Theriogenology xxx (2015) 1–16

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Putative embryonic stem cells derived from porcine cloned blastocysts using induced pluripotent stem cells as donors

Eunhye Kim [a], Seon-Ung Hwang [a], Hyunju Yoo [a], Junchul David Yoon [a], Yubyeol Jeon [a], Hyunggee Kim [c], Eui-Bae Jeung [a], Chang-Kyu Lee [b,**], Sang-Hwan Hyun [a,*]

[a] College of Veterinary Medicine, Chungbuk National University, Cheongju, Republic of Korea
[b] Department of Agricultural Biotechnology, Animal Biotechnology Major, and Research Institute for Agriculture and Life Science, Seoul National University, Seoul, Republic of Korea
[c] School of Life Sciences and Biotechnology, Korea University, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 6 July 2015
Received in revised form 20 September 2015
Accepted 28 September 2015

Keywords:
Embryonic stem cell
Induced pluripotent stem cell
Pluripotency
Pig

ABSTRACT

The establishment of porcine embryonic stem cells (ESCs) would have great impact in biomedical studies and preclinical trials through their use in genetic engineering. However, authentic porcine ESCs have not been established until now. In this study, a total of seven putative ESC lines were derived from porcine embryos of various origins, including *in vitro* fertilization, parthenogenetic activation, and, in particular, induced pluripotent stem (iPS) nuclear transfer (NT) from a donor cell with induced pluripotent stem cells (iPSCs). To characterize these cell lines, several assays including an assessment of intensive alkaline phosphatase activity, karyotyping, embryoid body formation, expression analysis of the pluripotency-associated markers, and the three germ layerassociated markers were performed. Based on quantitative polymerase chain reaction, the expression levels of *REX1* and *FGFR2* in iPS-NT lines were higher than those of cells of other origins. Additionally, only iPS-NT lines showed multiple aberrant patterns of nuclear foci elucidated by immunofluorescence staining of H3K27me3 as a marker of the state of X chromosome inactivation and a less mature form of mitochondria like naive ESCs, by transmission electron microscopy. Together, these data suggested that established putative porcine ESC lines generally exhibited a primed pluripotent state, like human ESCs. However, iPS-NT lines have especially unique characteristics distinct from other origins because they have more epigenetic instability and naive-like mitochondrial morphology than other putative ESC lines. This is the first study to establish and characterize the iPSC-derived putative ESC lines and compare them with other lines derived from different origins in pigs.

1. Introduction

Embryonic stem cells (ESCs) derived from mouse blastocysts have already been established [1] and have been used not only as a vehicle to generate knockout mice by homologous recombination [2] but also as a powerful tool that has provided valuable information on embryonic development [3]. However, these applications have been demonstrated only for mouse and rat ESCs [4,5], whereas other species, including pigs [6], cattle [7], rabbit [8], horse [9], and humans [10], have only been studied in limited capacities. Although pigs are regarded as the ideal biomedical model for human disease because of their immunological, physiological, and anatomical similarities with humans [11,12], only a few putative ESC lines have

* Corresponding author. Tel.: +82 43 261 3393; fax: +82 43 267 3150.
** Corresponding author. Tel.: +82 2 880 4805; fax: +82 2 873 4805.
E-mail addresses: leeck@snu.ac.kr (C.-K. Lee), shhyun@cbu.ac.kr (S.-H. Hyun).

0093-691X/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2015.09.051

been generated from preimplantation embryos, including *in vivo* embryos [13–16], and authentic porcine ESC lines have not been established.

It is thought that the most important key factors for obtaining stable authentic ESC lines are defining the optimum stage of embryonic development for derivation and ideal culture conditions for maintenance [17,18]. However, pigs have an extended period of preimplantation development compared with mice and humans; therefore, it is hard to determine the optimal timing to derive ESCs [19]. Furthermore, the key signaling pathways regulating early developmental processes in pigs that are essential for defining the culture conditions are not known, and there are some differences between species [20,21]. Indeed, the underlying porcine-specific mechanism that mediates the pluripotent state is still not well understood, and there are no specific tools for evaluating their state.

Meanwhile, induced pluripotent stem cells (iPSCs) have been generated by defined factors, such as Oct-4, Sox2, Klf4, and Myc (OSKM factors), in mice [22]. The success of this method had a profound impact on both basic biology and clinical applications by bypassing the ethical concerns related to ESCs. iPSCs have also been produced from other species, including humans [23,24] and pigs [25,26], and the ability to reprogram fibroblasts of different genetic backgrounds to generate iPSCs holds great promise for the development of autologous cell therapies. However, iPSCs also have a weakness compared with ESCs in terms of oncogene activation risks and developmental deficiencies that might occur because of insertional mutagenesis in iPSC-mediated clinical applications [27].

In recent years, there has been growing evidence demonstrating that pluripotent stem cells can be categorized according to their pluripotent state [28]. The distinct biphasic states, naive and primed, represent cells of the preimplantation embryo and later epiblast cells, respectively. It has been noted that the naive stage in human and porcine ESCs has been difficult to capture *in vitro*. Recently, several studies have reported the establishment of naive human ESC lines without the use of transgenes [29–32]. Despite the importance of the establishment of naive porcine ESC lines, which may be a critical experimental tool for the production of transgenic pigs and xenografting [33], research is lacking. Especially, patient-matched nuclear transfer-embryonic stem cells (NT-ESCs) are regarded as great sources for clinical applications, studies on the disease mechanism, and developing therapies [34]. However, porcine NT-ESCs have been reported only in a few published articles [14,35] and even those were not the naive NT-ESCs.

To overcome these obstacles, this study was conducted to transfer the nucleus of already reprogrammed iPSCs into enucleated oocyte and established porcine NT-ESC lines derived from cloned embryos (iPS-NT). Ultimately, we characterized iPS-NT lines compared with other embryonic origins, including *in vitro* fertilization (IVF) and parthenogenetic activation (PA) with regard to their pluripotent status.

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Veterinary and Quarantine Service. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Chungbuk National University (permit number: CBNUA-584-13-01). All sacrifice was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

2.2. Chemicals

Unless otherwise indicated, all chemicals and reagents used in the present study were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA).

2.3. Oocyte collection and in vitro maturation

Ovaries of prepubertal gilts in physiological saline supplemented with 100 IU/L penicillin G and 100 mg/mL streptomycin sulfate maintaining around 32 °C to 35 °C were collected at a local abattoir and transported to the laboratory within 2 hours from the time of collection. The cumulus oocyte complexes (COCs) were aspirated from 3- to 6-mm diameter of superficial follicles using an 18-gauge needle attached to a 10-mL disposable syringe and allowed to settle down as sediment in 15-mL conical tubes at 37 °C for 5 minutes. The supernatant was discarded, and the precipitate was resuspended with HEPES-buffered Tyrode's medium (TLH) containing 0.05% (wt/vol) polyvinyl alcohol (TLH-PVA). Then, the suspension was observed under a stereomicroscope to recover the COCs. Only COCs having three or more uniform layers of compact cumulus cells and a homogenous cytoplasm were selected and washed three times in TLH-PVA. Approximately, 60 COCs were placed into each well of a four-well Nunc dish (Nunc; Thermo Scientific, Leicestershire, UK) containing 500 μL of culture medium (tissue culture medium 199; Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 0.6 mM cysteine, 0.91 mM sodium pyruvate, 10 ng/mL epidermal growth factor, 75 μg/mL kanamycin, 1 μg/mL insulin, 10% (vol/vol) porcine follicular fluid, 10 IU/mL equine chronic gonadotropin, and 10 IU/mL human chronic gonadotropin (Intervet, Boxmeer, The Netherlands). The selected COCs were incubated at 39 °C with 5% CO_2 in 95% humidified air for *in vitro* maturation (IVM). After 21 to 22 hours of maturation with hormones, the COCs were washed two times in fresh hormone-free IVM medium and cultured in hormone-free IVM medium for an additional 21 to 22 hours.

2.4. In vitro fertilization

For IVF, the COCs were denuded 42 to 44 hours after IVM by gently pipetting with 0.1% hyaluronidase and were washed three times in TLH-PVA. Groups of 15 matured oocytes at the metaphase II (MII) stage were randomly placed into 40-μL droplets of modified Tris-buffered