High developmental potential in vitro and in vivo of cattle embryos cloned without micromanipulators

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Abstract

Purpose In order to simplify cloning, a new method that does not require micromanipulators was used. We aimed to evaluate the developmental potential of two bovine cell lines upon cloning.

Materials and methods In vitro matured bovine oocytes, were released from zona pellucida, enucleated, fused to foetal or adult somatic donor cells. The reconstructed embryos were reprogrammed, activated and cultured until blastocyst stage. No micromanipulators were used. Blastocyst rate and quality was scored. Some expanded (d7) blastocysts were transferred to recipient cattle and collected back at d17 to assess elongation.

Results High developmental potential in vitro of cloned embryos to expanded (d7) blastocysts was achieved (52.6%). In one cell line, 65.7% of blastocysts was scored. Most blastocysts (87.4%) were graded as excellent. In vivo development to elongation (day-17) in temporary recipient cows also showed a high developmental potential (11/18 transferred blastocysts elongated).

Conclusions Hand-made cloning is an efficient alternative for cloning in cattle.

Keywords Blastocysts · Cloning · Nuclear reprogramming · Zona free embryos

Introduction

Low efficiency of somatic cell nuclear transfer (SCNT), together with biological complexity of the process are at present the major drawbacks of the technology. Attempts to simplify cloning had been made [1–4] the main focus being to avoid the use of expensive equipment, such as micromanipulators, inverted microscopes and pulling and beveling devices for microinstruments. All these changes are included in the so called hand made cloning (HMC) approach developed originally in 2001 [5–7]. In spite of the benefits of HMC and of its potential applications to other fields of assisted reproduction, such as efficient derivation of embryonic stem cells [8] still few groups are using it. From our point of view, wide spread use of HMC and international communication of positive results would contribute to a more reliable, automatable and cost effective method of nuclear transfer in animals. The implications of this include the possibility to develop more efficiently embryonic stem cells, optimization of culture conditions for individual embryos and the contribution to potentially similar protocols in the human clinic practice [9]. In this paper we report the production of bovine cloned embryos from foetal or adult cells with unusual high efficiency and with striking repeatability. The ultimate developmental potential of the cloned embryos was assessed by bulk embryo transfer and recovery from temporary recipient cattle, in which also a high efficiency of development to elongated, peri-implantation stages was achieved, thus showing that HMC is indeed a reliable and simplified method for nuclear transfer in bovine.

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Table 1 In vitro development to blastocysts of nuclear transfer embryos produced from a foetal and adult cell line

Source of cells	Oocytes			Blastocysts (%)	# of repeats	Range of blastocysts (and of %)
	Enucleated	Fused	Cultured ^a			
Foetal (FCL)	465	221	212	81 (38.2)	8	5–13 (20–60)
Adult (AWC)	571	247	236	155 (65.7)	8	16–24 (48.5–75)

^a It is referred to reconstructed embryos cultured in vitro after fusion, reprogramming and activation

Materials and methods

Preparation of recipient cytoplasts Immature intrafollicular oocytes were aspirated from bovine ovaries and matured in vitro for 21 h in M199 supplemented with follicle-stimulating hormone (0.01 U/mL), luteinizing hormone (0.01 U/mL), 17 β estradiol (1 μ g/mL) and epidermal growth factor (10 ng/mL) at 39°C in 5% CO₂ atmosphere. At this time, oocytes were treated with demecolcine (0.4 μ g/ml) to induce protrusion of the mitotic cones, and released from Zona by pronase digestion. Enucleation was achieved by bisecting off the cones, 23 h after maturation.

Nuclear transfer, reprogramming and activation Donor fibroblast cell lines were aseptically isolated from tissue biopsies after overnight collagenase digestion from a 55 days old bovine foetus (FCL) and from ear of adult Japanese Black Cattle (Wagyu; AWC). Cells were cultured in Dulbecco's modified Eagle's medium:F12 with 10% fetal calf serum until confluence, then split or frozen. For cloning, cells were used at least 3d after reaching confluence in passages #3–7, non-serum starved. A single cell was placed on each enucleated half oocyte and attached to it by brief incubation with phytohemagglutinin (1 mg/ml). The cytoplasmic volume of a normal oocyte was reconstituted by adding a second enucleated half oocyte; the triplet was fused together by means of a single direct current pulse of 1 Kv/cm. The reconstructed embryo was reprogrammed for 2 h. Activation was induced by 7% ethanol (5 min) followed by 5 h incubation in cycloheximide (10 μ g/ml) and cytochalasin B (5 μ g/ μ l).

Embryo culture, transfer and cell counting Cloned embryos were cultured individually for 7 days in synthetic oviduct fluid in an atmosphere of 5.5% O₂, 5% CO₂ and 89.5% N, 100% humidity at 39°C in sealed aluminium foiled bags, using the well on the well (WOW) system [3]. After 7 days of culture, embryos were released from the WOWs, classified according to their stage of development and morphology and subjected to embryo transfer (ET) or cell counting (CC). For ET, 18 expanded blastocyst (day 7) were transferred non-surgically into three naturally cycling synchronized heifers (day of transfer=day 7 after standing oestrus). Ten days later, heifers were slaughtered and

elongated embryos were flushed from uterine lumen. For CC, day-7, embryos were fixed in 3:1 methanol to acetic acid and stained with aceto-orcein for cell counting under $\times 100$ magnification.

Results and discussion

Expanded cloned bovine blastocysts were obtained in all the replicate experiments performed ($n=16$) using two different primary cell cultures. The lowest efficiency in a single experiment was obtained for foetal cells (5 blastocysts out of 25 cultured embryos; 20%) and the highest, for adult cells (24 out of 32; 75%). Results of in vitro development to blastocysts using two different cell lines are provided in Table 1.

Morphologically, most of the cloned blastocysts (70/81; 86.41%; FCL) and (137/155; 88.38%; AWC) were classified as grade I. Mean cell number (aceto-orcein counting) was 101.22 ± 23.23 , (cloned; $n=10$ from FCL), and 112.14 ± 16.57 from AWC). The cell number of blastocysts obtained

Fig. 1 Elongated cloned bovine blastocyst from Wagyu adult cell line at day 17 of development. Embryonic disc is indicated by asterisk

in our HMC experiments did not differ from that of in vitro produced (IVP) embryos historically produced in our lab (not shown). Blastocysts of transferable quality were obtained at high rates for both cell lines. The overall efficiency of the SCNT procedure was 52.6% which is probably the highest published so far for cloned embryos using HMC [2–4, 7, 8]. In our hands, blastocyst rates of 11% to 20% were historically achieved when using conventional cloning [10–12]. For Wagyu cell line, the figures for blastocysts development (65.7%) are to our knowledge the highest ever published for any SCNT experiment reported so far and are similar to those reported for WOW culture of IVP embryos [3]. The reason for this highly efficient cloning could lie on the cell line itself, since the same operator performed all the cloning experiments. A “younger” primary culture derived from a nuclear transfer blastocyst, was used also for nuclear transfer, but did not yield the same figures as the adult cell line, but rather behaved like the foetal cell line, probably indicating a cell-specific defined reprogramming pattern (8 blastocysts out of 31 cultured embryos in two separate experiments; 38.1%, data not shown).

HMC can be a better technical alternative than conventional cloning for certain species and cell lines. Most of the concerns about HMC deal with the facts that the zona is removed and that oocytes are split in halves, thus leading to cytoplasm lost. The most commonly used enucleation method is the mechanical aspiration of the metaphase plate using micromanipulators, by means of which 4% to 50% of the total cytoplasm volume can be eliminated [13, 14]. In HMC approximately 25% to 30% of the cytoplasm is eliminated during enucleation; however up to 150% of the original volume is obtained upon fusing two halves with the somatic cell [7]. Using demecolcine to induce protrusion of the metaphasic plate, enucleation efficiency is close to 100% ([10] and unpublished). The lack of zona pellucidae as a result of the HMC does not affect neither the development to blastocysts (this paper) nor the birth of cloned bovine, swine, horse and sheep [1, 15–17]. Concerns about removing zona from embryos include the possibility of viral infection of them (however the risk is the same for conventional cloning, due to the rupture of the zona), the potential failures in DNA methylation in these embryos, as shown for mouse zona free embryos [18] and the impossibility for group culture of embryos due to aggregation of adjacent embryos; however this problem has been overcome by the use of WOW culture [3]. In the last years this method has been successfully used for production of bovine [2, 19–21], horse [22–23], mouse [24] and pig [25] offspring. Finally, and probable one of the most important features of HMC is its simplicity, speed of work and low cost when compared to traditional cloning using micromanipulators.

In the only published report of cloned Wagyu cattle, the efficiency of blastocysts production was 19%, from a muscle cell line; however in vivo, 20% of calving was achieved after transfer of 26 cloned embryos [26]. The high developmental potential of that Wagyu cell line is in agree with our in vivo data. Nevertheless, there are still too few data as to withdraw conclusions about an increased developmental potential of cloned embryos derived from this breed of cattle.

The ultimate criterion of efficient reprogramming is the in vivo development of the embryos, ideally the birth of cloned animals. We bulk transferred 18 cloned embryos from adult cell line (Wagyu cattle) to three synchronized recipients and recovered 11 embryos after slaughter of the transferred cows at day 17 of pregnancy. One cattle, which received seven excellent expanded blastocysts, did not become pregnant and no embryos could be recovered from this animal, 11 elongated blastocyst out of the 11 remained transferred embryos were recovered from the other two cows (Fig. 1). The overall recovery was 11/18 (61.1%) which compares favourably to data reported by others [27, 28] thus confirming the developmental potential of the adult cell line derived from Wagyu cattle used in our experiments.

Our data show that HMC is a reliable method for the production of cloned embryos with high developmental potential.

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