1. Introduction

Human induced pluripotent stem cells (hiPSCs) provide a unique platform for treatment of various human diseases without ethical issues. The ability of patient-specific iPSCs to differentiate into relevant autologous tissues or organs highlights their potential as a cell source for exploration of disease mechanisms, to identify novel therapeutic targets, and ultimately, for possible autologous cellular therapy [1,2]. In parallel with the rapid progress in iPSC research, the number of tissue banks set up using patient-specific fibroblasts has been steadily increasing, while the therapeutic relevance and potential of iPSCs has been recognized [3–5].

Derivation, expansion and differentiation of hiPSCs under xeno-free conditions are an essential prerequisite towards realizing iPSC-based clinical application. Generally, the somatic cell reprogramming process requires initial cell proliferation, after which a fraction of the cell progeny successfully converts into an embryonic stem cell (ESC)-like state with different time latencies [6,7]. Due to the poor efficiency of existing reprogramming methods, it is often necessary for reprogramming to be performed in the presence of mouse embryonic fibroblast (MEF) feeder cells along with the use of serum and xeno-containing products, to maximize colony formation [8]. To overcome this safety issue, xeno-free iPSC derivation using human feeder cells has recently been reported [9,10]. However, the use of human feeder cells adds complexity and set up time to the reprogramming procedure, introduces technical variability, and interferes with the monitoring and analysis of the reprogramming process. In the search for methods of hiPSC derivation under feeder-free condition, several research groups have reported

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reprogramming studies based on Matrigel- or Vitronectin-coated substrates along with the use of chemically defined media [11–13]. However, a big challenge still exists in developing well-defined and safe xeno-free systems which can integrate efficient derivation, expansion and direct relevant tissue differentiation of disease-specific iPSCs for transplantation or disease modeling.

Maintaining genomic integrity is critical for therapeutic application of hiPSCs. hiPSCs were first derived in 2007 from human fibroblasts through viral introduction of a set of stemness factors: Oct3/4, Sox2, Klf4 and c-Myc with about 0.01% efficiency [14]. Due to its reliability and relatively high efficiency at delivering the reprogramming factors to the cells, the viral-based approach remains the most widely used method for iPSC derivation [15], and patient-specific iPSCs from a variety of diseases, including amyotrophic lateral sclerosis, Parkinson's disease (PD), type 1 diabetes mellitus, Huntington's disease, and Down's syndrome have been reported [16,17]. However, viral vector-mediated reprogramming results in integration of both the vector backbone and transgenes into the genome [14,18], presenting a formidable obstacle to therapeutic use of iPSCs. In the search for methods to induce pluripotency without incurring genetic change, several integration-free methods have been employed to reprogram murine cells or neonatal normal human cells only, by using purified proteins, mRNAs, or plasmids and xenogeneic culture conditions [19–25]. Although these methods have the potential to generate genetically unmodified iPSC lines, their main disadvantage lie in the low efficiency of reprogramming, preventing reliable application for reprogramming disease-specific adult human somatic cells [22–25]. To improve the reprogramming efficiency, several studies reported iPSC derivation on MEF layer using reprogramming enhancing molecules [26,27]. Of note, many of these molecules have pleiotropic effects that could result in transient or permanent epigenetic or genetic alterations, hindering the use of chemically induced iPSCs for therapeutic purposes [28,29]. Thus, an efficient and safe reprogramming method with a compliant xeno-free culture system is still needed in order to achieve the widespread derivation of disease-specific transgene-free iPSCs from humans with inherited or degenerative diseases.

The goal of our study is to develop a defined xeno-free and feeder-free culture system for efficient transgene-free patient-specific iPSC derivation, expansion and subsequent differentiation. To achieve this, we chose a single, excisable polycistronic lentivirus vector for human adult fibroblast reprogramming [30]. This approach allows efficient introduction of the reprogramming factors into human adult fibroblasts, followed by easy excision of the transgenes from the iPSCs when treated with Cre recombinase. In addition, we chose xeno-free recombinant human laminin 521 (LN521) for the fibroblast reprogramming substrate because of the role of laminins in early embryonic development [25,31]. Laminins are the central components of basement membranes, containing 15 different isoforms in human tissues [32]. Recent studies revealed a striking difference in the effects of various laminin isoforms on ESC culture. Isoform LN411 was not capable of supporting ESC adhesion or survival, and LN111 supported ESC attachment, while LN511 and laminin fragment 8 supported human pluripotent stem cell culture in the chemically defined medium [19,33,34]. Similar to LN511, LN521 is normally expressed and secreted by human embryonic stem cells (hESCs) [35]. We hypothesized that LN521 would support fibroblast growth and reprogramming. Chemically defined xeno-free media including E8, NutriStem and TeSR2 were tested in the reprogramming experiments, and compared with the xenogeneic media, mTeSR1 and MEF conditioned medium (CM). Four adult fibroblast lines from human individuals of different ages, including one PD patient fibroblast line, were reprogrammed using this culture system, and tested for long-term expansion as well as directed differentiation under totally defined xeno-free conditions.

2. Materials and methods

2.1. Culture of human adult fibroblasts (HDF)

Four human adult fibroblast lines (Table 1) were used in this study: HDF-A (PCS-201, ATCC, USA), HDF-L (CC-2511, Lonza, USA), HDF-C (Cascade Biologics, Life Technologies) and PD patient fibroblasts HDF-PD (ND30116, Coriell, USA). Human fibroblast lines were cultured in standard culture media containing DMEM supplemented with 100 IU/mL penicillin-streptomycin (Life Technologies) and 10% fetal bovine serum (DMEM-FBS, Life Technologies) or human serum (DMEM-HS, ATCC, USA) and maintained at 37 °C in a 5% CO_2 incubator. Cells were allowed to expand to 80%–90% confluence before passaging with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA, Life Technologies). Early-passage HDF cells were used for virus transduction experiment.

2.2. Culture of human pluripotent stem cell lines

Human embryonic stem cell line HUES7 was obtained from Dr. Douglas Melton's Lab at Harvard University. The preparation of conditioned medium (CM) and the procedure for maintenance of HUES7 under feeder-free condition were essentially the same as described previously [36]. Briefly, HUES7 cells were cultured on Matrigel (BD Biosciences, San Jose, CA, USA)-coated culture wells in mTeSR1 (Stemcell Technologies, Vancouver, BC, Canada) at 37 °C in a 5% CO_2 incubator with daily medium changes. Cells were subcultured every 5–6 days with 1 mg/mL dispase (STEMCELL Technologies, Vancouver, Canada). The newly generated pre-excised and post-excised iPSCs were cultured under defined feeder-free culture conditions, as described below.

2.3. Production and infection of lentiviral vectors

Polycistronic reprogramming lentivirus vector Lenti-OSKM (pOSKM) was modified from pKP332 (Addgene #21627) by the insertion of a IRES-cMyc fragment at a unique PacI site. This modification dramatically improved the reprogramming efficiency of pKP332. Supplementary Fig. 1 shows the schematic representation of Lenti-OSKM vector. As a negative control for reprogramming, cDNA encoding copepod GFP (maxGFP, Lonza) was subcloned into pLenti6/V5-D-TOPO (Life Technologies) to give pLenti6maxGFP. For preparation of lentivirus particles, viral packaging was performed in 293FT cells using reagents and protocol from the ViraPower™ Lentiviral Packaging Kit (Life Technologies). Lentivirus particles were concentrated from cell culture supernatant according to the protocol of Deiseroth Lab (http://www.stanford.edu/group/dlab/resources/lvprotocol.pdf).

For determining the titer of the lentivirus, $\sim 0.1 \times 10^6$ human adult fibroblasts were plated in each well of a 6-well plate (BD falcon) in DMEM-FBS. On the day of transduction, 0 μl, 50 μl, 100 μl and 150 μl of freshly prepared lentivirus vectors were mixed with 1 mL of DMEM-FBS containing 8 μg/mL polybrene (Millipore) and added to the cells. The culture wells were gently shaken, and placed at 37 °C in a 5% CO_2 incubator overnight. The next day, virus-containing supernatant was replaced with 2 mL of DMEM-FBS. The cell numbers were assessed after 3 days transduction by phase contrast microscopy. After a pre-test, 100 μl of vectors/well was used for our study. For each reprogramming experiment, the transduced cells were trypsinized 48 h post-transduction, transferred to Matrigel-coated tissue culture plates, and cultured in stem cell culture media on the same well for 4 weeks with daily media changes. The stem cell media used in this study include MEF conditioned medium (CM), mTeSR1 (medium containing BSA), TeSR2 (xeno-free medium) (Stemcell technologies, Vancouver, BC, Canada), NutriStem (xeno-free medium, Stemgent, San Diego, CA, USA), and Essential 8 medium (E8, xeno-free medium, Life Technologies). Putative hiPSC colonies started to appear after 1–2 weeks. These colonies were

Table 1
Generation of hiPSCs by pOSKM vector under chemically defined condition, listing details of fibroblasts and annotations used for the fibroblasts and derived iPSC cell lines.

Human adult fibroblasts	Cell source	Sex	Age	Reprogramming efficiency*	iPSC Symbol	
					Before excise	Post excise
HDF-A	ATCC	Female	25	0.26%**	A-iPSC	EX-A-iPSC
HDF-C	Cascade Biologics	Female	31	0.30%**	C-iPSC	EX-C-iPSC
HDF-L	Lonza	Female	32	0.18%***	L-iPSC	EX-L-iPSC
HDF-PD (ND30116)	Coriell	Female	67	0.23%***	PD-iPSC	EX-PD-iPSC

Reprogramming efficiencies were calculated on the number of AP expressing colonies normalized to the number of cells seeded on matrigel- or LN521-coated plates. *: Reprogramming experiment was conducted in xeno-free E8 medium. **: Matrigel as substrates. ***: Xeno-free condition, and xeno-free LN521 as substrates.

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