Animal Reproduction Science xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Animal Reproduction Science

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

Influence of embryo handling and transfer method on pig cloning efficiency

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ARTICLE INFO

Article history:
Received 30 October 2014
Received in revised form
21 December 2014
Accepted 14 January 2015
Available online xxx

Keywords:
Cloned pigs
SCNT
Embryo transfer

ABSTRACT

The somatic cell nuclear transfer (SCNT) technique could be used to produce genetically superior or genetically engineered cloned pigs that have wide application in agriculture and bioscience research. However, the efficiency of porcine SCNT currently is very low. Embryo transfer (ET) is a key step for the success of SCNT. In this study, the effects of several ET-related factors, including cloned embryo culture time, recipient's ovulation status, co-transferred helper embryos and ET position, on the success rate of pig cloning were investigated. The results indicated that transfer of cloned embryos cultured for a longer time (22–24 h vs. 4–6 h) into pre-ovulatory sows decreased recipient's pregnancy rate and farrowing rate, and use of pre-ovulatory and post-ovulatory sows as recipients for SCNT embryos cultured for 22–24 h resulted in a similar porcine SCNT efficiency. Use of insemination-produced in vivo fertilized, parthenogenetically activated and in vitro fertilized embryos as helper embryos to establish and/or maintain pregnancy of SCNT embryos recipients could not improve the success rate of porcine SCNT. Transfer of cloned embryos into double oviducts of surrogates significantly increased pregnancy rate as well as farrowing rate of recipients, and the developmental rate of transferred cloned embryos, as compared to unilateral oviduct transfer. This study provided useful information for optimization of the embryo handling and transfer protocol, which will help to improve the ability to generate cloned pigs.

1. Introduction

Pigs are important livestock for agriculture and valuable animal models for bioscience research. Since the first production of cloned pigs by somatic nuclear transfer (SCNT) reported in 2000 by three groups (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000), the SCNT technique has been widely used to generate genetically modified or genetically superior cloned pigs, for bioscience research or agricultural purposes (Prather et al., 2008;

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http://dx.doi.org/10.1016/j.anireprosci.2015.01.006
0378-4320/

Klymiuk et al., 2010; Schmidt et al., 2010; Niemann and Lucas-Hahn, 2012; Vajta and Callesen, 2012). Nevertheless, the pig SCNT efficiency currently is still very low. The full-term developmental rate of cloned swine embryos is only at about 1–3% (Whitworth and Prather, 2010; Zhao et al., 2010; Liu et al., 2014), which is much lower than the approximately 75% full-term developmental ability of in vivo fertilized porcine embryos (Geisert and Schmitt, 2002). The low success rate limits practical application of pig SCNT technique in the swine industry and bioscience research.

For the whole SCNT procedure, embryo transfer (ET) is a key step that does determine the outcome of SCNT. Optimization of the ET protocol may significantly increase the ability to produce cloned pigs. It has been reported that some factors related to ET could affect the success rate of pig cloning, such as recipient breed (Koo et al., 2009), recipient's ovulation status (Petersen et al., 2008; Koo et al., 2010; Huang et al., 2013), in vitro culture time of transferred cloned embryos (Rim et al., 2013; Liu et al., 2014), transferred cloned embryo number per surrogate (Schmidt et al., 2010; Li et al., 2013; Rim et al., 2013), and embryo transfer position (Schmidt et al., 2010; Rim et al., 2013). Since the developmental ability of cloned procine embryos is extremely low, transfer of cloned embryos alone into surrogates may not be able to efficiently establish and/or maintain gestation of recipient, some studies have tried to use in vivo fertilized (IVV) or parthenogenetically activated (PA) embryos as helper embryos to assist transferred cloned embryos to establish and/or maintain pregnancy of recipients (Onishi et al., 2000; De Sousa et al., 2002; Lai et al., 2002). However, these studies were usually done unsystematically or by only using a relatively small number of recipients. In the current study, we aimed to systematically investigate the effects of cloned embryo culture time, recipient's ovulation status, co-transferred helper embryos and ET position on the success rate of pig SCNT, by using a very large number of recipient sows.

2. Materials and methods

2.1. Ethics statements

This study was carried out in Guangdong province (located at the south part of China) of China, and in strict accordance with "The Instructive Notions with Respect to Caring for Laboratory Animals," issued by the Ministry of Science and Technology of China. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of South China Agricultural University. All efforts were made to minimize animal suffering.

2.2. Experimental design

A total of 206,716 SCNT embryos generated from genetically superior adult boars were transferred into 830 recipient sows. Five experiments including 11 experimental groups were conducted in this study to test the effect of embryo culture time, recipient's ovulation status, IVV, PA, and IVF helper embryos, and embryo transfer position on the efficiency of pig cloning. The detailed embryo transfer protocols for each experimental group were summarized in Table 1.

2.3. Donor cell isolation, ovary collection, and oocyte maturation

Adult ear fibroblasts were isolated from about 2 year-old, genetically proven superior Pietrain boars (white-black spotted coat) or Duroc boars (red coat), as previously described (Deng et al., 2011). Isolated fibroblasts were frozen in liquid nitrogen. Before SCNT, fibroblasts were thaw and cultured for 8–10 days in DMEM (Gibco) supplemented with 10% (v/v) FBS (Gibco) at 39 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cultured cells at passages 3–8 were used for SCNT. The porcine ovaries used in this study were from white coat hybrid gilts (Duroc × Yorkshire × Landrace), and were collected from The Guangzhou Tianhe slaughterhouse located at Tianhe district, Guangzhou city, P.R. China. We obtained permission from this slaughterhouse to use the porcine ovaries for SCNT experiments in our study. Cumulus–oocyte complexes (COCs) were aspirated from the ovaries and matured in vitro for 42–44 h following the protocol described by Deng et al. (2011). Matured COCs were freed from cumulus cells by repeated pipetting in 0.1% hyaluronidase. Matured oocytes with the first polar body were selected for enucleation.

2.4. Preparation of somatic cell nuclear transfer (SCNT) embryos

The SCNT experiment was performed following the protocol described by us (Li et al., 2013). Briefly, the matured oocyte was sucked firmly onto the holding pipette (outer diameter = 100–120 μm, inner diameter = 20–30 μm) so that it does not move. The enucleation pipette (inner diameter = 15 μm) was inserted through the zona pellucida, and the first polar body and adjacent cytoplasm, presumably containing all the chromosomes, were aspirated into the enucleation pipette. Then a single fibroblast cell, which was separated by pipetting after digestion with 0.256% trypsin, was microinjected into the perivitelline space of the oocytes. The oocyte–donor cell complexes were cultured in porcine zygote medium 3 (PZM3) (Yoshioka et al., 2003) at 39 °C, 5% CO_2, 5% O_2, 90% N_2, and 100% humidity for 1.5 h and then activated to fuse in a medium containing 250 mM mannitol, 0.1 mM $CaCl_2 \bullet 2H_2O$, 0.1 mM $MgCl_2 \bullet 6H_2O$, 0.5 mM Hepes, and 0.01% PVA, by two successive DC pulses at 1.2 kV/cm for 30 μs using an electro-fusion instrument (model: CF-150/B, BLS company, Budapest, Hungary). The activated cloned embryos were then cultured in PZM3 medium containing Cytochalasin B (5 μg/ml) for 4 h. After the post-activation treatment, the reconstructed embryos were then cultured in PZM3 medium at 39 °C, 5% CO_2, 7% O_2, 88% N_2, and 100% humidity.

2.5. Preparation of parthenogenetically activated (PA) embryos

In vitro matured oocytes from white coat hybrid gilts were activated by a single 80 μs DC pulse at 1.4 kV/cm,

Please cite this article in press as: Shi, J., et al., Influence of embryo handling and transfer method on pig cloning efficiency. Anim. Reprod. Sci. (2015), http://dx.doi.org/10.1016/j.anireprosci.2015.01.006