—Mini Review—

Handmade Somatic Cell Cloning and Related Studies in Farm Animals

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Abstract: *The method of enucleating recipient oocytes by bisection with a metal-blade has been adapted to somatic cell nuclear transfer (SCNT) using zona-free mammalian oocytes, and the process named handmade cloning (HMC). Besides the simplification of enucleation without using a manipulator, this technique also provides for a reduction in the amount or the elimination of expensive equipment that is usually indispensable for conventional SCNT. In this review, we will highlight some recent studies on HMC and related studies in farm animals, especially cloning using enucleation by the gradient centrifugation of zona-free oocytes to produce a large number of recipient cytoplasts at once.*

Key words: *Somatic cell nuclear transfer, Hand made cloning, Oocyte, Enucleation*

Introduction

A sheep [1] was the first animal in the world to be cloned from somatic cells. Since then, other farm animals (cattle and pigs) have been cloned using somatic cell nuclear transfer (SCNT) [2–6]. One advantage of using somatic cells as nuclear donor cells is that the resulting SCNT animals have the same genotype as the donor [7]. Although there are concerns regarding susceptibility to epigenetic errors, especially in fetuses derived from SCNT embryos [8], and heavy birth weights and low survival rates in SCNT calves [9, 10], surviving cloned cattle appear to be normal and healthy in terms of growth and reproduction [11–14]. Thus, SCNT technology offers the opportunity to reproduce domestic animals with desired genotypes, thereby making a substantial contribution to efficient animal production [11, 12]. For example, SCNT technology would be very useful to the livestock industry, particularly in breeding by increasing the number of genetically proven valuable animals as copies. Additionally, use of SCNT animals in various comparative studies in physiology, nutrition, embryology, genetics, and breeding would substantially reduce the number of animals required for generating statistically valid data due to the elimination of genetic variation [13]. However, in SCNT, besides the low rate of development to term of cloned embryos and lack of understanding of the mechanism(s) of reprogramming of the transferred somatic cell nucleus, nuclear transfer techniques that rely on laborious works, technical skills, and complicated equipment, which need precise control by experienced workers, may explain the limited applications of this technique in animal reproduction.

To produce cloned mammalian embryos, at least two major processes have to be completed prior to the activation or stimulation of the reconstructed oocytes; 1) the enucleation of recipient oocytes (preparation of a cytoplast), and 2) the insertion or transplantation of a donor somatic cell (nucleus) into the cytoplast by fusion [15] or direct injection [16]. Most bovine cloning has been done using the fusion method. In contrast, porcine cloning was reported first to be achieved by Onishi *et al.* [6] using the direct injection method and then by Polejaeva *et al.* [17] using the fusion method.

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Since then, several attempts have succeeded in producing cloned piglets by the fusion [18-20] or direct injection [21] method. This process is not complicated compared with the enucleation of recipient oocytes, and only the two methods exist. In contrast, various methods of enucleation have been used for mammalian cloning (reviewed by Li *et al.*, in 2004) [22]; blind enucleation by aspiration of a small volume of oocyte cytoplasm including the first polar body [23], oocyte bisection [24], enucleation with hoechst staining and UV light [25], enucleation by herniation of the first polar body and the surrounding cytoplasm [26], DNA fluorochrome SYBR14-assisted enucleation [27], non-invasive enucleation using pol-scope microscope [28], and sugar pretreatment enucleation [29]. However, the majority of these enucleation techniques depend on the presence of a manipulator on the stage of the inverted microscope.

Recently, the method of enucleation by bisection with a metal-blade has been adapted for cloning using zona-free mammalian oocytes [30] and the process named handmade cloning (HMC) [31]. In addition, the bulk-enucleation of zona-free mammalian oocytes using centrifugation through a discontinuous percoll gradient was achieved by Tatham *et al.* [32]. This method uses the differential density of chromatin and cytoplasmic content to enucleate an oocyte by exerting a centrifugal force to expel genetic material out of the cytoplasm as an oocyte cytoplasm fragment having the genetic material.

Although both methods have the advantage of not requiring a manipulator and reducing time and works that needed to reconstruct embryos, they have the disadvantage of using zona-free embryos which have a low rate of development to the blastocyst stage compared with zona-intact embryos [33]. To obtain blastocysts from zona-free embryos, inducing the appropriate three-dimensional arrangement of the blastomeres in the embryos is essential [33]. To achieve this arrangement of the blastomeres, the well of the well (WOW) culture system using needle depressions was invented [34]. The system was reported to be also effective for culturing zona-free bovine SCNT embryos [30] and zona-free porcine SCNT embryos [35] to increase their viability.

In this review, we will discuss recent studies about HMC and related studies in farm animals, especially cloning methods using enucleation by the gradient centrifugation of zona-free oocytes to produce a large number of recipient cytoplasts at once.

Handmade Somatic Cell Cloning (HMC)

Originally established for embryonic nuclear transfer [36], HMC has proven the simplest way to produce cloned embryos in bovine [30, 31, 37–42], murine [43], and porcine [35, 44–46] species. HMC is unique in that it uses two halved oocytes without a nucleus as recipient cytoplasts. The method has produced many calves [37–42], and most recently, the first piglets [46]. Furthermore, the method was reported to be twice as efficient as conventional methods and only one-tenth as costly (New Scientist: Handmade cloning success uses a chopped egg. http://www.newscientist.com/channel/sex/mg19125604.800-handmade-cloning-success-uses-a-chopped-egg-.html.).

The procedures for HMC are illustrated in Fig 1. According to Vajta *et al.* [38], *in vitro* matured bovine oocytes were incubated with 1.5 mg/ml of pronase for 10–15 min at 39°C to remove the zona pellucida. Zona-free oocytes were lined up in a petri dish and bisection was performed manually under stereomicroscopic control with Ultra Sharp Splitting Blades (AB Technology, Pullman, WA, USA). After completion of the bisection, all halved-oocytes were stained with 10 μg/ml of fluorochrome Hoechst 33342 for 5 min. Using an inverted microscope and UV light, halved-oocytes without chromatin staining (cytoplasts) were identified, and then collected under a stereomicroscope. Fusion was performed 23–24 h after the start of maturation. For the first electrical fusion, a cytoplast (the 1st cytoplast) was transferred to a dish containing 500 μg/ml of phytohaemagglutinin (Sigma L 8754) for 3 s, then quickly dropped over a single somatic cell (fibroblast) settled to the bottom of the dish. Following attachment, the 1st cytoplast-somatic cell pair was again picked up, and transferred to a fusion chamber (BTX microslide 0.5 mm fusion chamber, model 450, cat. no. 01-000209-01). Wires were covered with 2 ml of 26–27°C fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$, and 0.05 mM $CaCl_2$). After incubation for 2–3 min in the fusion medium, the pair was attached to one of the wires using an alternating current (AC) of 15 V and 700 kHz (Genaust Electrofusion Machine, Australia). Fusion was performed with a double direct current (DC) pulse of 65 V, each pulse lasting for 20 μsec and being 0.1 sec apart. The pair was then carefully removed and transferred to another well of the dish, where it was incubated for 15–30 min to determine whether fusion had occurred to form a demi-clone embryo. For the second electrical fusion, another cytoplast (the 2nd cytoplast) and demi-clone embryo were transferred to

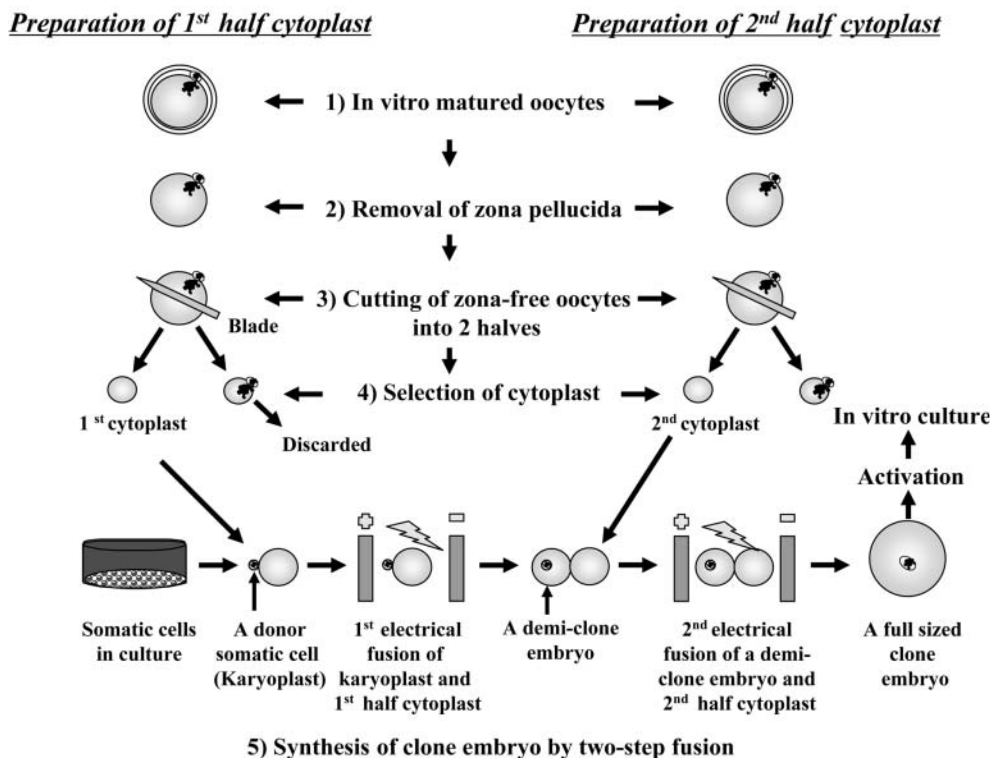

Fig. 1. Procedures for hand made cloning (HMC).

the fusion medium covering the fusion chamber. After incubation for approximately 2 min, the 2nd cytoplast was aligned to one electrode using the same AC as for the first fusion. Subsequently one fused pair (demi-clone embryo) was attached to the 2nd cytoplast. A double fusion pulse with the same parameters as above but with 45 V DC was applied, and then the full-sized (double cytoplasts-fibroblast cell triplets) clone embryos were incubated for 20 min in a culture medium. These reconstructed full-sized embryos were transferred into a WOW system prepared in a four-well dish containing 400 μl of culture medium consisting of SOFaa medium [47] supplemented with 5% calf serum covered with mineral oil and incubated at 39°C in a humidified mixture of 5% CO_2, 5%O_2, and 90% N_2. Activation was initiated 28 h after the start of maturation (approximately 4 h after the fusion); the full-sized clone embryos were incubated in a culture medium containing 2 mM of the Ca ionophore A23187 for 5 min at room temperature. Then they were cultured *in vitro* using the WOW system [34].

Although this technique requires more oocytes as a source of recipient cytoplasts than the conventional nuclear transfer technique since only half of the oocytes can be used as recipient cytoplasts [36, 41], a high rate of development of SCNT embryos using HMC could compensate for the number of oocytes needed for experiments [41]. To date, in order to increase the efficiency of HMC, several modifications of this technique have been attempted; a chemically induced enucleation [48], or use of a single fusion step [41], in which the fusion of a donor karyoplast and a cytoplast was carried out in a single step rather than two consecutive steps for couplets. The history, principles, disadvantages, and potential benefits of this procedure have been reviewed recently [49].

SCNT Using Cytoplasts Enucleated by Gradient Centrifugation of Zona-Free Oocytes and Fused (Centri-Fusion Cloning)

Another method of enucleation using the gradient centrifugation of zona-free oocytes has also been introduced [32, 50]. This method has the advantage of being able to enucleate many oocytes at once (bulk-enucleation), therefore it could reduce the time needed for the enucleation, compared with conventional SCNT or HMC. In a previous study, we examined the

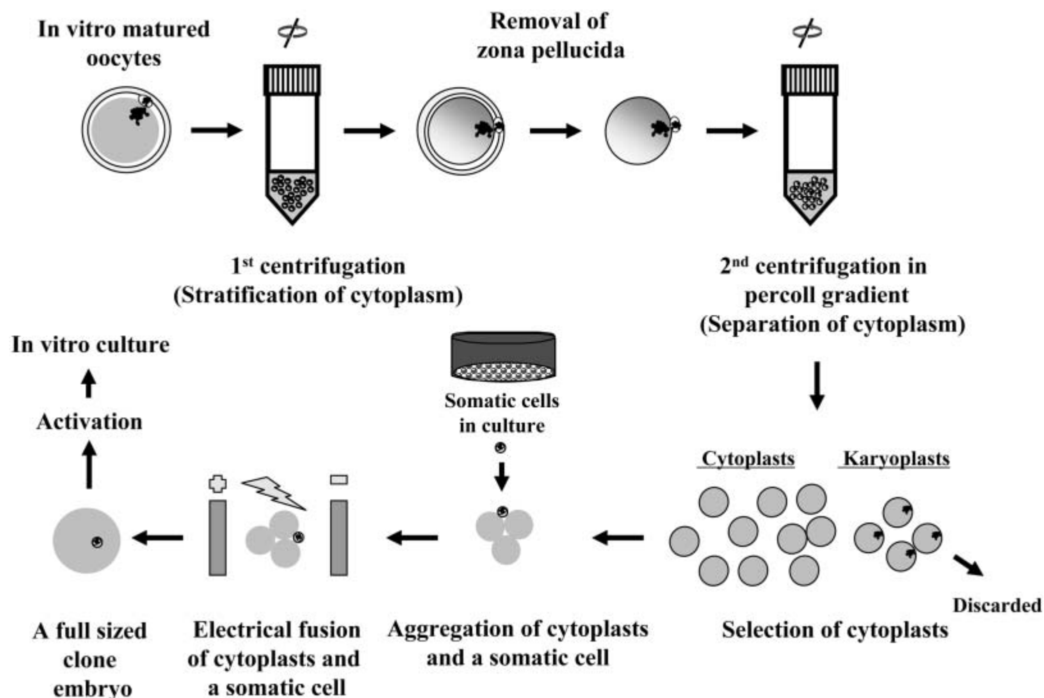

Fig. 2. Procedures for SCNT using enucleation by centrifugation of zona-free oocytes (Centri-fusion cloning).

possibility of performing cloning with zona-free oocytes in pigs using enucleated fragments of oocyte cytoplasm prepared by gradient centrifugation in Percoll solution as recipient cytoplasts after their fusion (Centri-fusion cloning), and examined their ability to support remodeling and the further development *in vitro* of SCNT embryos to the blastocyst stage [51].

The procedures used for the Centri-fusion cloning are illustrated in Fig 2. The recipient cytoplasts were prepared as follows; *in vitro*-matured (IVM) porcine oocytes were enucleated with the gradient centrifugation method as described by Savard *et al.* [50] with minor modifications. Briefly, IVM oocytes were denuded of cumulus cells through exposure to 150 IU/ml of hyaluronidase for 2–3 min in a test tube followed by vortexing of the tube for 1 min to dissociate the remnant cumulus cells. The cumulus-free oocytes were collected under the dissection microscope, transferred to 1.5 ml microcentrifuge tubes, and then centrifuged at 13,000 × *g* for 9 min to stratify the cytoplasm (the first centrifugation). Only matured oocytes with a visible first polar body were used for the experiments. The zona pellucida was then partially dissolved by exposing the oocytes to 0.5% (w/v) pronase for 2–3 min [52]. Oocytes with a thin zona pellucida were incubated in culture medium for approximately 10 min at 39°C. This was followed by gentle pipetting to completely remove the zona pellucida completely. After several washes, several groups of 30 zona-free oocytes were layered on a 300 μl discontinuous gradient (100 μl of 45, 30, and 7.5%, respectively) of Percoll (Amersham Biosciences, Uppsala, Sweden) in culture medium supplemented with 5 μg/ml of cytochalasin B in microcentrifuge tubes. These gradients were then subjected to centrifugation at 5,000 × *g* for 4 sec (the second centrifugation). After several washes to remove excess Percoll, oocyte cytoplasmic fragments (OCFs) larger than 50 μm in diameter (larger than one third of the diameter of an intact oocyte) were selected. The OCFs were then stained with 5 μg/ml of Hoechst- 33342 (Calbiochem, La Jolla, CA) for 20 min, and briefly examined under an epifluorescence microscope to select OCFs without a nucleus (cytoplasts).

The reconstruction of SCNT embryos was carried out by aggregating three cytoplasts with a single cumulus cell (karyoplast) in phytohemagglutinin solution (PHA, 300 μg/ml) dissolved in PBS. Briefly, the three cytoplasts were aggregated in a drop of PHA, and then transferred to a drop of TCM-199 containing singly dispersed cumulus cells. A single somatic cell was positioned between the cytoplasts [41]. The formation must be stable enough to be maintained during the

fusion process. Within approximately five minutes, ten pairs of aggregated cytoplasts with a karyoplast were pooled in a well of the four-well dish until the initiation of fusion. Prior to fusion, they were equilibrated in culture medium with a fusion solution (0.28 M mannitol solution supplemented with 0.05 mM $CaCl_2$ and 0.1 mM $MgSO_4$); 1:1 before the final equilibration in the fusion solution. Following manual alignment in the fusion chamber (FTC-23, Shimadzu, Kyoto, Japan), two DC pulses of 1.5 kV/cm lasting for 20 μsec were delivered from a fusion machine (SSH-10, Shimadzu) to induce fusion. During alignment, the area of surface between the somatic cell and cytoplasts should be parallel to the electrodes, since a somatic cell forms only a narrow contact area with a cytoplast, whereas the area of contact between the cytoplasts is wide enough to form a field parallel to the electrodes. They were then briefly washed and incubated in a culture medium supplemented with 5 μg/ml of cytochalasin B for 1 h at 38.5°C until the onset of activation.

Following activation or stimulation with two DC pulses of 0.8 kV/ cm for 30 μsec, the reconstructed embryos were incubated for 2 h in a culture medium, glucose-free NCSU-37 containing 4 mg/ml of BSA supplemented with 0.17 mM sodium pyruvate and 2.73 mM sodium lactate [53] containing 5 μg/ml of cytochalasin B [54]. The reconstructed embryos were briefly washed with a culture medium, and then cultured individually in small wells that were made in the well of four-well dishes (WOW) [35]. The wells were filled up with 400 μl of glucose-free NCSU-37 from Days 0 to 2 (the day of nuclear transfer is designated as Day 0). From Days 2 to 7 they were cultured in NCSU-37 supplemented with 5.55 mM D-glucose and 5% (v/v) FBS. On Day 2, the medium replacement was replaced without removing embryos from the WOW system.

When SCNT embryos were reconstructed by the fusion of three cytoplasts and one cumulus cell in Centri-fusion cloning, about 10% of reconstructed embryos developed to the blastocyst stage. This percentage is lower than the 44.4% obtained using HMC [46]. The reason for the low developmental rate is not clear. However, during the gradient centrifugation of zona-free oocytes, a large portion of the cytoplasm having lipids is lost or discarded in Centri-fusion cloning. This loss may cause problems later in the embryonic development. Furthermore, it has been reported that centrifugation altered the spindle pattern [55] or damaged spindle in the bovine oocytes [56].

The Well of the Well (WOW) Culture System Using Needle Depressions

For the successful production of SCNT embryos using HMC and Centri-fusion cloning, the WOW culture system is very important, because resultant SCNT embryos are free of zona-pellucidae. In mice, the zona pellucida was reported to be needed to prevent the separation of blastomeres in cleaving embryos in the oviduct of the recipients, and actually, difficulty in obtaining newborns from pre-compacted zona-free embryos transferred into the oviduct was reported [57]. Moreover, it was reported that the rates at which zona-free blastomeres separated at the 2-cell stage developed to the blastocyst stage and to full term were lower than those of zona-intact 2-cell embryos [33]. Graham and Lehtonen [58] compared the blastomere arrangement of zona-free mouse half embryos separated at the 2-cell stage with that of the same half embryos enclosed by the zona pellucida and observed the same phenomena as found in the present study. Furthermore, Suzuki *et al.* [59] reported that the rates of development to the blastocyst stage were lower for zona-free mouse zygotes than zona-intact controls. They suggested that while the majority of controls formed a three-dimensional blastomere arrangement with 6 points of contact between the 4 blastomeres at the 4-cell stage, the majority of zona-free zygotes that developed to 4-cell embryos had a flat arrangement of blastomeres with fewer cell contacts. They also reported that the cell number of ICM in blastocysts derived from zona-free 4-cell embryos was lower than that of controls resulting in low rates of implantation and live fetus production after transfer, and suggested that the blastomere arrangement of zona-free embryos at the 4-cell stage influenced their differentiation into ICM and subsequent embryonic development to term. Thus, to induce the three-dimensional blastomere arrangement in zona-free embryos, the WOW culture system using needle depressions has been used for the *in vitro* culture of zona-free SCNT embryos. Needle depressions were prepared on the bottom of a culture dish where a microdroplet of culture medium was placed and covered with mineral oil. Conical needle depressions were created by pressing the bottom of dishes with sterilized rods or aggregation needle (DN-09, BLS Ltd., Hungary). Embryos were cultured individually in needle depressions [34].

Conclusions and Perspectives

It can be concluded that SCNT animals can be obtained from the HMC embryos and cytoplasts obtained by two consecutive rounds of centrifugation, including one gradient centrifugation, of zona-free oocytes in the Cenri-fusion cloning can support the remodeling of a transferred somatic nucleus resulting in the development of reconstructed porcine embryos to the blastocyst stage. These cloning methods offer many cytoplasts for SCNT at once compared to conventional SCNT. Also considering that recently advanced micro-fluidic technology combined with micro-mechatolonics will allow for the integration of different steps of the SCNT process such as the transfer and alignment of karyoplasts and cytoplasts and their fusion within the same equipment with appropriate exchange of media as reported for each IVP step [60], the bulk production of SCNT embryos will be possible in the near future by using the Centri-fusion cloning method because the size of cytoplasts can be adjusted easily to be applicable to micro-mechatolonics.

However, the developmental ability and normality of the cloned embryos obtained by these methods should be evaluated after the transfer to recipients. It should be noted that zona-free mammalian embryos can not be traded between countries under the regulations of the International Embryo Transfer Society (http://www.iets.org/).

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