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Piglets born from handmade cloning, an innovative cloning method without micromanipulation

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Abstract

Porcine handmade cloning (HMC), a simplified alternative of micromanipulation based traditional cloning (TC) has been developed in multiple phases during the past years, but the final evidence of its biological value, births of piglets was missing. Here we report the first births of healthy piglets after transfer of blastocysts produced by HMC. As a cumulative effect of technical optimization, 64.3 ± 2.3 (mean ± S.E.M.) reconstructed embryos from 151.3 ± 4.8 oocytes could be obtained after 3–4 h manual work, including 1 h pause between fusion and activation. About half (50.1 ± 2.8%, *n* = 16) of HMC reconstructed embryos developed to blastocysts with an average cell number of 77 ± 3 (*n* = 26) after 7 days *in vitro* culture (IVC). According to our knowledge, this is the highest *in vitro* developmental rate after porcine somatic cell nuclear transfer (SCNT). A total of 416 blastocysts from HMC, mixed with 150 blastocysts from TC using a cell line from a different breed were transferred surgically to nine synchronized recipients. Out of the four pregnancies (44.4%) two were lost, while two pregnancies went to term and litters of 3 and 10 piglets were delivered by Caesarean section, with live birth/transferred embryo efficiency of 17.2% (10/58) for HMC. Although more *in vivo* experiments are still needed to further stabilize the system, our data proves that porcine HMC may result in birth of healthy offspring. Future comparative examinations are required to prove the value of the new technique for large-scale application.
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1. Introduction

Production of genetically modified pigs attracts increasing interests in medical research. Pigs seem to be superior to traditional laboratory animals for human

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medical research. Due to the great similarity in organ size, physiology, metabolism and also genetics between human and pig, genetically modified pigs could provide animal models for human diseases and may become sources for tissues/organs for xenotransplantation. Among the various approaches [1,2], currently the most efficient way to perform targeted genetic modifications in pigs is SCNT. However, serious obstacles hamper the application of SCNT including low efficiency and high costs for TC because of the need for micromanipulation.

An alternative cloning technology called HMC has been developed in cattle [3–6]. It provides a much simpler and quicker procedure for bovine SCNT with comparable *in vitro* and *in vivo* efficiencies. HMC may even offer the possibility for automation of the cloning procedure since almost every crucial step of HMC can be performed by microfluidic technique [7]. During the past years, attempts have been made to establish an efficient porcine HMC system, as well [8–10]. The seemingly simple application of an existing technology to another species encountered many technical difficulties related to the fragility of porcine MII phase oocytes and their lower buoyant density related to the higher lipid content.

Another potential obstacle for porcine HMC lies in embryo transfer. In this species, more good quality embryos (for example, at least 20 *in vitro* fertilized blastocysts) are required for establishing early pregnancy [11] compared to the one or two required in cattle. However, IVC of porcine embryos is a demanding task, therefore most research group prefer transfer of large number (50–150) of 0–2 days embryos after SCNT performed with TC [12]. However, the zona-free approach requires extended IVC and blastocyst transfer to avoid separation of blastomeres from precompacted stage embryos in the oviduct. For these reasons, achieving pregnancies and offspring seems to be more demanding after porcine than bovine HMC.

However, Wu et al. [13] published the birth of piglets after transferring IVC blastocysts produced from *in vitro* fertilized (IVF) zona-free oocytes. Another zona-free SCNT approach - still based on micromanipulation for enucleation - has also resulted in high numbers of offspring in cattle [14], sheep [15], horses [16], mouse [17] and very recently pigs [18]. These successes offered a realistic perspective of producing piglets from blastocysts produced by HMC.

The objectives of this study were to (1) establish efficient and reliable *in vitro* porcine HMC system based on our previous achievements, and (2) to produce viable offspring after porcine HMC. For positive control, blastocysts produced with TC by using donor cells from a different breed were also transferred into the same recipients.

2. Materials and methods

Except where otherwise indicated all chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

All animal care-related procedures described in present study were reviewed and approved by the Danish Animal Inspectorate.

2.1. Oocyte collection and in vitro maturation (IVM)

Cumulus-oocyte complexes (COCs) were aspirated from 2 to 6 mm follicles from slaughterhouse-derived sow ovaries. COCs were matured in groups of 50 in 400 μl bicarbonate-buffered TCM-199 (GIBCO BRL) supplemented with 10% (v:v) cattle serum (CS), 10% (v:v) pig follicular fluid, 10 IU/ml eCG, 5 IU/ml hCG (Suigonan Intervet; Skovlunde, Denmark) at 38.5 °C in the "Submarine Incubation System" (SIS) [19] in 5% CO_2 in humidified air for 41–44 h.

2.2. Handmade cloning (HMC)

Forty one hours after the start of IVM, the cumulus investment of the COCs was removed by repeated pipetting in 1 mg/ml hyaluronidase in Hepes-buffered TCM-199. From this point (except where otherwise indicated) all manipulations were performed on a heated stage adjusted to 39 °C, and all drops used for handling oocytes were of 20 μl covered with mineral oil. Zona removal and oriented enucleation were performed with a procedure based on partial digestion of the zona pellucida, as described earlier [10,20]. Oocytes were shortly incubated in 3.3 mg/ml pronase dissolved in T33 (T for Hepes-buffered TCM 199 medium; the number means percentage (v:v) of CS supplement, here 33%) for 20 s, then quickly washed in T2 and T20 drops. Oocytes with partially digested but still visible zona were lined up in drops of T20 supplemented with 2.5 μg/ml cytochalasin B (CB). With a finely drawn and fire-polished glass pipette, oocytes were rotated to find the polar body on the surface, and oriented bisection was performed manually under stereomicroscope control with a microblade (AB Technology, Pullman, WA, USA). Thus, less than half of the cytoplasm close to the polar body was removed from the remaining putative cytoplast. Cytoplasts were washed twice in T2 drops and collected in a T10 drop.

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