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Annals of Biological Research, 2012, 3 (6):3043-3048
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

Handmade cloning: an alternative technique for somatic cell nuclear transfer

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ABSTRACT

Handmade cloning (HMC), a simplified alternative of micromanipulation based traditional cloning (TC) has been developed in multiple phases during the past years. HMC radically decreases costs and the need for a skilled workforce; furthermore, it increases productivity, enables cryopreservation, and results in birth rates comparable, or even higher, than those achievable by micromanipulation-based traditional cloning (TC). The new technique can accelerate technology transfer and standardization and, eventually, might contribute to the widespread application of cloning. Additionally, HMC offers unique possibilities for the automation of somatic cell nuclear transfer. The HMC will allow the scientists to produce cloned animals with simple non-expensive equipment. Consequently, enormous data concerning all the facets of the nuclear transplantation experiments could be retrieved from various laboratories. This will allow a better future application of the cloning technique for the welfare of human, through production of animals with high genetic traits, rescue of endangered animal species and production of transgenic animals that can produce medicine for certain human diseases.

Keywords: Handmade cloning, somatic cell nuclear transfer (SCNT), Animal cloning

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INTRODUCTION

Since Dolly's birth in 1997 by the somatic cell nuclear transfer (SCNT) technique (1), animal clones from various species such as cattle (2), mice (3), goats (4), pigs (5), cats (6), rabbits (7), horses (8), rats (9), and dogs (10) were generated in a consecutive manner. Traditional SCNT technology has been well-established and used extensively in cloning laboratories. However, the technology requires expensive equipments and high maintenance costs, not to mention highly qualified and skillful personnel, as well as extensive time involving training.

Recently, the SCNT technology skipped the use of pricey micromanipulators and simplified the manipulation of bisecting zona pellucida (ZP)-free oocytes by bare hand, i.e. the so-called handmade cloning (HMC) (11). Therefore, HMC can be accomplished at an apparently lower cost by a simplified and rapid procedure yet with comparable or even higher efficiency.

Animal production by somatic cell nuclear transfer offers a range of opportunities in basic and applied research, in agriculture, genetic conservation and human medicine. However, to fulfill much of this potential a simple, repeatable and robust methodology is required. The production of animals by SCNT involves multiple steps (Fig. 1) and each of these may influence the successful outcome.

To date the frequency of development has not dramatically increased since the original reports, however, modifications and improvements in techniques have had a number of effects depending upon species including: (1) simplifying the methodology, (2) reducing costs, and (3) improving survival following birth (Box 1).

BOX 1. Advantages of handmade cloning (HMC)

(i) Equipment: one order of magnitude less expensive than that required for micromanipulation-based cloning.
(ii) Procedure: simple, rapid, easy to learn and perform.
(iii) Efficiency: required time, workforce and investment are lower than in traditional cloning. Transferable embryo per oocyte rates are approximately the same, although two oocytes are used for reconstruction of one embryo.
(iv) Embryo cryopreservation: possible to produce healthy offspring produced in cattle and pig.
(v) Pregnancy and calving and/or farrowing rates: according to the few available data, at least identical with those reported after micromanipulation-based traditional cloning.
(vi) Special benefits: possibility for automation with the microchannel–microfluidics technology.

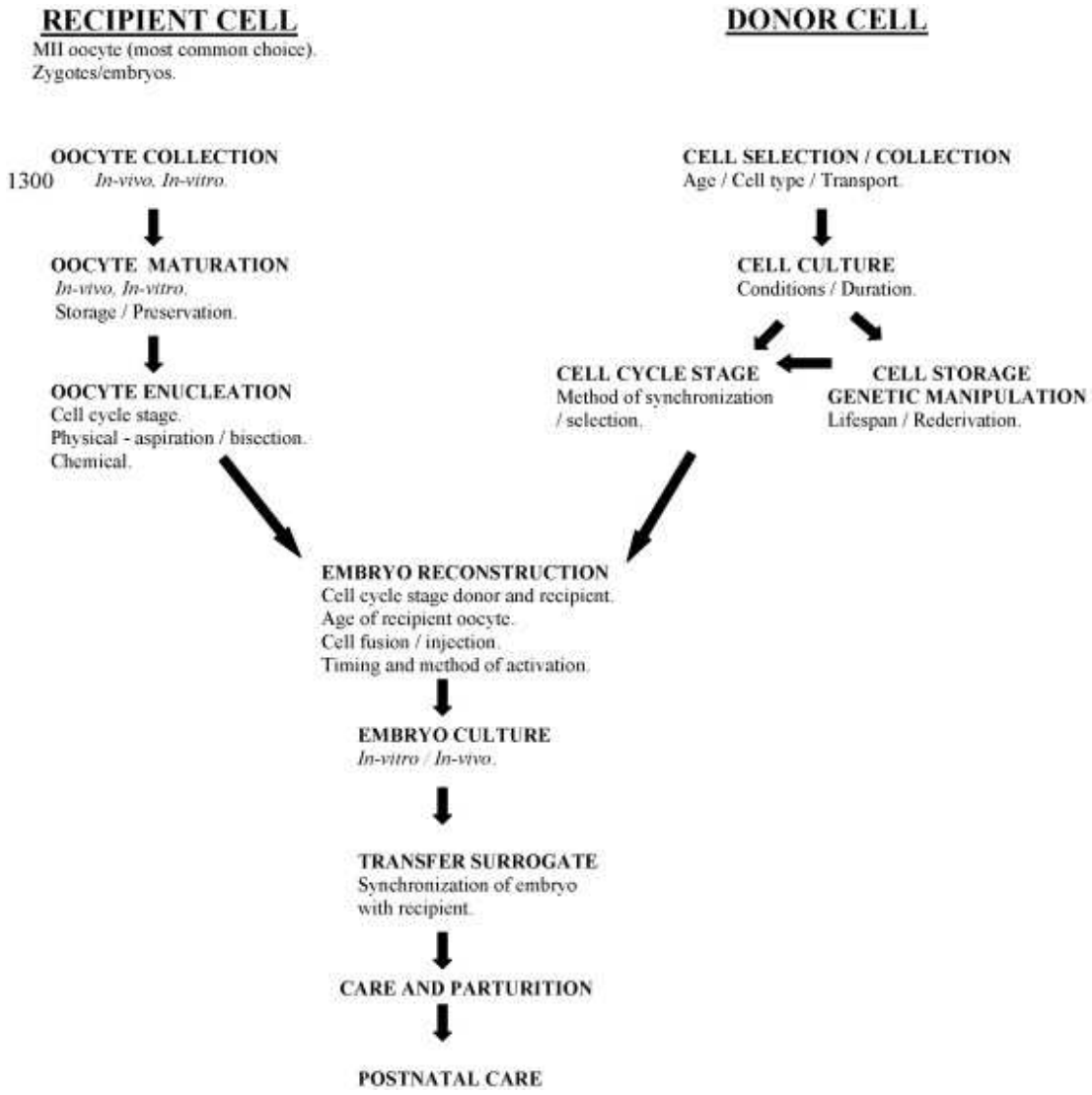

Fig. 1. Individual steps involved in the production of offspring by somatic cell nuclear transfer (SCNT).

Handmade cloning approach

The principle of the new (although more than a decade old) approach is simple. The general assumption that the zona pellucida (analogous to an eggshell) is indispensable for the normal development of early mammalian embryos has restricted the creative thinking necessary to improve *in vitro* reproductive technologies in mammals. Until recently, only sporadic attempts have been made to break this supposed frontier; however, slow-growing evidence regarding the possibility of zona-free *in vitro* fertilization (12) and (13) and parthenogenesis activation and embryo culture (14), (15) and (16) in cattle and pigs has incrementally opened the way for zona-free manipulations.

The first known zona-free nuclear transfer approach was performed by Tatham *et al.*(17). Unfortunately, their method for enucleation (density-gradient centrifugation of zona-free oocytes) was unreliable and no calves were obtained after fusion with embryonic cells. However, the ingenious invention of a handmade enucleation, with a not-too-sharp blade, and by gluing the polar body to the oolemma with phytohaemagglutinin, as an orientation point, Peura *et al*. (18) have mastered enucleation and established a reliable system for reconstruction by fusing two enucleated oocytes to one blastomere. Unfortunately, after obtaining several calves, even from second generation cloning (19), this group turned to other approaches, leaving the final problems (the application of the method for nuclear transfer with somatic cells as donors and improving the efficiency of *in vitro* culture to the transferable stage) to be resolved by others.

In actual fact, the solution turned out to be simple (Figure 2). The somatic cell was glued to the surface of the cytoplast – again with phytohaemagglutinin – before fusion, and the reconstructed embryos were placed, individually, into capillaries or microwells (20), (21) and (11) for culture. Curiously, some cloners still prefer to use micromanipulators for enucleation, either with or without the zona pellucida (15), (16), (22), (17), (24) and (25), although the entire procedure can be performed by hand without sophisticated tools – this is where the name handmade cloning (HMC) originated from. For the culture of individual embryos, which is required to avoid aggregation of zona-free reconstructed embryos before compaction, the modified microwell – well-of-the-well (WOW) – system was the most efficient (14) and (15). In contrast to the commonly used microdrops, the inverted sugar-loaf-shaped WOW offers unique benefits for zona-free embryos by keeping blastomeres together and providing a stabile microenvironment for the developing embryo.

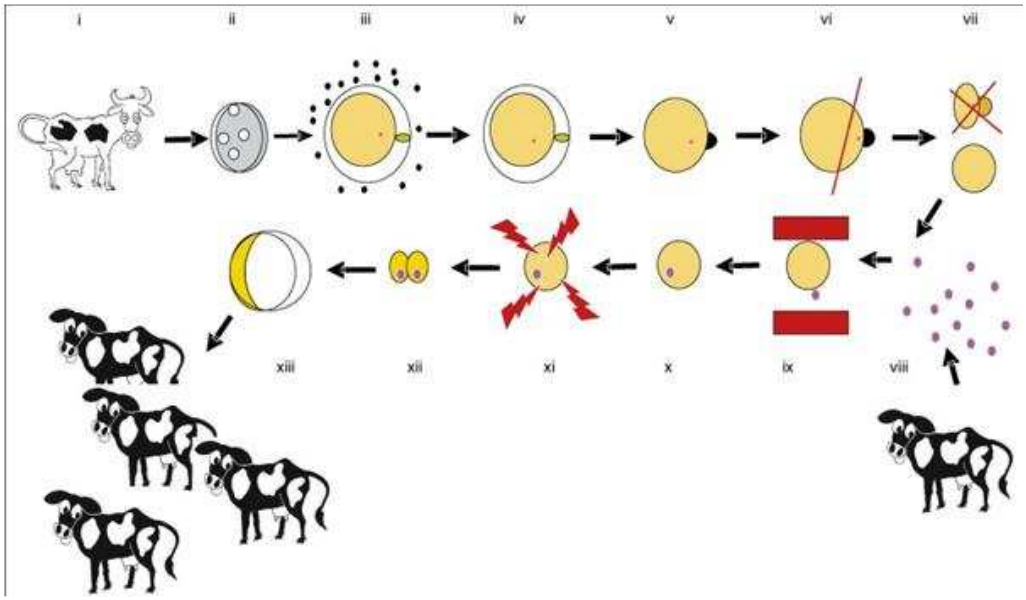

Figure 2. The process of bovine HMC with chemically assisted enucleation. Ovaries are collected from slaughtered animals (i), transported to the laboratory, and oocytes are aspirated from the visible 2–7 mm diameter follicles (ii). After a 22 h maturation, cumulus cells are removed by vortexing (iii), denuded oocytes are incubated for a further 1 to 2 h in demecolcine (iv), then the zonae pellucidae are digested by pronase (v). Through the joint effect of demecolcine and pronase, an extrusion cone occurs on the surface, which serves as an orientation point for enucleation by hand with a disposable blade (vi). Karyoplasts containing the chromatin are discarded, whereas cytoplasts are used as recipients (vii). Somatic cells, derived from another cattle, calf or fetus, are cultured on monolayers (viii). After trypsinisation, these cells are individually attached to cytoplasts that have been submerged, briefly, into phytohemagglutinin to make their surface sticky, then the pairs of cells are transferred to between the electrodes of a fusion chamber (ix). After electrofusion,

reconstructed embryos (x) are subjected to chemical activation (xi) and then cultured *in vitro*(xii) for one week. Emerging blastocysts (xiii) are transferred into recipients to produce animals (almost) identical with the somatic cell donor. (Cow cartoons drawn by Poul Maddox-Hyttel).

Benefits and drawbacks

The unquestionable benefits of this system have been proven in two domestic species: cattle and pig. With the use of oriented, unequal bisection (based on the position of the polar body or the extrusion cone that occurs from the joint effect of a cytoskeleton relaxant, demecolcine, and the pronase used for removal of the zona pellucida), approximately one-third of the cytoplasm is removed under a stereomicroscope (Figure 2) (26) and (27). The efficiency is high because the procedure can be performed successfully in almost all metaphase II oocytes. Moreover, the reliability is 96–98%; therefore, no further staining and selection of chromatin-free cytoplasts is required. Accordingly, there is no need for expensive inverted fluorescent microscopy and potentially harmful staining and UV illumination. For fusion, a simplification of the procedure has reduced the need for two stereomicroscopes to one, and the most reliable fusion machine, specially designed for the purpose, can be purchased for approx. US$3000. The drastic drop in the cost of instruments (in contrast to the sophisticated tools, micromanipulators, microscopes, tool-making instruments or expensive micropipettes required for TC, only one stereomicroscope and one fusion machine are required for HMC) might reduce the required investment by an order of magnitude, to transform a simple, routine, diagnostic laboratory into an up-to-date cloning facility.

Time and productivity are crucial factors in cloning, not only to decrease the costs but also to increase the quality of the produced embryos. Most cloners agree that the time oocytes, cytoplasts and embryos spend outside the incubator inversely correlates with their quality. With HMC, an experienced cloner can produce between 30 and 50 transferable-stage embryos from 200 slaughterhouse-derived oocytes (two oocytes are required for one reconstructed embryo, and the average blastocyst per reconstructed embryo rate is around 50% in both species) every 3–4 hours. This is excluding the incubation times but including all related preparative and cleaning work. In one workday, one cloner can produce enough embryos for one surgical transfer into pigs, and enough for between 15 and 50 transfers into cattle. Paradoxically, although most criticisms addressed at somatic-cell nuclear transfer refer first to the low overall efficiency, the productivity of HMC has, so far, not met with a real market requirement; accordingly, most embryos produced in the laboratory might end up in the garbage. Fortunately, both cattle and (with some additional manipulation) pig HMC embryos can be cryopreserved successfully with vitrification. Preliminary data suggest no decrease in pregnancy rates after cryopreservation.

The transfer of zona-free embryos does not present a technical challenge. In fact, the zona-free situation might help to overcome the problems related to hatching, which are aggravated by the zona hardening as a consequence of *in vitro* embryo culture. Pregnancy rates of 50% can be achieved with cloned zona-free embryos, both in cattle and pigs (24), (28) and (29). According to the limited available data, no significant difference in the rate of developmental anomalies between TC and HMC was observed in cattle, and there are no serious developmental problems after HMC in pigs. HMC contributed decisively in producing the greatest litter from one sow (10 piglets) after somatic cell cloning and in the greatest offspring per transferred embryo in pig (22%) (29). Similar observations were published regarding transfer of cloned zona-free embryos in horse and mouse (23), (25) and (28). The only negative feature is that zona-free oocytes, cytoplasts and reconstructed embryos can attach to each other; their subsequent separation requires time and occasionally results in some losses. With a little care, and by using media with elevated macromolecule content, this problem can be entirely eliminated. Another frequently mentioned concern is the lack of the protective sheet – the zona pellucida – resulting in the potential for disease transmission.

Future perspectives

The greatest potential benefit of HMC is the potential this approach offers for automation. Microchannel or microfluidics technology (eventually offering a microchip where wires are replaced by channels filled with solutions) is widely used now for different purposes, including biology, and its application has already been tested in embryology. In fact, almost all the steps required for HMC can be performed, or have already been performed, in microchannels (30). This is in sharp contrast to TC, where automation seems to be impossible. The only major problem that remains to be resolved is the integration of the individual steps into a production line. Unfortunately, efforts in this field are sparse, and the proponents are mostly restricted to ambitious embryologists, who are not really qualified for this task, resulting in painfully slow advancement. When the experts of the microchannel and/or microfluidic technology enter the field and help to overcome the existing fundamental drawbacks (e.g. the occurrence of gas bubbles in the channels during incubation, hampering the passage of solutions and deforming the

embryos) and provide an up-to-date technological background to control, fine-tune and integrate processes, the cloning machine can become a reality. This would offer a completely new dimension for somatic cell nuclear transfer and subsequently to almost all embryo technologies, enabling the production of top quality embryos by highly standardized and repeatable procedures, technology transfer and rapid advancement in the field.

CONCLUSION

Since the production of Dolly in 1996 significant advances have been made in the number of animals cloned and also in understanding the molecular processes underlying normal and abnormal development. Nuclear transfer is a multifactorial process and advances in all areas will contribute to simplifying and improving the efficiency of the technique.

HMC has been proved to be an efficient and simplified alternative to traditional, micromanipulator-based SCNT in animals with promising prospects of low cost production of genetically modified animals.

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