Animal Reproduction Science 178 (2017) 40–49

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Evaluation of porcine stem cell competence for somatic cell nuclear transfer and production of cloned animals

Jan O. Secher[a,*], Ying Liu[b], Stoyan Petkov[c], Yonglun Luo[d], Dong Li[f], Vanessa J. Hall[f], Mette Schmidt[a], Henrik Callesen[b], Jacob F. Bentzon[e], Charlotte B. Sørensen[e], Kristine K. Freude[f], Poul Hyttel[f,*]

[a] Veterinary Reproduction and Obstetrics, Department of Large Animal Sciences, University of Copenhagen, DK1870 Frederiksberg C, Denmark
[b] Department of Animal Science, Aarhus University, DK8830 Tjele, Denmark
[c] Institute for Farm Animal Genetics (FLI), Neustadt, Germany
[d] Department of Biomedicine, Aarhus University, DK8000 Aarhus C, Denmark
[e] Department of Clinical Medicine, Aarhus University, DK8200 Aarhus N, Denmark
[f] Department of Veterinary Clinical and Animal Sciences, University of Copenhagen, DK1870 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 12 September 2016
Received in revised form 11 January 2017
Accepted 15 January 2017
Available online 19 January 2017

Keywords:
Pluripotency
Induced pluripotent stem cells
Embryonic germ cell
Transgenic pigs
Somatic cell nuclear transfer
Handmade cloning

ABSTRACT

Porcine somatic cell nuclear transfer (SCNT) has been used extensively to create genetically modified pigs, but the efficiency of the methodology is still low. It has been hypothesized that pluripotent or multipotent stem cells might result in increased SCNT efficacy as these cells are closer than somatic cells to the epigenetic state found in the blastomeres and therefore need less reprogramming. Our group has worked with porcine SCNT during the last 20 years and here we describe our experience with SCNT of 3 different stem cell lines. The porcine stem cells used were: Induced pluripotent stem cells (iPSCs) created by lentiviral doxycycline-dependent reprogramming and cultered with a GSK3β- and MEK-inhibitor (2i) and leukemia inhibitor factor (LIF) (2i LIF DOX-iPSCs), iPSCs created by a plasmid-based reprogramming and cultured with 2i and fibroblast growth factor (FGF) (2i FGF Pl-iPSCs) and embryonic germ cells (EGCs), which have earlier been characterized as being multipotent. The SCNT efficiencies of these stem cell lines were compared with that of the two fibroblast cell lines from which the iPSC lines were derived. The blastocyst rates for the 2i LIF DOX-iPSCs were 14.7%, for the 2i FGF Pl-iPSC 10.1%, and for the EGCs 34.5% compared with the fibroblast lines yielding 36.7% and 25.2%. The fibroblast- and EGC-derived embryos were used for embryo transfer and produced live offspring at similar low rates of efficiency (3.2 and 4.0%, respectively) and with several instances of malformations. In conclusion, potentially pluripotent porcine stem cells resulted in lower rates of embryonic development upon SCNT than multipotent stem cells and differentiated somatic cells.

1. Introduction

Dolly the sheep was the first mammal created by somatic cell nuclear transfer (SCNT) (Wilmut et al., 1997). This achievement paved the way for research within cell plasticity and cell reprogramming, which have been major

* Corresponding authors.
E-mail addresses: jbsecher@sund.ku.dk (J.O. Secher), poh@sund.ku.dk (P. Hyttel).

http://dx.doi.org/10.1016/j.anireprosci.2017.01.007
0378-4320/

research areas over the past two decades. SCNT has been implemented with great success to create non-murine genetically modified animals in several species, including the pig (Al-Mashhadi et al., 2013; Callesen et al., 2014; Onishi et al., 2000; Polejaeva et al., 2000). Unfortunately, the efficiency of SCNT is rather low (Liu et al., 2014). In the pig, only 1–5% of the reconstructed embryos transferred to surrogate sows result in live offspring (Lee et al., 2013; Liu et al., 2014; Schmidt et al., 2010). Additionally, a considerable proportion of the born piglets exhibits abnormalities (Schmidt et al., 2015) thereby reducing the efficiency even further (Schmidt et al., 2015, 2010).

Earlier studies have indicated that cells derived from early embryos result in increased developmental rates following SCNT (Oback, 2009). Thus, it was hypothesized that the use of pluripotent stem cells (PSCs) for SCNT would result in higher efficiencies and less abnormalities. Considerable efforts have been invested in deriving PSCs in the large domestic species including the pig, which is generally considered to be a valid model for humans due to physiological and anatomical similarities (Bassols et al., 2014; Lelovas et al., 2014). It has, however, proven to be a great challenge to maintain *bona fide* porcine embryonic stem cells (ESCs) and also the quest for derivation of porcine iPSCs has been associated with several complications including lack of transgene silencing and incomplete reprogramming and activation of the endogenous pluripotency network (reviewed by (Telugu et al., 2010)). Some of the problems with piPSC have been sought explained by comparing piPSC to naïve and primed murine embryonic stem cells (mESC). Naïve mESCs can be isolated from the murine ICM just before implantation and depend on leukemia inhibitor factor (LIF) and MEK- and GSK3β-inhibitors (2i) *in vitro* (Nichols and Smith, 2009) whereas primed murine ESCs can be isolated from the murine epiblast just after implantation and can be maintained *in vitro* by addition of fibroblast growth factor (FGF) to the media (Nichols and Smith, 2009). Only the naïve mESC can contribute to germline chimeras and they yield a higher efficiency of SCNT than primed mESC (Nichols and Smith, 2009). The first piPSCs generated (Esteban et al., 2009; Ezashi et al., 2009; Wu et al., 2009) resemble the primed type mESCs in regards to colony morphology and dependence on FGF. Subsequently, several groups have established piPSCs and cultured them supplemented with LIF and 2i to mimic the conditions which resulted in naïve murine ESCs (Cheng et al., 2012; Fujishiro et al., 2013; Telugu et al., 2011) and in cattle this has resulted in promising results (Kawaguchi et al., 2015). The use of both naïve-like and primed-like piPSCs for SCNT has been reported (Du et al., 2015; Fan et al., 2013; Yuan et al., 2014). Du et al. used naïve-like porcine iPSCs reprogrammed with a non-integrating episomal vector and cultured with LIF as well as GSK3β- and MEK-inhibitors (2i) but was unable to produce live piglets following SCNT (Du et al., 2015). Similar results were obtained by Fan et al. who used primed-like iPSCs reprogrammed with a doxycycline regulated lentiviral construct and cultured with FGF (Fan et al., 2013). Interestingly, Fan and co-workers also found that if they submitted the iPSCs to spontaneous differentiation prior to SCNT, they were able to achieve live piglets. This is in contrast to the results obtained in mice where the SCNT efficiency has been demonstrated to increase with differentiation potency of the cells used as nuclear donors (Nichols and Smith, 2009; Okita et al., 2011).

Here, we provide a description of our experience with SCNT using a transgenic male Large White/Landrace perinatal fibroblast line (WL-pNFs) expressing the Venus (green fluorescence) protein, a iPSC line derived from the WL-pNFs using a doxycycline regulated lentiviral construct and cultured with 2i and LIF (2i LIF DOX-iPSCs), a male wild type Göttingen embryonic fibroblast line (G-PEFs), a iPSC line derived from the G-PEFs (2i FGF Pl-iPSC) using the non-integrating episomal method (Okita et al., 2011) and cultured with FGF, and finally a Yucatan female transgenic porcine embryonic germ cell line (EGC) overexpressing the human wildtype PCSK9 (for an overview over the experiments and the cell lines, please see Fig. 1A–C and D). We compare initial embryonic development and results from embryo transfer to the hypothesis that the capacity of porcine cells to sustain embryonic development upon SCNT is positively correlated with their developmental potency, i.e. that PSCs are more capable of being reprogrammed than somatic cells and that multipotent stem cells might have an intermediate capacity.

2. Material and methods

2.1. Cells and iPSC reprogramming

Large White/Landrace porcine neonatal fibroblasts (WL-PNFs) carrying a transgene encoding the green fluorescent protein Venus with a constitutive promotor (Garrels et al., 2011) were kindly provided by Wilfried Kues and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM AQ; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% Penicillin/Streptomycin (Pen/Strep; Sigma Aldrich) and 10% fetal bovine serum (FBS; Hyclone, GE Health Care Life Sciences, Chicargo, IL, USA). The WL-PNFs were reprogrammed using a lentiviral construct as described previously (Secher et al., 2015). In brief, a doxycycline-regulated CAG promoter containing porcine *OCT4, c-MYC, SOX2 and KLF4* was used for lentiviral reprogramming of WL-PNFs into iPSCs (Nethercott et al., 2011). The resulting iPSC (2i LIF DOX-iPSCs) were maintained on mitomycin-C-treated mouse embryonic fibroblasts (MEFs) in DMEM/F12 medium (Sigma Aldrich) supplemented with 20% KnockOut Serum Replacement (Invitrogen, Waltham, MA, USA), 1x Pen/Strep (Sigma Aldrich), 1x nonessential amino acids (Sigma Aldrich), 100 μM b-mercaptoethanol (Life Technologies), 10 ng/mL leukemia inhibitory factor (LIF) (Millipore, Billerica, MA, USA), two kinase inhibitors (1 μM PD0325901 [Sigma Aldrich], 3 μM CHIR99021 [Sigma Aldrich]), and 2 μg/mL doxycycline (Sigma Aldrich) (piPSC medium). Cells were dissociated with 1x TrypLE (Gibco, Waltham, MA, USA) and passaged 1:6 every 7 days. Please notice that this cell line differs from the iPSC line reprogrammed with a doxycycline regulated lentivirus construct made by Fan et al. (Fan et al., 2013) and used for their SCNT experiments by the fact that we used 2i LIF culture conditions and they used FGF. A full characterization of the 2i LIF DOX-iPSC is in press (Secher et al., 2017).