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Generation and characterization of bat-induced pluripotent stem cells

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ABSTRACT

Induced pluripotent stem cells (iPSCs) were first generated from mouse embryonic fibroblasts in the year 2006. These cells resemble the typical morphology of embryonic stem cells, express pluripotency markers, and are able to transmit through germlines. To date, iPSCs of many species have been generated, whereas generation of bat iPSCs (biPSCs) has not been reported. To facilitate in-depth study of bats at the molecular and cellular levels, we describe the successful derivation of biPSCs with a *piggyBac* (PB) vector that contains eight reprogramming factors Oct4, Sox2, Klf4, Nanog, cMyc, Lin28, Nr5a2, and miR302/367. These biPSCs were cultured in media containing leukemia inhibitory factor and three small molecule inhibitors (CHIR99021, PD0325901, and A8301). They retained normal karyotype, displayed alkaline phosphatase activity, and expressed pluripotency markers Oct4, Sox2, Nanog, TBX3, and TRA-1-60. They could differentiate *in vitro* to form embryoid bodies and *in vivo* to form teratomas that contained tissue cells of all three germ layers. Generation of biPSCs will facilitate future studies on the mechanisms of antiviral immunity and longevity of bats at the cellular level.

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1. Introduction

Embryonic stem cells (ESCs) can proliferate indefinitely without differentiation and are capable of chimera formation and germline transmission [2]. Currently, ESCs with such properties have only been derived from the mouse and rat [1]. Mouse ESCs were established from the inner cell mass of mouse embryos in the year 1981 [3,4]. They can differentiate into multiple tissue cells [5] or form chimeric mice [6]. Rat ESCs were successfully isolated and propagated in the presence of small molecules in the year 2008 [7]. The derived rat ESCs also contribute to germline chimeric rats [7,8].

In the year 2006, Yamanaka [9] used four pluripotency factors Oct4, Sox2, cMyc, and Klf4 to reprogram mouse fibroblasts into mouse-induced pluripotent stem cells (miPSCs) *via* retroviral infection. Human-induced pluripotent stem cells (hiPSCs) were generated successfully in the year 2007 [10,11]. Subsequently, iPSCs were generated from monkeys [12], rats [13,14], pigs [15–17], rabbits [18], bovines [19,20], dogs [21], horses [22], buffalo [23], and sheep [24–26]. This opened up a new era of disease modeling, animal traits improvement, and translational medicine.

Induced pluripotent stem cells have similar biological properties to ESCs, such as infinite proliferation, expression of pluripotency markers, and the ability to differentiate into three germ layers, as well as germline transmission *in vivo* [9]. Induced pluripotent stem cell technology has been widely used to create disease models and to generate individual-specific hiPSCs that can differentiate into various cell types [27–29]. For livestock, iPSCs can be used to create gene-modified animals through nuclear transfer or generating chimeras. Gene targeting has been successfully performed with both miPSCs [28] and hiPSCs [30]. Chimeras of miPSCs [31] and rat-induced pluripotent stem cells (riPSCs) [14] were also generated successfully. As a

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result, iPSCs are a good substitute for both ESCs and somatic cells for researches and applications in other mammalian species [32].

As an ancient mammal, bats are the second largest group of mammals and live all over the world. Compared with other mammalian species of similar body size, the lifespan of bats is much longer [33,34]. It is thought that hibernation, high body metabolic rates, low radical generation, and low reproductive rates are the causes of the longevity of bats [33,35]. DNA mismatch repair systems, microsatellite instability, and antioxidant activity may be associated with bat longevity at the molecular level [36]. Sequencing analysis of the bat *Myotis brandtii* genome and transcriptome discovered that altered growth hormone/insulin-like growth factor-1 axis may have effect on longevity [37].

Bats are considered as natural virus reservoirs and support the replication of high titers of viruses *in vivo* without any clinical signs [38]. In the year 2013, severe acute respiratory syndrome coronavirus (SARS-CoV) ancestor was isolated and characterized in Chinese bats [39]. Whole-genome sequencing and comparative analyses of two distantly related bat species have been reported recently [40]. All these findings indicate that bats possess unique physiological characteristics and immune systems that can be utilized in biomedical research.

Because bats are not suitable for artificial rearing and bat embryonic fibroblasts (BEFs) can only be proliferated with limited passages, the successful generation of bat-induced pluripotent stem cells (biPSCs) would provide ideal and original materials to facilitate bat-related research at the cellular and molecular levels.

In this study, we have successfully derived biPSCs with a *piggyBac* (PB)-based vector, pSTEM-h103, containing eight stem cell factors. These cells have typical characteristics of high-quality ESCs or iPSCs. They should have broad applications for bat-related researches.

2. Materials and methods

2.1. Cell culture

Bat embryonic fibroblasts isolated from bat of *Myotis lucifugus* were obtained as a gift from Dr Mario Capecchi. They were cultured at 37 °C with 5% CO_2 in mouse embryonic fibroblast (MEF) medium. Mouse embryonic fibroblast medium consisted of Dulbecco's modified eagle medium, 15% fetal bovine serum, 0.5% GlutaMax, 1.0% non-essential amino acid, 1 mM sodium pyruvate, and 0.5% penicillin and streptomycin. Embryonic stem cell medium consisted of Dulbecco's modified eagle medium, 15% ESC grade fetal bovine serum, 0.5% GlutaMax, 1.0% non-essential amino acid, 1 mM sodium pyruvate, 0.5% penicillin and streptomycin, 1000 units/mL mouse leukemia inhibitory factor (LIF, Chemicon); 0.1 mM β-mercaptoethanol, and 50 μg/mL vitamin C. The 3i medium consisted of equal volume of Neurobasal medium and Dulbecco's modified eagle medium/F12, 0.5% N_2 supplement, 1.0% B27 supplement, 0.5% penicillin and streptomycin, 0.1 mM β-mercaptoethanol, 1 mM PD0325901 (Selleck); 3 mM CHIR99021 (Selleck); 0.5 μM A8301 (Tocris Biosciences), and 1000 units/mL mouse LIF. The concentration of G418 sulfate (Calbiochem) for BEFs after nucleofection was 500 μg/mL. The final concentration of Fialuridine (FIAU, Moravek) was used at 0.3 μM. All reagents used above were purchased from Gibco, unless otherwise noted.

2.2. Generation of biPSC lines

Vector construction. To construct PB-based pSTEM-h103, human cDNA for OCT4, KLF4, SOX2, cMYC, NR5A2, NANOG, LIN28, and bat-specific miR302/367 gene cluster were assembled onto the pSP72 (Promega) plasmid backbone using polymerase chain reaction (PCR), restriction enzyme–based cloning, and recombining. The CAG promoter-driven PBase was from the CAG-PBase vector, and the PB 5′ and 3′ terminals were from the ZGs vector. pCAG-PBase vector that expresses the PB transposase can insert the PB sequences into the biPSC genome [41]. Detailed maps and sequences are available on request.

The BEF culture was replenished with MEF medium every 24 hours until about 75% confluency. Lonza Amaxa Nucelofector II device electroporation program A024 for fibroblasts was used to deliver 4 μg of the inducing vector pSTEM-h103 and 2 μg PBase expressing vector pCAG-PBase simultaneously (Fig. 1A) into 10^5 BEFs. After transfections, BEFs were spread on 10-cm cell culture dishes with MEF feeder layers. The cells were cultured with ESC medium with G418 selection for 6 days. From the seventh day onward, the medium was substituted with 3i medium without G418. The cells were fed with fresh medium every 24 hours until iPSC colonies appeared (Fig. 1B). The colonies were picked with Pasteur pipettes under dissection microscope. Colonies were subsequently dissociated with 0.1% trypsin and passaged onto 24-well plates. When the cells reached about 60% to 75% confluency, they were individually passaged onto new six-well dishes and later expanded to 10-cm dishes.

2.3. Optimization of electroporation conditions for biPSCs

Bat-induced pluripotent cells were cultured on MEF feeder layers and passaged by trypsinization. About 1×10^5 iPSCs were suspended by Lonza electroporation buffer and transfected with 2 μg pMax-GFP (Fig. 2A) with Lonza Amaxa Nucelofector II device. We chose nine programs (A012, A013, A023, A024, A030, B016, T013, T016, and U013) to test electroporation efficiency for biPSCs.

2.4. Removal of PB sequences from biPSCs

pCAG-PBase vector was used to remove exogenous insertional sequences from the biPSC genome [41]. Bat-induced pluripotent cell lines were electroporated with pCAG-PBase vector and cultured with 3i medium supplemented with FIAU as negative screening drug. After 7 days, the survived cells formed colonies that could be picked and proliferated into dozens of cell lines. Southern blot analysis and PCR were performed to examine the removal effect.

2.5. Southern blot analysis and PCR

Southern blot analysis was performed with a PB probe using a protocol described earlier [40]. The genomic DNAs

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