Livestock Science 121 (2009) 234–238

Contents lists available at ScienceDirect

Livestock Science

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

Somatic cell nuclear transfer with recipient oocytes derived from ovum pick up

Xiao-Yu Yang [a,c,d,1], Ming-kai Ji [b,1], Xia Liu [c], Yan-Fang Huang [b], Geng Zhang [a], Rong-Qing Xu [a], Jian-Feng Yao [a], Jian-yin Lin [a], Ming Zheng [a,*]

[a] College of Preclinical Medicine, Fujian Medical University, Fuzhou 350004, PR China
[b] The Affiliated First Hospital, Fujian Medical University, FuZhou 350005, PR China
[c] The Maternal and Child Health Care Hospital of JiaXing, Jiaxing University, Zhejiang 314000, PR China
[d] Center of Cell Developmental Biology, Fujian Medical University, Fuzhou 350004, PR China

ARTICLE INFO

Article history:
Received 2 April 2008
Received in revised form 19 June 2008
Accepted 20 June 2008

Keywords:
Heifer
Nuclear transfer
Ovum pick up
Embryo

ABSTRACT

The aim of this experiment was to determine whether it was feasible to produce embryos using a combination of somatic cell nuclear transfer (SCNT) and ovum pick up (OPU) and to determine whether use of transfected donor cells for cloning alters developmental potential of embryos. Heifers were subjected to OPU once weekly. In total, 1861 oocytes were recovered from 2436 follicles and recovery rate was 76.4% (7.8±0.4 oocytes/session per animal). Oocytes were then subjected to SCNT (cumulus cell-NT, CC-NT; transfected cumulus cell-NT, TCC-NT) and *in vitro* fertilization (IVF). Cloned embryos derived from transfected donor cells had a similar *in vitro* developmental competence and a lower *in vivo* developmental competence compared with non-transgenic cloned embryos. Three cloned calves were obtained and survived for more six months. In conclusion, the combination of SCNT and OPU is feasible and genetic modification may exert a negative effect on pregnancy rates derived using the present protocol.

1. Introduction

Although various species have been cloned using somatic cell nuclear transfer (SCNT), the overall efficiency of somatic cloning is low. Incomplete epigenetic reprogramming of the somatic nucleus has been proposed as one cause of this inefficiency because of developmental failure (Jeon et al., 2008). Two elements of the SCNT process probably have an effect on reprogramming efficiency: the state of the donor cell/nucleus and the suitability of the recipient cytoplast (Aston et al., 2006). The fundamental importance of oocyte cytoplasmic factors for reprogramming of transferred nuclei and early embryonic development is well recognized (Kang et al., 2003).

Nuclear transfer is a useful tool for basic research into cell biology and reprogramming. The bovine embryo is a good model for this research because of the late activation of its genome and late implantation as compared to the mouse model (Heyman, 2005). Also, bovine somatic cloning has some potential applications in biomedicine in association with transgenesis and in agriculture for improving livestock. Since 1998 when the first calves derived from somatic cell nuclear transfer were born (Cibelli et al., 1998), many experiments of bovine somatic nuclear transfer have been performed (Thongphakdee et al., 2008; Yang et al., 2008). In nearly all of these experiments, oocytes used for cloning were obtained from the ovaries of slaughtered cows so that genetic background of the oocytes was usually unknown. Such an approach would be unsuitable when oocytes with defined genomes or defined sources are required. A good method for obtaining oocytes from a defined source is to use the ovum pick up (OPU) technology which allows harvesting of oocytes from defined individual cattle (De Roover et al., 2008).

The effectiveness of OPU for *in vitro* production of calves has been demonstrated by the large number of calves that have been born derived from oocytes recovered by OPU and

* Corresponding author. Fujian Medical University, NO.88 Jiao Tong Road, Fuzhou 350004, PR China. Tel.: +86 591 83569021; fax: +86 591 22862031.
E-mail address: zhengming1108@yahoo.cn (M. Zheng).
[1] Xiao-Yu Yang and Ming-kai Ji contributed equally to this work.

1871-1413/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.livsci.2008.06.029

used *in vitro* fertilization (IVF) (van Wagtendonk-de Leeuw, 2006). The objective of the present study was to determine whether oocytes obtained by OPU can be used to produce cloned bovine. Another objective was to determine whether use of transfected cells as donor karyoplasts affects developmental capacity *in vitro* and *in vivo* of cloned embryos. Transgenic cells could represent a powerful tool for the production of transgenic livestock with higher production efficiency compared with calves derived by traditional pronuclear injection (Lai et al., 2002).

2. Materials and methods

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.1. Animals

Ten Holstein heifers, between 13 and 14 months of age with a similar weight and health condition, were used for OPU. The heifers were housed in a barn and fed a mixed ration consisting of hay and a commercial feed concentrate. The investigation was conducted in accordance with the protocols and guidelines for agricultural animal research imposed by the Committee for Ethics of Shanghai.

2.2. Ovum pick up

All cattle were subjected to OPU once weekly. The OPU sessions were performed for 20 weeks for the NT experiment (cumulus cell-NT, CC-NTgroup; transfected cumulus cell-NT, TCC-NT group, ten weeks respectively) and 4 weeks for the IVF experiment. OPU in a week for which groups is decided at random. The OPU was done with a portable ultrasound machine (SSD-500; Aloka Co., Tokyo Japan) with a sector scanner transducer (7.5 MHz) and needle guide. Briefly, after emptying the rectum and thoroughly cleaning the vulva and perineal area, the transducer was advanced to the external os of the cervix. When the targeted follicles were stabilized on the puncture line, an 18-gauge needle was inserted in the guide, advanced through the vaginal wall and into the follicle antrum. Follicles (>2 mm in diameter) were aspirated using continuous negative pressure (about 95 mm Hg). The system was rinsed with D-PBS (Gibco, Grand Island, NY, USA; pH 7.4) medium containing 3% BSA and 2 IU/ml heparin.

2.3. Oocyte categorization and in vitro maturation

Fluids recovered from the OPU procedure were filtered through an embryo filter (Fujihiraindustry Co., Tokyo Japan) and cumulus–oocyte complexes (COCs) were identified with the aid of a stereomicroscope. The COCs were classified into three categories (Grades A, B, and C) as described by Neglia et al. (2003). Briefly, Grades A or B oocytes had a uniform cytoplasmic appearance and were enclosed within three (Grade B) or more (Grade A) layers of viable compact granulosa cells, whereas Grade C oocytes had less than three layers of granulosa cells or were partially denuded.

All oocytes (including grades A–C) were matured in TCM-199 (Gibco, Grand Island, NY, USA), supplemented with 10% fetal calf serum (FCS), 10 μg/ml luteinizing hormone(LH), 1 μg/ml estradiol (E2), and 1 μg/ml follicle-stimulating hormone (FSH) under a humidified atmosphere of 5% CO_2 in air at 38.5 °C.

2.4. Culture and transfection of donor cells

Oocytes were collected from a single Holstein cow. Twenty-hours after the onset of maturation, cumulus cells were isolated by pipetting in PBS supplemented with 2.5 mg/ml hyaluronidase transferred to DMEM/F12 (Gibco) containing 10% (v/v) FCS for use as donor cells. The fresh cumulus cells were cultured for several passages for transfection and cryopreserved for future use.

Cumulus cells (2–4 passages) were transfected with a DNA vector containing 1) a human factor IX (hFIX) gene under the control of the β-casein gene promoter and 2) a neomycin resistance gene downstream of hFIX. The neomycin resistance gene allowed the screening of the transgenic cell lines with G418. The linearization of DNA vector was digested with Sal I and then used for transfection.

Cumulus cells were harvested by trypsinization at 70–80% confluence and resuspended to $2–3\times10^6$ cells/ml with medium HeBS (140 mmol/L NaCl, 5 mmol/L KCl, 0.75 mmol/L Na_2HPO_4, 6 mmol/L glucose, 25 mmol/L Hepes) containing 10 μg/ml DNA. Cells were exposed to one double electric pulse of 480 v/cm for 4 ms using the BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA) for transfection. After 48 h of transfection, cells were placed in medium containing G418 (800 μg/ml) for 10–14 d until the appearance of the "mono-colony" cell population. Each cell population was expanded by consecutive passages while cultured in medium containing 300 μg/ml G418, and tested with PCR at second passage after transfection.

2.5. Nuclear transfer, activation and embryo culture

All matured oocytes were used for nuclear transfer. Cumulus cells and transfected cells were cultured at 70–80% confluences in DMEM/F12 under a humidified atmosphere of 5% CO_2 in air at 38.5 °C, and then rendered quiescent by culture in DMEM/F12 containing 0.5% (v/v) FCS for 2 to 4 d. After 20 h of *in vitro* maturation, oocytes were transferred into microdrops of TCM-199 supplemented with 5 μg/ml cytochalasin B and 10% FCS. Enucleation was performed with a 20 μm (i.d.) glass pipette by aspirating the first polar body and a small amount of surrounding cytoplasm. After enucleation, the donor cell was introduced through the same slit in the zona pellucida and wedged between the zona pellucida and the cytoplasm

Table 1
Characteristics of aspiration sessions, aspirated follicles and recovered oocytes

Characteristics	CC-NT	TCC-NT	IVF	Total
Number of aspiration session	100	100	40	240
Number of aspiration follicles	1010	1012	414	2436
Categories of follicles (%)				
2–3 mm	455(45)	435(43)	186(45)	1076(44)
4–7 mm	444(44)	445(44)	168(41)	1057(43)
>8 mm	111(11)	132(13)	60(14)	303(12)
Total number of recovered oocytes	768	781	312	1861
Recovery rate %	76.0	77.2	75.3	76.4
oocytes per session	7.7±0.3	7.8±0.5	7.8±0.4	7.8±0.4

CC-NT: cumulus cell-NT; TCC-NT: transfected cumulus cell-NT; IVF: *in vitro* fertilization.