Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 71 (2009) 1026–1034
Theriogenology
www.theriojournal.com

First birth of an animal from an extinct subspecies (*Capra pyrenaica pyrenaica*) by cloning

J. Folch [a,1,*], M.J. Cocero [b,1], P. Chesné [c], J.L. Alabart [a,1], V. Domínguez [b], Y. Cognié [d], A. Roche [a], A. Fernández-Árias [a], J.I. Martí [a], P. Sánchez [a], E. Echegoyen [a], J.F. Beckers [e], A. Sánchez Bonastre [f], X. Vignon [c]

[a] *Centro de Investigación y Tecnología Agroalimentaria de Aragón, Av. Montañana 930, 50059 Zaragoza, Spain*
[b] *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Av. Puerta de Hierro s/n, 28040 Madrid, Spain*
[c] *INRA, Biologie du Développement et Reproduction, 78352 Jouy-en-Josas, France*
[d] *INRA, Physiologie de la Reproduction, 37380 Nouzilly, France*
[e] *Physiology of Reproduction, Faculty of Veterinary Medicine, University of Liège, Belgium*
[f] *Departament de Patologia i Producció Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra, Spain*

Received 8 September 2008; received in revised form 14 November 2008; accepted 19 November 2008

Abstract

Two experiments have been performed to clone the bucardo, an extinct wild goat. The karyoplasts were thawed fibroblasts derived from skin biopsies, obtained and cryopreserved in 1999 from the last living specimen, a female, which died in 2000. Cytoplasts were mature oocytes collected from the oviducts of superovulated domestic goats. Oocytes were enucleated and coupled to bucardo's fibroblasts by electrofusion. Reconstructed embryos were cultured for 36 h or 7 d and transferred to either Spanish ibex or hybrid (Spanish ibex male × domestic goat) synchronized recipients. Embryos were placed, according to their developmental stage, into the oviduct or into the uterine horn ipsilateral to an ovulated ovary. Pregnancy was monitored through their plasmatic PAG levels. In Experiment 1, 285 embryos were reconstructed and 30 of them were transferred at the 3- to 6-cells stage to 5 recipients. The remaining embryos were further cultured to day 7, and 24 of them transferred at compact morula/blastocyst stage to 8 recipients. In Experiment 2, 154 reconstructed embryos were transferred to 44 recipients at the 3- to 6-cells stage. Pregnancies were attained in 0/8 and 7/49 of the uterine and oviduct-transferred recipients, respectively. One recipient maintained pregnancy to term, displaying very high PAG levels. One morphologically normal bucardo female was obtained by caesarean section. The newborn died some minutes after birth due to physical defects in lungs. Nuclear DNA confirmed that the clone was genetically identical to the bucardo's donor cells. To our knowledge, this is the first animal born from an extinct subspecies.

Keywords: Bucardo; Interspecies; Nuclear transfer; Spanish ibex; Wild goat

1. Introduction

The bucardo (Pyrenean ibex; *Capra pyrenaica pyrenaica*) was one of the four subspecies of the Spanish ibex (Cabra Montés—*Capra pyrenaica*) identified in 1910 by Cabrera [1] according to morphological characteristics. Two subspecies of Spanish ibex are at

* Corresponding author. Tel.: +34 976 71 6436; fax: +34 976 71 6335.
E-mail address: jfolch@aragon.es (J. Folch).
[1] These authors contributed equally.

0093-691X/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.theriogenology.2008.11.005

present free-living in Spain: *Capra pyrenaica victoriae*, living in Central and Northwest Spain and *Capra pyrenaica hispanica* living both in the South and Eastern Spanish Mediterranean Mountains. The Portuguese ibex (*Capra pyrenaica lusitanica*) became extinct in 1892 [1]. The bucardo population was abundant in the Pyrenees, but decreased very quickly along the last two centuries supposedly due to high hunting pressure. In the second half of the 20th century only a scarce population was living in the National Ordesa Park situated in the Spanish Central Pyrenees. All the *in situ* attempts to stop the declining of the population by natural methods, such as strategies of food supply, were ineffective. As a consequence, in 1989, the EU and the local Aragon Government underwent a Project to capture all available population of bucardos, with the aim to multiply them by assisted reproduction in captivity, but at this time only three old females and no males were living. Genetic studies of this population showed an extremely low variability in the MHC [2], which may in part explain the bucardo's decline. Natural hybridization of the three remaining females with *C. p. hispanica* fertile males was attempted. Although the levels of faecal estrogens and progesterone indicated that pregnancies took place in two females (Alabart, unpublished data), no live kids were observed. In 1999 only one bucardo female of about 12-year old was living. In a final attempt to preserve the bucardo's genetic resources, we captured this last specimen and cells from a skin biopsy were obtained, multiplied and kept frozen in liquid nitrogen. This animal was *in situ* released just after biopsy and died in 2000. Therefore, the Spanish Government recently declared the bucardo extinct [3]. It is also currently listed as extinct by the IUCN Red List.

Previous studies on interspecies nuclear transfer (NT) followed by embryo transfer into domestic recipients resulted on live offspring, both in gaur (*Bos gaurus*) [4] and mouflon (*Ovis orientalis musimon*) [5]. It has not been proved that the obtained clones can reach the adult stage. The cloned Gaur survived for only few days and the cloned mouflon survived for at least 7 months [6], but no more information is available to our knowledge. In spite of this, we attempted a similar approach for the bucardo, using the cryopreserved cells, since cloning is the only possibility to avoid its complete disappearance. This paper presents the results obtained in this study.

2. Materials and methods

Embryos were reconstructed by fusion of epithelial bucardo's cells and enucleated oocytes of domestic goats, and transferred to pure Spanish ibex or hybrids (Spanish ibex × domestic goats).

Unless otherwise specified, all materials were obtained from Sigma–Aldrich. All experimental procedures are in accordance with the current European Directive 86/609/EEC (DOCE number 358).

2.1. Preparation of karyoplasts

Donor cells were derived from a skin biopsy of the last specimen and grown from explants as described in Ref. [7]. The cells grown out of the explants were trypsinized and seeded in new culture dishes after 2 weeks (passage 1). In the following steps, cells were passaged at subconfluency and a portion was frozen for long-term storage. Fibroblasts were characterized by indirect immunofluorescence using an anti-vimentin antibody (V9 clone; Chemicon). All cultures were conducted in DMEM supplemented with 10% foetal calf serum at 38.5 °C and 5% CO_2 in humidified air. Nuclear transfer experiments were performed using cells at passage 3, which were maintained confluent for at least 72 h in DMEM–10% FCS before NT. Cells for NT were collected by trypsinisation and kept in suspension in culture medium at room temperature for 20–120 min before being transferred to the manipulation chamber.

2.2. Superovulation and collection of oocytes

Mature oocytes were collected from domestic goats that were superovulated using highly purified porcine FSH and LH (Laboratory of Endocrinology, Faculty of Veterinary Medicine, University of Liège, Belgium). Thirty adult, mixed breed goats were synchronized by 45 mg fluorogestone acetate (FGA) sponges (Intervet) during 11 d. Superovulation was achieved with 6 intramuscular doses of pFSH (4, 4, 2, 2, 2 and 2 mg) at 12 h intervals, starting 48 h before sponge withdrawal. Cloprostenol (75 μg; Estrumate, Schering-Plough) was administered at the first FSH injection and two doses of 66 μg pLH were applied at the 5th and 6th FSH injections. Ovulations were synchronized by an intravenous injection of 50 μg of LHRH (SIGMA, L-7134) applied 32 h after sponge withdrawal. Oocytes were collected under general anaesthesia by retrograde flushing of the oviducts with Dulbecco's Modified PBS, supplemented with BSA (2 g l^{-1}), 28–34 h after LHRH injection [8]. Oocytes were transferred to M199 medium supplemented with 10% FCS. Oocytes with attached cumulus cells were denuded by exposure to 0.5 mg ml^{-1} hyaluronidase in M199–HEPES (20 mM)