

Articles

Fertilizable oocytes reconstructed from patient's somatic cell nuclei and donor ooplasts



Jan Tesarik obtained his MD degree in 1979 and PhD in 1982. He realized the first successful gamete intra-Fallopian transfer (GIFT) and the first childbirths after oocyte fertilization with round spermatids (1995) and with in-vitro cultured spermatids from a man with meiotic maturation arrest (1998). He developed an original technique for nuclear transfer in mature human oocytes (2000). He is author or co-author of >250 scientific publications. At present he is director of MAR&Gen (Molecular Assisted Reproduction & Genetics) in Granada (Spain) and scientific consultant for the Laboratoire d'Eylau (Paris, France) and European Hospital (Rome, Italy).

Dr Jan Tesarik

J Tesarik^{1,4}, ZP Nagy, M Sousa², C Mendoza³, R Abdelmassih

Clinic and Research Centre for Human Reproduction Roger Abdelmassih, São Paulo, Brasil

¹Laboratoire d'Eylau, 55 rue Saint-Didier, 75116 Paris, France; ²Laboratory of Cell Biology, Institute of Biomedical Sciences, University of Porto, Porto, Portugal; ³Molecular Assisted Reproduction and Genetics, Gracia 36, 18002 Granada, Spain

⁴Correspondence: Fax: +34 958 265043; e-mail: cmendoza@ugr.es

Abstract

The only assisted reproduction treatment now available for women with ovarian failure or irreparable oocyte defects is oocyte donation. However, some women experience psychological barriers to the recourse to donor oocytes, related to the lack of contribution of their proper genes to the progeny. A pilot study in humans suggests that this problem may be overcome by the development of techniques for haploidization of somatic cell nuclei, allowing the formation of new oocytes bearing the complete nuclear genome of the patient. Somatic cell nuclei were obtained from cumulus cells of a patient who failed to produce fertilizable oocytes and were transferred into enucleated oocytes (ooplasts) from a donor. Out of six ooplasts injected with the somatic cell nuclei and fertilized with spermatozoa from the patient's husband, signs of haploidization were detected in three oocytes, two of which subsequently started embryonic development and were cryopreserved for eventual future transfer to the genetic mother. These data show that human oocytes can be used for both reprogramming and haploidization of somatic cell nuclei, allowing reconstruction of genetically own oocytes for patients without, or with seriously disturbed, ovarian function.

Keywords: oocyte donation, oocyte reconstruction, ovarian failure, somatic nucleus haploidization

Introduction

Oocyte donation is now the only efficient treatment for restoring fertility in women who lack ovarian function (Sauer *et al.*, 1991) or whose ovaries produce oocytes of poor quality (Rosenwaks, 1987). However, this approach is not acceptable for certain individuals because it leads to a complete loss of the maternal genetic contribution to the progeny (Englert and Govaerts, 1998). One possibility to be proposed to women whose ovaries can still produce oocytes is ooplasmic transfer from donor oocytes (Cohen *et al.*, 1997, 1998). Recently, nuclear transfer techniques – which are increasingly used in animal cloning experiments (Campbell, 1999) – have been suggested for reconstruction of defective patients' oocytes by transferring their nuclei into donor ooplasts (Zhang *et al.*, 1999; Tsai *et al.*, 2000; Tesarik *et al.*, 2000). However, none of these techniques is applicable in women from whom no oocyte can be recovered.

Previous work suggests that the cytoplasm of enucleated metaphase II oocytes (ooplasts) may not only reprogramme blastomere nuclei, rendering them totipotent, but also enable the doubling of the reprogrammed nuclei after subsequent oocyte activation in the presence of the microtubule-blocking agent nocodazol (Kwon and Kono, 1996). The idea that the metaphase II ooplast may be used to force a diploid cell nucleus to undergo a reduction division (haploidization) similar to that occurring during meiosis in gametogenesis has been corroborated empirically by the finding that nuclei of mouse secondary spermatocytes injected into metaphase II mouse oocytes enter metaphase and are dealt with subsequently by the oocytes as if they were their own nuclei, leading to haploidization and extrusion of an extra haploid set of chromatids into a structure resembling the polar body (pseudopolar body) (Kimura and Yanagimachi, 1995). Though encouraging, those data were obtained with germ cell nuclei

that had already undergone the first meiotic division. In fact, despite having the same DNA content as somatic cell nuclei in G0/G1 phase of the cell cycle, secondary spermatocyte nuclei may react in a different way to oocyte cytoplasmic factors because of differences in the molecular mechanisms governing mitosis and meiosis. Notwithstanding, pioneering experiments in the mouse (Kubelka and Moor, 1997; Tsai *et al.*, 2000) have suggested that the above strategy of oocyte production from somatic cells is viable.

Here we report a case where a woman who unexpectedly failed to produce viable oocytes after ovarian stimulation consented to her cumulus cells, which were identified in the follicular aspirates, being used in an attempt to reconstruct oocytes by haploidizing them in ooplasts prepared from donated oocytes. After obtaining informed consent from the couple, the reconstructed oocytes were fertilized, and the resulting embryos were cryopreserved for eventual later transfer.

Materials and methods

Ovarian stimulation and follicle aspiration

After pituitary desensitization with triptorelin (Decapeptyl, Ipsen Pharma, Paris, France), beginning in the midluteal phase of the menstrual cycle, the growth of ovarian follicles was stimulated by daily injections of recombinant FSH (rFSH) (Puregon, Organon, Oss, The Netherlands) whose dosage was being adjusted during the stimulation according to serum oestradiol concentrations and the size of ovarian follicles as determined by ultrasonography. When at least three follicles reached the diameter of >18 mm, ovulation was induced with 10 000 IU of human chorionic gonadotrophin (HCG) (Profasi, Serono, Rome, Italy). All antral follicles detected in both ovaries were aspirated transvaginally under ultrasonographic control 36 h after the HCG injection.

Micromanipulation techniques

Oocytes were separated from their surrounding mass of cumulus oophorus by digesting the cumulus extracellular material for 30 s with 50 IU/ml hyaluronidase (Sigma, St. Louis, MO, USA) dissolved in Gamete-100 medium (Scandinavian IVF Science, Gothenborg, Sweden). The cumulus-free oocytes were first incubated for 10 min at 37°C with 5 µg/ml cytochalasin B (Sigma) in Gamete-100 medium and then transferred immediately to drops of 2 µl Gamete-100 medium located on cell-culture-grade plastic Petri dishes under embryo-tested mineral oil (Scandinavian IVF Science). The dishes were placed in an inverted microscope equipped with Hoffman modulation contrast optics (Nikon, Tokyo, Japan), a Narishige micromanipulator set and non-contact laser microsurgery equipment (Fertilase, Medical Technologies Montreux, Montreux, Switzerland). A hole of ~20 µm was laser-drilled in the polar body area, and oocytes were enucleated with the use of a blastomere-separation pipette (Humagen Fertility Diagnostics), as described in Tesarik *et al.*, 2000.

The suspension of cumulus cells remaining after cumulus oophorus enzymatic digestion was washed free of hyaluronidase by two cycles of centrifugation (500 g, 5 min) and resuspension in Gamete-100 medium. For nuclear transfer,

washed cumulus cells were added to drops of Gamete-100 medium containing the enucleated oocytes (ooplasts). After oocyte immobilization on the holding micropipette, nuclei were isolated mechanically from individual cumulus cells (Wakayama *et al.*, 1998) and injected into oocytes using intracytoplasmic sperm injection (ICSI) micropipettes (Humagen Fertility Diagnostics, Charlottesville, VA, USA) and a non-activating micro-injection technique previously developed for injection of leukocytes (Sousa *et al.*, 1996). After nuclear transfer, ooplasts were incubated for 14 h in IVF medium (Scandinavian IVF Science) under previously described conditions (Tesarik *et al.*, 2000) and subsequently injected with spermatozoa using a standard ICSI technique (Tesarik and Sousa, 1995).

Embryo in-vitro culture and cryopreservation

Sperm-injected reconstituted oocytes and early cleaving embryos were cultured at 37°C in IVF medium (Scandinavian IVF Science) equilibrated with 5% CO₂ in air (Tesarik and Greco, 1999). The duration of culture was 48 h from the time reconstituted oocytes were injected with spermatozoa. Embryos that had undergone at least one cleavage division by the end of the culture period were cryopreserved by using the embryo freezing kit Freeze-kit 1 (Scandinavian IVF Science) and the manufacturer's instructions.

Microscopical analysis

All analyses performed in this study were non-invasive, in order to preserve oocyte and embryo viability for eventual future transfer to the mother. Observations on reconstituted oocytes, pronuclear- (PN-) stage zygotes and cleaving embryos were performed in an inverted research microscope equipped with Hoffman modulation contrast optics (Nikon), using 20x and 40x objectives. These conditions allowed an accurate evaluation of the male and female nucleus remodelling at the PN stage and prediction of the embryonic developmental potential (Tesarik and Greco, 1999).

In-situ hybridization

Pseudopolar bodies that separated from ooplasts after nuclear transfer and subsequent sperm injection were isolated and prepared for in-situ hybridization by using the technique previously developed for analysis of polar bodies of human oocytes (Durban *et al.*, 1998). Hybridizations were performed with AneuVysion multicolour DNA probe kit (CEP 18, X, Y-alpha satellite, LSI 13,21; Vysis, Downers Grove, IL, USA), as recommended by the manufacturer.

Case report

A case of female infertility due to poor ovarian response to hormonal stimulation was treated in this IVF programme. After pituitary desensitization and ovarian stimulation, only two oocytes were identified in the follicular aspirates, and both of them showed signs of advanced degenerative alterations combined with immaturity, making their use for IVF impossible. In contrast, both oocytes were surrounded by numerous cumulus oophorus cells, most of which were viable, as determined by supravital eosin staining.

Coincidentally, 14 metaphase II oocytes were recovered on the same day from another woman undergoing IVF treatment for male factor (no female pathology detected), who agreed to make use of only eight of the oocytes for her own treatment and to donate the remaining six. After obtaining informed consent from the couple in whom no healthy oocytes had been recovered, it was decided to try to reconstruct new oocytes for this couple by using the patient's cumulus cells and the six donor oocytes. The choice of cumulus cells as the source of the patient's somatic cell nuclei was based on the previous observations that most of these cells are spontaneously in the G0/G1 phase of the cell cycle and on their relatively high efficiency in mouse cloning (Wakayama *et al.*, 1998).

The donor oocytes were enucleated, and each resulting ooplast was injected with a human cumulus cell nucleus. After injection, the oocytes were maintained in culture for 14 h. The injected cumulus cell nuclei became invisible by 1 h after injection, indicating that metaphase-promoting factor persisting in the ooplasts had driven the nuclei to metaphase. This state was maintained throughout the 14 h culture period. Thereafter, each reconstructed oocyte was injected with a spermatozoon from the patient's husband and then cultured for an additional 40 h. A structure similar to the second polar body (pseudopolar body) was extruded in three oocytes by 5 h after sperm injection (Figure 1). The pseudopolar body was located close to the first polar body in one oocyte (Figure 2) and at a larger distance in the other two. This structure was removed and analysed by fluorescence in-situ hybridization (FISH) with a kit of directly labelled fluorescent probes for chromosomes 13, 18, 21, X and Y. One of the specimens was lost during preparation. A single fluorescence signal for chromosomes 13, 18, 21 and X was found in the remaining two pseudopolar bodies, suggesting that the ooplast had not only forced the G0/G1 cumulus cell nucleus to a premature metaphase but had also treated the cumulus cell-derived chromosomes as if they were the oocyte's own chromosomes, by transporting them to a peripheral cytoplasmic location, attaching them to meiotic spindle and, after activation by the injected spermatozoon, splitting them into individual chromatids distributed between the oocyte and the pseudopolar body. By 10 h after sperm injection, two normal-looking PN (Figure 2) appeared in two oocytes. The two PN observed in each oocyte had the same size and morphology and showed the typical PN structure, quite different from the cumulus cell nucleus, with characteristic nucleolar precursor bodies aligned near the interpronuclear contact (Figure 2). This finding suggests an extensive remodelling of the cumulus cell-derived nuclei in the oocyte cytoplasm. Both 2PN zygotes underwent the first cleavage division by the end of the culture period (Figure 1) and were cryopreserved for eventual future transfer to the patient's uterus, awaiting the availability of elements necessary for a realistic estimation of the potential hazards associated with this unorthodox method of oocyte production.

Discussion

The results of this study show that human somatic cell nuclei, presumably in G0/G1 phase, can be rapidly driven to metaphase when injected into metaphase II human ooplasts. The rapidity of the nuclear breakdown (<1 h) suggests that the injected G0/G1 nuclei enter metaphase without previous S-phase, thus escaping a cell cycle checkpoint, which prevents

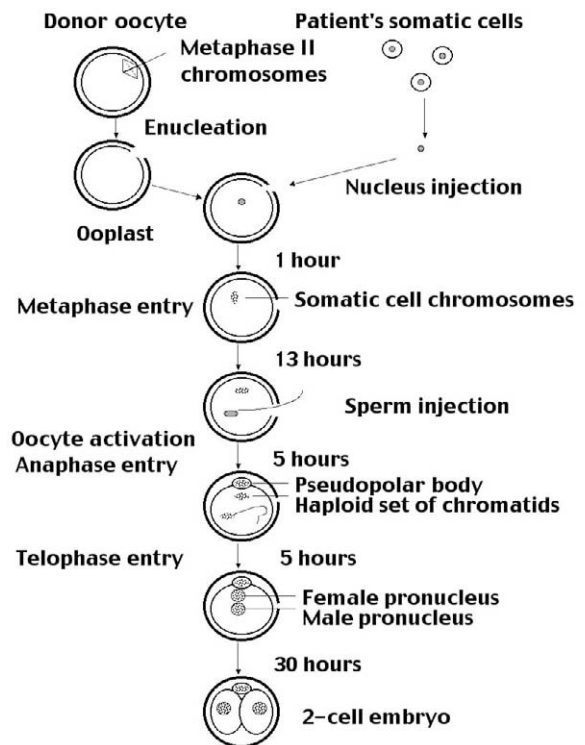


Figure 1. Schematic representation of the manipulations used for somatic cell nucleus haploidization and reconstruction of a fertilizable oocyte.

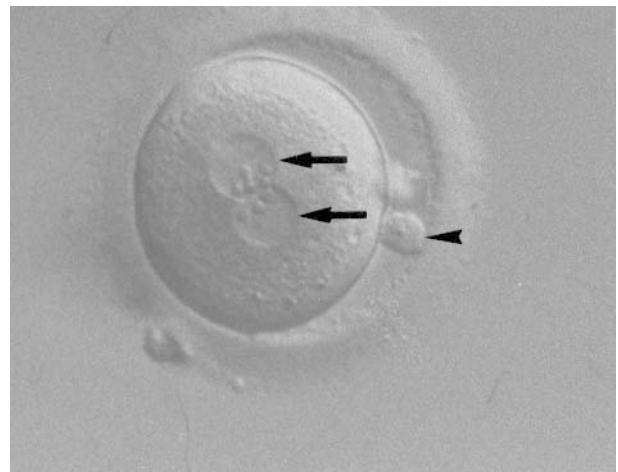


Figure 2. Two-pronucleated zygote obtained by intracytoplasmic injection of a husband's spermatozoon into an oocyte reconstructed from a patient's cumulus cell and a donor ooplast. The pseudopolar body (arrowhead) is located close to the first polar body (slightly out of focus). The two pronuclei (arrows) have the same size and the typical pronuclear morphology, with nucleolar precursor bodies accumulated in the region of each pronucleus facing the other pronucleus.

such a direct transition in intact somatic cells. In this respect, the cell cycle of the reconstructed oocytes appears to follow the developmental programme of the metaphase II ooplasts, forcing the injected somatic nucleus to a direct entry to metaphase without previous S-phase, similar to the second meiotic division in intact oocytes. In fact, the cell cycle checkpoint preventing G1 nuclei from entering metaphase before achieving S-phase is largely dependent on factors present in the cytoplasm (Fulka *et al.*, 2000). Most of such factors originating from the nuclear donor cell are likely to be removed during nucleus preparation for transfer, and those which may remain associated with the nucleus can be expected to be removed or diluted by the voluminous oocyte cytoplasm after the transfer. Studies are under way in two animal models (mouse, cow) to confirm the absence of an intercalated S-phase before the entry of the injected G0/G1 somatic nuclei to metaphase.

The skipping of S-phase with subsequent extrusion of one set of G0/G1 chromatids into the pseudopolar body would lead to a complete haploidization of the somatic cell nucleus. Notwithstanding, the way this nuclear status is achieved is different during artificial somatic cell haploidization as compared with normal meiosis (**Figure 3**). When meiosis is initiated, the oocyte contains two sets of chromosomes, each

chromosome consisting of two chromatids resulting from the last premeiotic S-phase. After the first meiotic division only one set of chromosomes (still consisting of two chromatids) is present, followed by chromosome splitting into individual chromatids during the second meiotic division, which is achieved only after oocyte activation by the fertilizing spermatozoon. Immediately after syngamy, the zygote has two sets of chromatids (one from the oocyte and the other from the spermatozoon), which are subsequently doubled in the respective PN during the first embryonic S-phase (**Figure 3**).

The nuclear status of the construct resulting from the transfer of a G0/G1 somatic nucleus to a metaphase II ooplast is comparable to the secondary oocyte during normal meiosis so far as its DNA content is concerned (**Figure 3**). However, instead of one set of chromosomes each consisting of two chromatids, these constructs contain two sets of single-chromatid chromosomes. The activation of the constructs by the fertilizing spermatozoon can thus lead to segregation of one whole set of single-chromatid chromosomes into the pseudopolar body, so that the embryos resulting from the fertilized reconstructed oocytes can be euploid (**Figure 3**). The ability of the cytoplasm of mouse meiotic oocytes to split chromosomes of mouse fibroblasts into single chromatids has been demonstrated previously (Kubelka and Moor, 1997). A

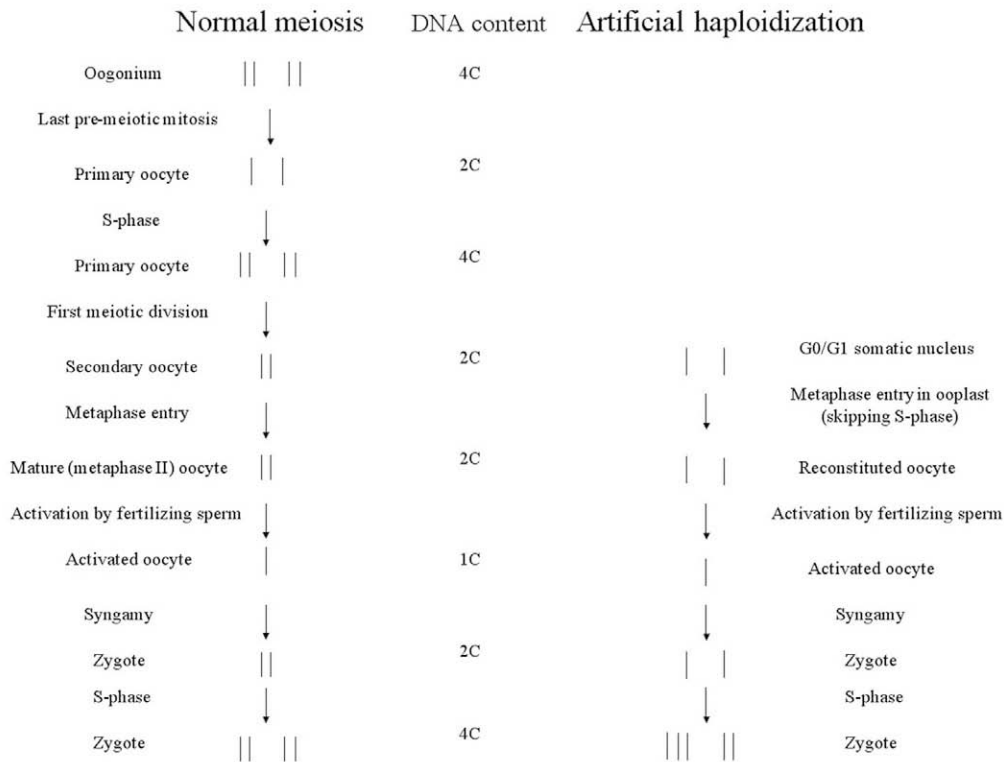


Figure 3. Schematic representation of cell cycle events occurring during artificial somatic cell haploidization, as compared with normal meiosis. Only one chromosome is represented. Vertical bars represent individual chromatids. In different stages of normal meiosis and of artificial haploidization the represented chromosome occurs in two versions or in only one version, each version consisting of one or two chromatids. In both normal meiosis and artificial haploidization the reduction division is achieved after oocyte activation by the fertilizing spermatozoon so that the set of single chromatids of maternal origin can be complemented by a sperm-derived chromatid set to restore the normal chromosome number after the subsequent first zygotic S-phase.

more comprehensive review of the questions related to the inheritance of chromatids and centromeres after various forms of nuclear transfer has been published recently (Edwards and Beard, 1998).

These data show that oocyte cytoplasm can be used to haploidize somatic cell nuclei. A number of points, including the regularity of chromatid separation during this artificial reduction division, the impact of the presumed shortened telomere length in the somatic cell nuclei used for oocyte reconstitution as well as the possible telomere elongation by oocyte and zygote telomerase, interactions between somatic cell- and sperm-derived centrosomes, and the possible anomalies of genomic imprinting, remain to be elucidated. All these questions are important, but approaches to their solution are known and may be tested immediately in an appropriate animal model.

The mechanism of chromatid separation may be manipulated by changing the time elapsed between nuclear transfer and activation of the reconstituted oocyte, or by modulating the activity of protein kinases and phosphatases regulating the cell cycle (Fissore *et al.*, 1999). Shorter telomeres have been reported in Dolly the sheep, the first mammal cloned from a somatic cell (Shiels *et al.*, 1999), but a recent study in bovines has shown that, under certain (yet not completely understood) conditions, shortened somatic cell-derived telomeres can be restored after nuclear transfer to metaphase II ooplasts (Lanza *et al.*, 2000). Incompatibility between the sperm- and oocyte-derived centrosomes may be overridden by fertilizing the reconstructed oocytes with isolated sperm heads without the centrosomal material.

As to genomic imprinting, there is growing evidence for a certain degree of plasticity in this process (Campbell, 1999). For instance, a dominant trans-modification activity was detected in germ cell cytoplasm, and it was capable of inducing similar modifications in somatic nuclei in germ-somatic cell hybrids (Surani, 1999). Further epigenetic modifications may occur after fertilization, as demonstrated earlier (Reik *et al.*, 1993). Thus, the parent-specific mark on imprinted gene regions can be erased during extensive remodelling of somatic cells within metaphase II oocyte cytoplasm and during the first cell cycle after fertilization.

Clearly, all future breakthroughs in the field of mammalian cloning will be a source of valuable information to be used in the development of the technique of gamete reconstruction by somatic cell nuclear haploidization. Yet it has to be stressed that somatic cell nuclear haploidization is not cloning, because it does not lead to asexual production of an embryo. In fact, the endpoint of this technique is a gamete that bears the entire nuclear genome of the female parent and that still needs to be fertilized by a gamete of the opposite sex to start embryonic development. After resolving all pending questions, this technique may enable the restoration of the full biological motherhood to women without ovaries or with complete loss of oocyte production. A modification of this technique can also be envisaged to haploidize male somatic cell nuclei and thus enable genetic fatherhood to men with a complete lack of the germ line.

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