Opinion TRENDS in Biotechnology Vol.xxx No.x

Handmade cloning: the future way of nuclear transfer?

Gábor Vajta

Department of Genetics and Biotechnology, Danish Institute of Agricultural Sciences, DK-8830 Tjele, Denmark

The topic of this review is an alternative technique for somatic cell nuclear transfer. Removal of the zona pellucida facilitates manipulations of mammalian oocytes and early embryos, and problems related to their subsequent culture are commonly overestimated. This approach enables radical modifications to somatic cell nuclear transfer, and the handmade cloning (HMC) technique is now successfully applied to an increasing numbers of species. 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.

Introduction

The cloning of mammals by nuclear transfer is commonly regarded as a revolutionary approach and the ultimate cutting-edge technology; however, the principles were outlined 70 years ago [1], the technique was successfully applied in amphibians in the early 1950s [2], and the technology now used by most laboratories for mammalian nuclear transfer was published in 1984 [3]. Accordingly, many students, amazed by the futuristic atmosphere that surrounds nuclear transfer, actually use a technology that was established before they were born, essentially with the same instruments and same principles. From a technical point of view, the only real change during the past 20 years is negligible: early embryo cells have been replaced as donors by somatic cells [4]. Considering the rate of development in other fashionable areas of science (e.g. molecular biology, computing and nanotechnology), the advancement in nuclear transfer methods is far from impressive. Only a small (but growing) group of scientists have been looking for different technical solutions; and after many dead ends, the new route is now, more or less, outlined and might offer a real alternative. Controversially, the main element of this new approach is a radical simplification (Box 1): the decrease in the requirements of time and investment in a skilled workforce is considerable. Moreover, the required instruments are so simple that somatic cell cloning could easily have been realized 100 years ago, before the first scientist was even dreaming of it. The results achieved are at least competitive with those of the commonly used nuclear transfer procedure – traditional cloning (TC) – and the new technique offers a unique perspective: the full automation of somatic cell nuclear transfer. This latter possibility might eliminate the growing gap between our achievements and dreams, and the frustrating realities we face every day in the laboratory.

The alternative 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 [5,6] and parthenogenesis activation and embryo culture [7–9] 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.* [10]. 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.* [11] 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 [12], 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 1). 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 [13–15] for culture. Curiously, some cloners still prefer to use micromanipulators for enucleation, either with or without the zona pellucida [8,9,16–19], although the entire procedure can be performed by hand without sophisticated tools – this is

Corresponding author: Vajta, G. (gabor.vajta@agrsci.dk).
Available online xxxxxx.

  doi:10.1016/j.tibtech.2007.04.004

Please cite this article in press as: Vajta, G., Handmade cloning: the future way of nuclear transfer?, Trends Biotechnol. (2007), doi:10.1016/j.tibtech.2007.04.004

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 micro-channel–microfluidics technology.

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 [7,8]. 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.

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 1) [20,21]. 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,

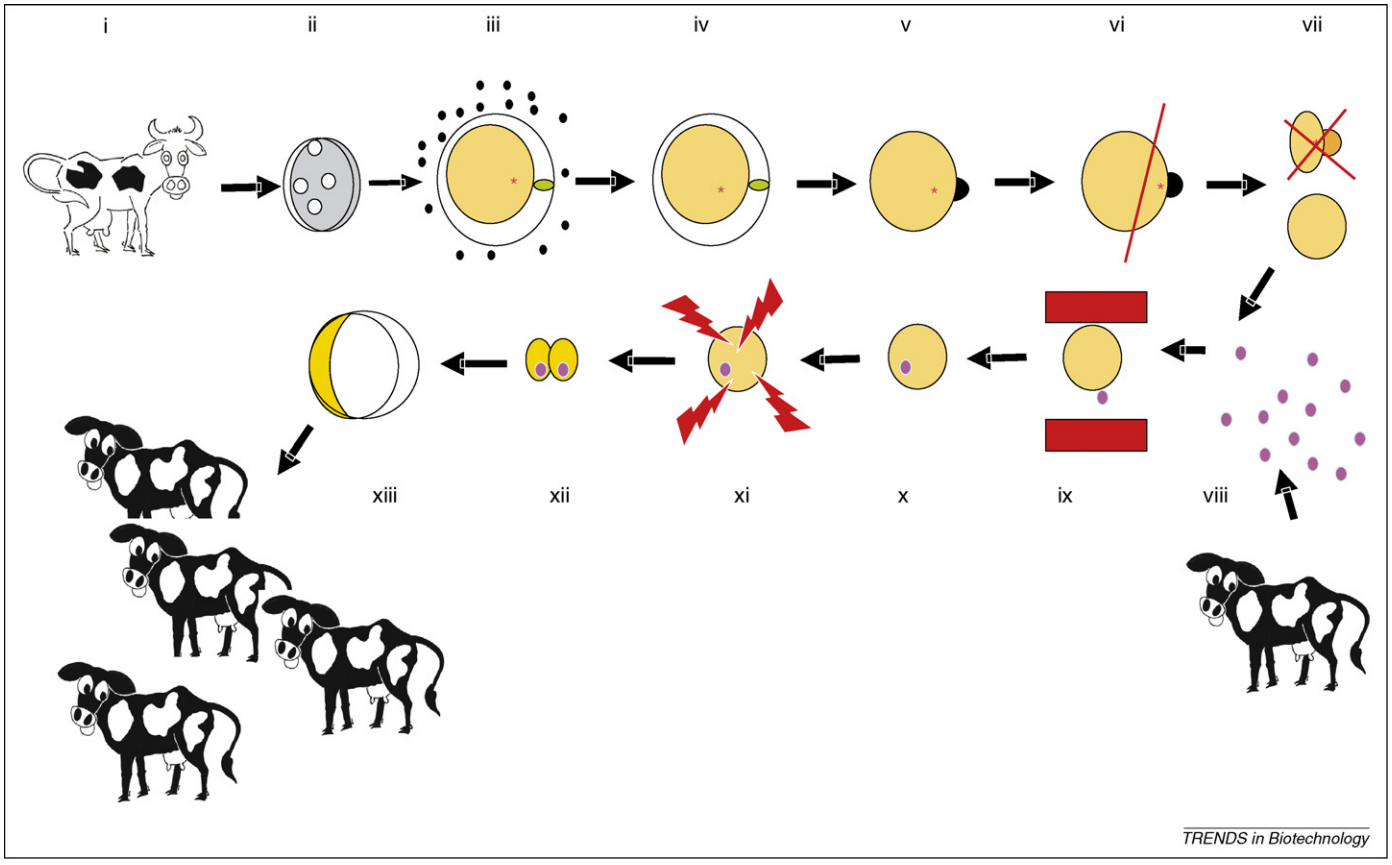

Figure 1. The process of bovine HMC with chemically assisted enucleation. Ovaries are collected from slaughtered animals **(i)**, transported to the laboratory, and oocytes are aspirated from the visible 2–7 mm diameter follicles **(ii)**. After a 22 h maturation, cumulus cells are removed by vortexing **(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).

Please cite this article in press as: Vajta, G., Handmade cloning: the future way of nuclear transfer?, Trends Biotechnol. (2007), doi:10.1016/j.tibtech.2007.04.004

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.

In our experience, anyone with basic technical knowledge in embryo handling can learn bovine HMC in one week, and the more demanding porcine HMC in two or three weeks (the difference is due to the higher sensitivity and lower buoyant density of porcine oocytes, the latter of which makes appropriate orientation difficult), and can produce blastocysts regularly [22,23]. To reach the highest productivity might require two to three months intensive practice, but this is a fraction of the time required to master TC.

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 [18,24,25]. 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 (Figure 2). 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%) [25]. Similar observations were published regarding transfer of cloned zona-free embryos in horse and mouse [17,19,24].

One should, of course, mention the disadvantages of HMC compared with TC; however, if performed appropriately, it is hard to find any definite drawback. 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. These critics, however, disregard the fact that the zona is not intact in TC either. Moreover, many embryos are at hatching or hatched stage at transfer; therefore, the difference of the (theoretical) danger for disease transmission is approximately the same for both methods. Heteroplasmy caused by three different sources of mitochondria might raise concerns; however, so far, no experimental or practical disadvantages of such heteroplasmy have been proved. One practical problem might be that two oocytes are used for the reconstruction of a single embryo, increasing the requirements for oocytes. However, this disadvantage is compensated by the cumulative effect of the efficiency of the steps in the process (all morphologically intact oocytes can be used, regardless of the presence of the polar body; the efficiency and reliability of enucleation is high, >90% in cattle and pig; fusion rates are close to 100%; blastocyst per reconstructed embryo rates as well as pregnancy rates are ≥50%). Conversely, for many domestic species, slaughterhouse-derived ovaries provide an abundant source of oocytes.

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 [22]. 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

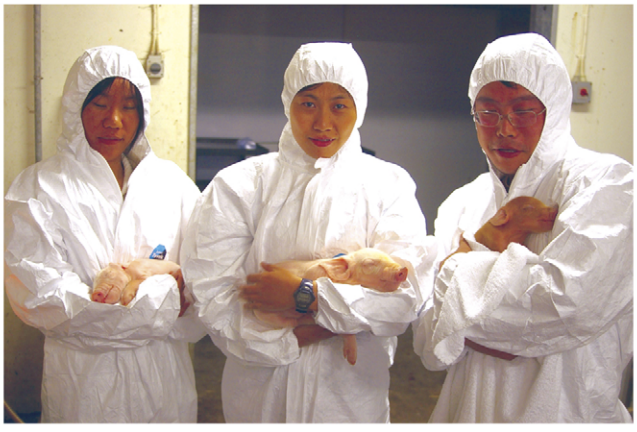

Figure 2. The first somatic cell cloned animals of Scandinavia: 4-hour-old piglets born after HMC (left and middle) and TC (right) embryos transferred into the same recipient sow.

Please cite this article in press as: Vajta, G., Handmade cloning: the future way of nuclear transfer?, Trends Biotechnol. (2007), doi:10.1016/j.tibtech.2007.04.004

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.

Acknowledgement

The author thanks the contribution of all his colleagues who participated in the establishment of the handmade cloning technique in various laboratories around the world. Special thanks to Poul Maddox-Hyttel for the fruitful collaboration, as well as the cow cartoons in Figure 1.

References

1 Spemann, H. (1938) *Embryonic Development and Induction.* Yale University Press, p.211
2 Briggs, R. and King, T.J. (1952) Nuclear transplantation studies on the early gastrula (*Rana pipiens*). *Dev. Biol.* 2, 252–270
3 Willadsen, S.M. (1986) Nuclear transplantation in sheep embryos. *Nature* 320, 63–65
4 Wilmut, I. *et al.* (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* 385, 810–813
5 Xu, K.P. *et al.* (1990) Immature zona-free bovine oocytes can be matured, fertilized and cultured to the blastocyst stage *in vitro*. *J. Reprod. Fertil.* 5, 57
6 Wu, G-M. *et al.* (2004) Birth of piglets by *in vitro* fertilization of zona-free porcine oocytes. *Theriogenology* 62, 1544–1556
7 Vajta, G. *et al.* (2000) New method for culture of zona-included or zona-free embryos: the well-of-the-well (WOW) system. *Mol. Reprod. Dev.* 55, 256–264
8 Booth, P.J. *et al.* (2001a) Simplification of bovine somatic cell nuclear transfer by application of a zona-free manipulation technique. *Cloning Stem Cells* 3, 139–150
9 Booth, P.J. *et al.* (2001b) Application of the zona-free manipulation technique to porcine somatic nuclear transfer. *Cloning Stem Cells* 3, 191–197
10 Tatham, B.G. *et al.* (1995) Enucleation by centrifugation of *in vitro*-matured bovine oocytes for use in nuclear transfer. *Biol. Reprod.* 53, 1088–1094
11 Peura, T.T. *et al.* (1998) The effect of recipient oocyte volume on nuclear transfer in cattle. *Mol. Reprod. Dev.* 50, 185–191
12 Peura, T.T. *et al.* (2001) Development of bovine embryo-derived clones after increasing rounds of nuclear recycling. *Mol Reprod. Dev.* 58, 384–389
13 Thouas, G.A. *et al.* (2003) The GO system – a novel method for microculture for *in vitro* development of mouse zygotes to the blastocyst stage. *Reproduction* 126, 1–9
14 Vajta, G. *et al.* (2001) Somatic cell cloning without micromanipulators. *Cloning* 3, 89–95
15 Vajta, G. *et al.* (2003) Handmade somatic cell cloning in cattle: analysis of factors contributing to high efficiency *in vitro*. *Biol. Reprod.* 68, 571–578
16 Oback, B. *et al.* (2003) Cloned cattle derived from a novel zona-free embryo reconstruction system. *Cloning Stem Cells* 5, 3–12
17 Lagutina, I. *et al.* (2005) Somatic cell nuclear transfer in horses: effect of oocyte morphology, embryo reconstruction method and donor cell type. *Reproduction* 130, 559–567
18 Lagutina, I. *et al.* (2006) Birth of cloned pigs from zona-free nuclear transfer blastocysts developed *in vitro* before transfer. *Cloning Stem Cells* 8, 283–293
19 Ribas, R. *et al.* (2006) Modifications to improve the efficiency of zona-free mouse nuclear transfer. *Cloning Stem Cells* 8, 10–15
20 Vajta, G. *et al.* (2004) Highly efficient and reliable chemically assisted enucleation method for handmade cloning (HMC) in cattle and pig. *Reprod. Fertil. Dev.* 17, 791–797
21 Li, J. *et al.* (2006) Chemically assisted handmade enucleation of porcine oocytes. *Cloning Stem Cells* 8, 241–250
22 Vajta, G. *et al.* (2005) Handmade Cloning approach: potentials and limitations. *Reprod. Fertil. Dev.* 17, 97–112
23 Kragh, P.M. *et al.* (2005) Efficient *in vitro* production of porcine blastocysts by handmade cloning with combined electric and chemical activation. *Theriogenology* 64, 1536–1545
24 Lagutina, I. *et al.* (2007) Comparative aspects of somatic cell nuclear transfer with conventional and zona-free method in cattle, horse, pig and sheep. *Theriogenology* 67, 90–98
25 Du, Y. *et al.* (2007) Piglets born from handmade cloning. *Reprod. Fertil. Dev.* 19, 135

Please cite this article in press as: Vajta, G., Handmade cloning: the future way of nuclear transfer?, Trends Biotechnol. (2007), doi:10.1016/j.tibtech.2007.04.004