Articles

Oocyte-induced haploidization*

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Gianpiero D Palermo developed ICSI, the revolutionary procedure that alleviates male infertility. He established the ICSI programmes with André Van Steirteghem at the Brussels Free University in Belgium and later with Zev Rosenwaks at Cornell University in New York. ICSI is progressively superseding standard in-vitro insemination: over 40,000 babies have been born from this procedure worldwide. Dr Palermo completed his clinical training in Obstetrics and Gynaecology at the University of Bari in Italy, attended the Masters and PhD programmes at the Brussels Free University and is currently completing additional post-doctoral training in New York and Melbourne. Dr Palermo has won many prestigious prizes and awards for his pioneer work in Reproductive Biology and has delivered more than 100 lectures before international audiences on topics of mammalian fertilization. He is also a prolific author. Since 1993 he has been Director of the ICSI Program at the Cornell Institute for Reproductive Medicine and Associate Professor at the Weill Medical College of Cornell University. He leads a team of talented researchers actively involved in molecular and genetic aspects of fertilization, follow-up of ICSI babies, genetic aspects of male infertility as well as devising new procedures to treat age-related female infertility.

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Abstract

This paper describes the technical approach to treatment of age-related oocyte aneuploidy. Although one solution can be oocyte/embryo selection, another is represented by the nuclear transplantation procedure. The efficiency of nuclear transplantation into immature oocytes is described as a way of generating embryos, and the possibility that viable female gametes can be constructed by transfer of diploid somatic cell nuclei into enucleated oocytes. Germinal vesicle (GV)-stage mouse oocytes were collected from unstimulated ovaries and somatic nuclei were obtained from mouse cumulus cells obtained after ovarian stimulation. Spare human GV-stage oocytes were donated from consenting patients undergoing intracytoplasmic sperm injection (ICSI) treatment, and human somatic cells were stromal cells coming from uterine biopsies performed on consenting patients undergoing endometrial cell co-culture. GV ooplasts, prepared by enucleation, were transplanted with either GV or somatic nuclei by micromanipulation. Grafted oocytes were electrofused and cultured to allow maturation, following which they were selected at random for insemination or cytogenetic analysis. GV transplantation was accomplished with an overall efficiency of ~80 and 70% in the mouse and the human respectively. The maturation rate of 96% (mouse) and 62% (human) following reconstitution was comparable to that of control oocytes, as was the incidence of aneuploidy among the reconstituted oocytes. The reconstituted human oocytes were successfully fertilized by ICSI at a rate of 52%. After the transfer of mouse cumulus or human endometrial cell nuclei into enucleated immature oocytes, a polar body was extruded in >40%. In a limited number of observations where the nucleus of an aged oocyte was transferred into a younger ooplasm, the chromosomes segregated normally at the time of polar body extrusion. The technique of nuclear transplantation itself did not increase the incidence of chromosomal anomalies in the mouse or human, since their oocytes reconstituted with homologous donor GV resumed meiosis to metaphase II and maintained a normal ploidy. In addition, immature mouse ooplasts induced haploidization of transplanted somatic cell nuclei. Although further evaluation of their genetic status is needed, the procedure may offer a realistic way of producing normal oocytes in cases of aged-related infertility. While the procedure is technically similar to cloning, it would generate a unique individual as a result of the contribution of both parental genomes.

Keywords: *aneuploidy, cell fusion, in-vitro maturation, nuclear transplantation, oocyte micromanipulation*

Introduction

In a large majority of infertile couples, oocytes may harbour metabolic or genetic abnormalities, one sometimes being a consequence of the other (Van Blerkom, 1996). Some infertile couples consistently produce embryos with morphological abnormalities supposedly linked to respiratory or metabolic oocyte defects, some of which may be treated by supplementation with ooplasm aspirated from a fertile donor oocyte (Cohen *et al.*, 1998). Nevertheless, some genetically abnormal oocytes can develop into embryos that, morphologically, would be considered suitable for uterine transfer (Munné *et al.*, 1995). The risk of conceiving an aneuploid fetus has been reported to increase from 6.8% for women of 35–39 years to ~50% in women ≥45 years (Hassold and Chiu, 1985). The large majority of chromosomally

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abnormal embryos probably do not implant, however, and even when they do so the fetus generally does not reach term.

Genetic problems (oocyte aneuploidy) also appear to be the pricing factor inducing the decline in maternal fertility that is evident by 40 years (Tietze, 1957; Dailey *et al.*, 1996). In accord with this, 37.2% of morphologically normal 8-cell embryos in the 40–45 maternal age group were found to express chromosomal aberrations (Munné *et al.*, 1995), again suggesting that aneuploidy is often responsible for implantation failure. Older women have become pregnant after replacement of embryos derived from oocytes donated by younger women, confirming that the reduced ability to procreate with advanced maternal age is due to the genetic constitution of the oocyte and not the uterine environment (Rosenwaks, 1987; Legro *et al.*, 1995).

The abnormalities of the oocyte karyotype associated with ageing relate primarily to abnormal segregation during meiosis I (Eichenlaub-Ritter, 1998; Hassold and Hunt, 2001), and due to effects on the meiotic spindle (Battaglia *et al.*, 1996; Volarcik *et al.*, 1998). Why ageing affects this step is still unknown, although free oxygen radicals (Tarín, 1995; Tarín *et al.*, 1996) and/or a compromised perifollicular microcirculation may be responsible (Gaulden, 1992; Van Blerkom *et al.*, 1997).

The allo-transfer of ooplasm into eggs or embryos has been shown to improve their developmental potential (Muggleton-Harris *et al.*, 1982; Pratt and Muggleton-Harris, 1988; Flood *et al.*, 1990; Levron *et al.*, 1996), and in the case of putatively low grade mature human oocytes the transfer of cytoplasm from fertile donor oocytes has resulted in successful deliveries (Cohen *et al.*, 1997, 1998; Barritt *et al.*, 2001). However, since this procedure was performed on mature oocytes, it has no bearing on the prior events of meiosis I.

Attempts to improve the chance of achieving a pregnancy in women who are at increased risk for oocyte aneuploidy have involved selection of oocytes and embryos by preimplantation genetic diagnosis (PGD) (Gianaroli *et al.*, 1997, 1999; Munné *et al.*, 1999; Verlinsky *et al.*, 1999). While selection of oocytes and embryos with a normal chromosomal content might enhance the implantation rate, this leaves fewer embryos for transfer.

An alternative solution for aneuploidy prevention would be the transfer of a germinal vesicle (GV) from an aged oocyte into a younger (donor) ooplast (Zhang *et al.*, 1999). However, nuclear transplantation needs to be performed at the GV stage where the younger cytoplasm would foster healthy spindle formation, so allowing normal meiosis. Fortunately, nuclear transplantation to the mammalian oocyte can in principle be accomplished efficiently without affecting its maturation or increasing the incidence of chromosomal abnormalities (Takeuchi *et al.*, 1999a, 2001a).

The downside of the nuclear transplantation procedure is the availability of oocytes. Those of women ≥40 years are 'compromised', and generally, only a few can be recovered. Therefore, even when optimized, nuclear transplantation procedures have to deal with an initially low number of eggs, the inefficiency of the in-vitro maturation process, and the efficacy of the transplantation technique itself.

Another more radical approach would be the induction of meiosis-like reduction divisions in diploid cell nuclei within an enucleated donor oocyte (Takeuchi *et al.*, 1999b, 2000). The availability of such 'manufactured' oocytes would be dictated by the number of donated eggs and not by the efficiency of the technique.

Preliminary results from nuclear transplantation of mouse and human oocytes

Immature GV oocytes were retrieved by puncturing follicles of unstimulated ovaries of B6D2F1 mice. In order to prevent spontaneous germinal vesicle breakdown (GVBD), cumulus-free oocytes were cultured until their use in medium (M199) supplemented with a phosphodiesterase inhibitor (0.2 mmol/l 3-isobutyl-1-methylxanthine; Sigma Chemical, St Louis, MO, USA).

All the micromanipulation and electrofusion procedures were performed in a shallow plastic Petri dish on a heated stage, placed on an inverted microscope equipped with hydraulic micromanipulators (Takeuchi *et al.*, 1999a). The zona pellucida was breached with a glass microneedle, then oocytes were exposed to 25 μg/ml of cytochalasin B (CCB). The GV nucleus surrounded by a small amount of cytoplasm (GV karyoplast) was removed by a micropipette of 20 μm inner diameter, and was inserted into the perivitelline space of another oocyte previously enucleated at the GV stage (GV ooplast). Each grafted oocyte was aligned with a micromanipulator between two micro-electrodes perpendicular to their axes. To induce fusion, a single 1.0 kV/cm direct current fusion pulse was delivered for 99–100 μs in an electrolyte medium (M2) by an Electro Cell Manipulator (BTX 200 and 2001, Genetronics Inc., San Diego, CA, USA). Then, after washing and culture for 30 min in a CCB-free medium, these oocytes were examined to confirm cell survival and fusion. Successfully reconstituted oocytes were incubated for 12–16 h after fusion to allow maturation, which was assessed according to extrusion of a first polar body (PB).

Spare human GV oocytes were retrieved after pituitary desensitization with gonadotrophin-releasing hormone (GnRH) agonist and ovulation induction with gonadotrophins as described previously (Palermo *et al.*, 1995, 1996) from consenting patients undergoing ICSI. Immediately prior to ICSI, cumulus–corona cells were removed by enzymatic and mechanical treatments. The denuded oocytes were then examined under an inverted microscope to assess their integrity and nuclear status (Palermo *et al.*, 1995, 1996). Nuclear transplantation in humans was performed essentially as described for the mouse (Takeuchi *et al.*, 2001a), following which the oocytes were examined at 24 and 48 h after electrofusion to evaluate nuclear maturation, characterized by GVBD and subsequent extrusion of the first PB.

In the mouse, nuclear transplantation into GV-stage oocytes, followed by extrusion of a PB, has been achieved with an overall efficiency of 80%. Interestingly, this aggressive technique appears not to increase the incidence of chromosomal abnormalities (Takeuchi *et al.*, 1999a). Human oocytes were reconstituted similarly with an efficiency of 73%. The maturation rate of 62% following their reconstitution

was comparable to that of control human GV oocytes, as was the 20% incidence of aneuploidy. The fertilization rate of those reconstituted human oocytes was 52%, but although they underwent early cleavage, their survival and quality was less optimal than that of in-vivo matured controls (Takeuchi *et al.*, 2001a). In other recent studies, lower maturation rates and impaired embryo development have been the rule, probably attributable to the suboptimal in-vitro culture conditions currently used for human oocyte maturation (Zhang *et al.*, 1999; Takeuchi *et al.*, 2001a).

In a limited number of observations where the nucleus of an aged oocyte was transferred into a younger ooplasm, there was a normal first meiotic division accompanied by the extrusion of the first PB.

While further cytogenetic information is needed regarding such reconstituted oocytes, this approach appears to be the only treatment option for age-induced aneuploidy. Although further improvements need to be made, especially in regard to the in-vitro maturation process, the technique may open the way to prevention of chromosomal defects associated with oocyte ageing.

An alternative source of oocytes

While better culture conditions might enhance the limited ability of immature reconstituted human oocytes to mature *in vitro*, the low number of such oocytes remains another limiting factor. More might be created by a form of cloning – transplantation of a patient's somatic cell nucleus into an enucleated ooplast obtained from a younger donor. This approach would benefit older women, women with premature ovarian failure, or those considered as 'poor responders' (Tsai *et al.*, 2000; Tesarik *et al.*, 2001). Indeed, several such attempts to 'manufacture' gametes are currently underway for humans and animals (Takeuchi *et al.*, 2001b; Tesarik *et al.*, 2001; Lacham-Kaplan *et al.*, 2001; Nagy *et al.*, 2001).

Since GV oocytes often tend to complete the first meiotic division spontaneously *in vitro*, it seemed possible that GV-stage ooplasm might be able to induce the haploidization of diploid somatic nuclei (Kubelka and Moor, 1997), with a transition to metaphase II (Takeuchi *et al.*, 1999b; Tsai *et al.*, 2000). Therefore, the transfer of somatic nuclei to GV ooplasts and their ensuing haploidization may provide a source of viable mammalian oocytes, particularly for patients who are candidates for oocyte donation.

To obtain such nuclei from consenting patients undergoing endometrial cell co-culture during IVF, endometrial stromal cells were isolated from uterine biopsies by enzymatic digestion using 0.2% collagenase type II with differential sedimentation (Barmat *et al.*, 1998), and were cultured in a long-term culture medium supplemented with 10% fetal bovine serum (FBS). In the case of the mouse (B6D2 F1), cumulus–oocyte complexes were obtained after ovarian stimulation with pregnant mares' serum gonadotrophin (PMSG) and human chorionic gonadotrophin (HCG), and cumulus cells isolated by brief exposure to hyaluronidase were cultured for up to 30 days. GV oocytes were retrieved from the same strain by puncturing follicles of unstimulated ovaries and were denuded by mechanical removal of cumulus cells.

Table 1. Ability of the reconstituted mouse oocytes to extrude the first polar body. GV = germinal vesicle, IPB = first polar body.

No. of oocytes	*Origin of the somatic cell nucleus*	
	Human endometrium	*Mouse cumulus*
Intact GV	23	41
Enucleated	23	41
Grafted (%)	17 (73.9)	35 (85.4)
Reconstituted (%)	11 (47.8)	32 (78.0)
Extruded IPB (%)	9 (39.1)	16 (39.0)

All the micromanipulation and electrofusion procedures were carried out as described previously (**Figure 1**) (Takeuchi *et al.*, 1999a). Cultured human endometrial stromal and mouse cumulus cells were released from the dish with trypsin-EDTA, and one or the other was then inserted subzonally into an enucleated mouse GV oocyte (**Figure 2**). After, each grafted oocyte was then manually aligned between two microelectrodes for electrofusion (**Figure 3**). To allow maturation, reconstituted oocytes were incubated up to 14–16 h until extrusion of the first PB. To evaluate the distribution status of nuclear chromatin, some mature oocytes were stained with a DNA specific staining (DAPI), while others were anchored between a microslide and coverslip, fixed with methanol/acetic acid (3:1; v/v), and stained with 1% orcein in 45% acetic acid.

Out of a total of 64 enucleated GV oocytes fused with somatic cells, the overall efficiency of the sequence from an intact GV oocyte to a reconstituted oocyte with an extruded PB (**Figure 4**) was 39.1% for endometrial cells and 39.0% for cumulus cells, respectively (**Table 1**). Further in-vitro culture for up to 24 h did not improve these results.

Other studies were performed to evaluate the karyotypes of reconstituted mature oocytes by gradual fixation and subsequent Giemsa staining (Takeuchi *et al.*, 1999a). A total of 77 enucleated GV oocytes were successfully grafted with a single cumulus cell, 72% (56) being reconstituted successfully by electrofusion, and 29 of the reconstituted oocytes (51.8%) extruding their first PB. Among the 13 analysable karyotypes of the latter, five (38.5%) had a set of haploid chromosomes in the ootid and PB (**Figure 5**), five showing structural aberrations such as pulverized chromosomes, and the remaining three were diploid with telophase-like structures.

The observation that immature mouse ooplasts can haploidize human or murine somatic cell nuclei suggests that this approach may provide an alternative source of viable oocytes. Clearly, somatic cell nuclei can be successfully haploidized at an acceptable rate in this way and reach MII with an apparently normal meiotic spindle. However, more detailed cytogenetic analysis of this outcome is needed, the behaviour of the somatic cell centrosome, during fertilization and later cleavage, must be elucidated. It is also unknown whether the imprinting pattern of such reconstructed gametes is comparable to that of a naturally produced haploid nucleus.

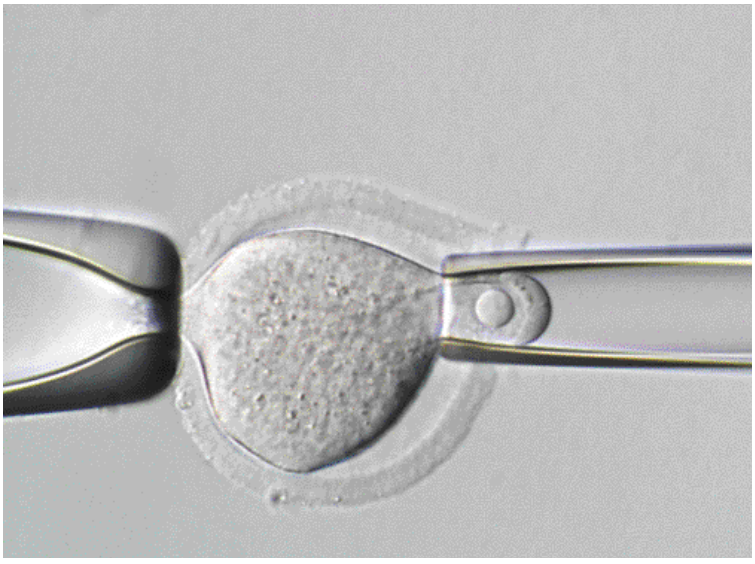

Figure 1. Removal of a germinal vesicle from a mouse oocyte.

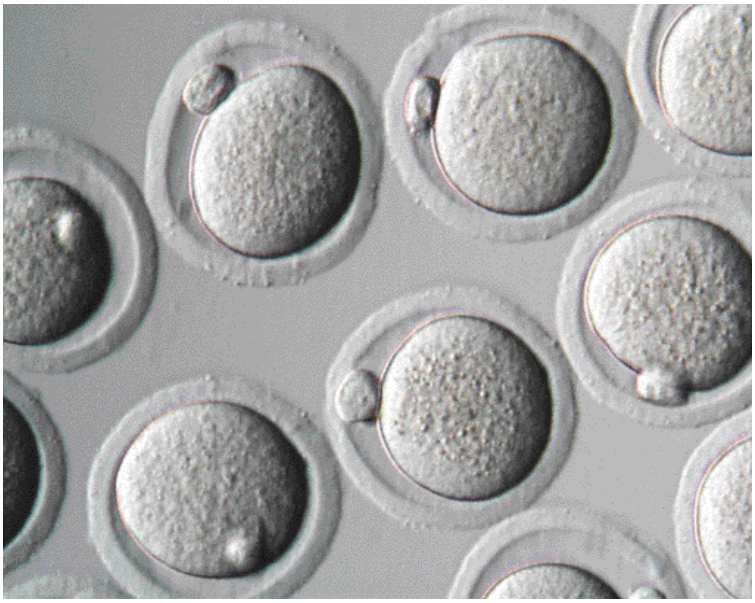

Figure 2. Grafted oocytes with mouse cumulus cells.

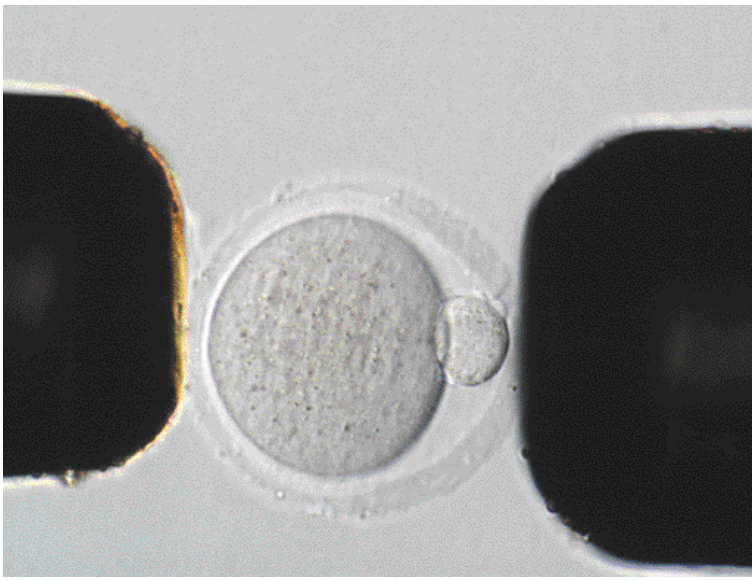

Figure 3. Grafted oocyte with a cumulus cell aligned between two microelectrodes.

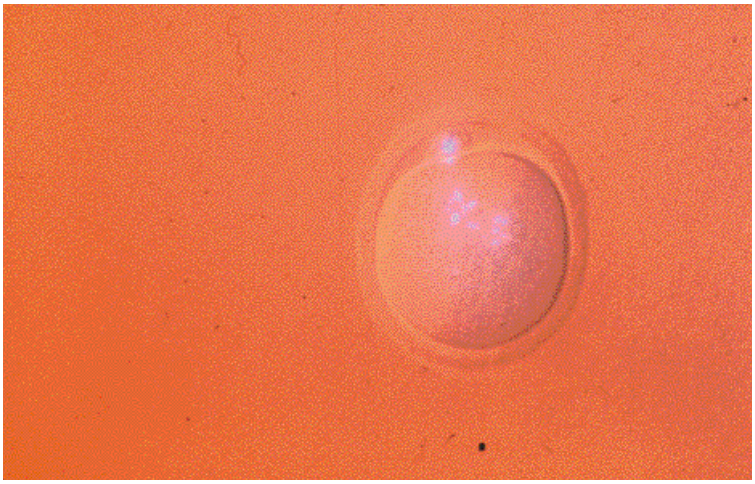

Figure 4. Mouse reconstituted oocyte with human endometrial cell showing segregated chromosomes in both the ootid and the polar body.

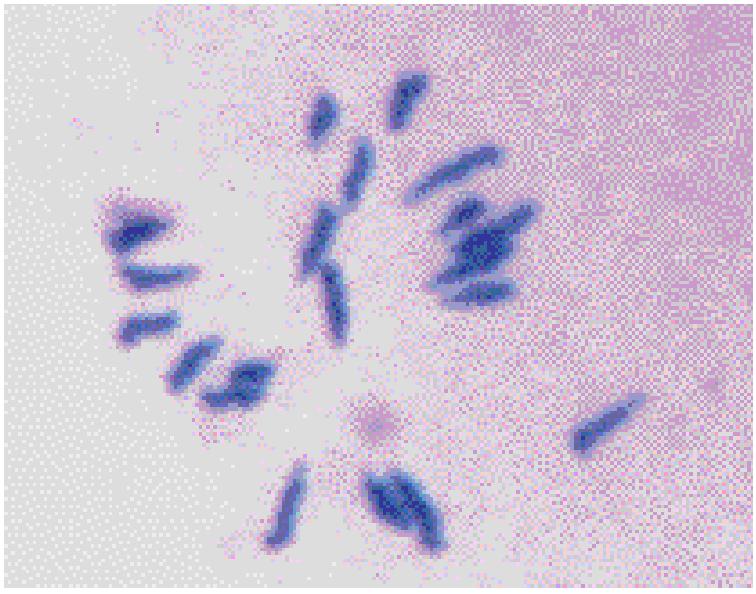

Figure 5. A normal haploid set of chromosomes stained with Giemsa in a reconstituted oocyte after extrusion of the first polar body.

Discussion

Techniques such as nuclear transfer being explored now in animal research may eventually offer a treatment option for some infertile couples, particularly those with age-related infertility. Transplantation of an isolated GV into an enucleated 'young' ooplast can be accomplished with relative efficiency, and such reconstituted oocytes can undergo nuclear maturation, fertilization, and early cleavage at least. However, oocyte availability remains as a limiting factor, since older patients generally produce only a small number.

In order to bypass this limitation, we have proposed a way to 'construct' oocytes by inducing haploidization of somatic nuclei within immature ooplasts. Fluorescent DNA staining was utilized to assess chromatin distribution between the polar body and ootid (Takeuchi *et al.*, 1999b; Lacham-Kaplan *et al.*, 2001) and also of chromosome spreads (Kubelka and Moor, 1997; Takeuchi *et al.*, 2000). Karyotyping shows that the transplanted somatic nucleus can undergo spontaneous segregation with extrusion of a PB. Oocytes that failed to extrude PBs displayed two sets of chromosomes with two spindle centres (Kubelka and Moor, 1997).

Preliminary experiments suggest that somatic cell haploidization can be achieved by a mature metaphase II stage ooplasm, which can force a G0/G1 somatic cell nucleus to undergo a premature M-phase, bypassing the S-phase with resulting segregation of one set of chromatids into either two distinct pronuclei or into one single pronucleus and polar body. Haploidization of human oocyte cumulus cells in this way was confirmed by fluorescence in-situ hybridization (FISH) analysis on either the pronucleus and/or the polar body (Takeuchi *et al.*, 2001b; Tesarik *et al.*, 2001). Thus haploidization of somatic nuclei can occur either in immature or in mature ooplasm, the latter requiring an additional activating stimulus.

When donor ooplasts are available, their construction as entire oocytes would represent a definitive advantage where only a few 'aged' oocytes are available, embryo development up to blastocyst stage in zygotes generated from haploidized somatic cells has been observed (Lacham-Kaplan *et al.*, 2001). Diploid cells may replace either the male or female gamete, making somatic cell haploidization a potential treatment for both male and female infertility. Although this procedure is technically similar to cloning, biologically the technique

assures a paternal genomic contribution to create a new individual. Nonetheless, more extensive cytogenetic information, such as spectral karyotyping and meiotic recombination assay, is needed to further ascertain just how normal is the haploidized nucleus. Although the safety of nuclear transplantation procedures can only be confirmed by obtaining healthy offspring, the results obtained so far certainly justify further research.

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