Article

Construction and fertilization of reconstituted human oocytes

Dr Takumi Takeuchi

Dr Takumi Takeuchi completed his MD and PhD training at Gunma University, Japan. At the same university, from June 1993 to March 1997, Dr Takeuchi was co-responsible for the ART programme. In April 1997, he was awarded a research fellowship at the Weill Medical College of Cornell University where together with Gianpiero Palermo he established and pioneered nuclear transplantation procedures in mouse and human oocytes. In September 2002, due to his outstanding research work, he became Assistant Professor of Reproductive Medicine and was also recruited into the Embryology laboratory. Dr Takeuchi routinely performs ICSI as well as genetic testing and epigenetic analysis on gametes and embryos. He has devoted himself to devising novel therapeutic options to treat age-related infertility, such as germinal vesicle transplantation and somatic cell haploidization. On this topic, Dr Takeuchi has published many reports and delivered several lectures before international audiences. Recently, he is working on embryonic stem cell harvesting and differentiation.

Takumi Takeuchi, Queenie V Neri, Gianpiero D Palermo[1]
Centre for Reproductive Medicine and Infertility, Weill Medical College of Cornell University, 505 East 70th Street, HT-336, New York, NY 10021, USA
[1]Correspondence: Tel: +1 212 7461689; Fax: +1 212 7468860; e-mail: gdpalerm@med.cornell.edu

Abstract

Construction of artificial gametes may be made possible by transferring somatic cells into enucleated oocytes and inducing chromosomal halving of their nuclei. This study examines the possibility of constructing viable human gametes, and their potential for participation in normal fertilization. Spare germinal vesicle-stage oocytes were donated by consenting patients undergoing intracytoplasmic sperm injection (ICSI). Approximately 62% of in-vitro matured oocytes survived enucleation and subsequent cumulus cell injection. Following micromanipulation and subsequent activation, about 40% of the reconstituted oocytes yielded two pronuclear-like entities. This was not accompanied by extrusion of a polar body, but resulted in the formation of two 'putative haploid' pronuclei. Therefore selective removal of a female pronucleus marker was required to restore a balanced ploidy. Male pronuclei were identified by association with sperm mitochondria. Additional pronuclei were then removed, allowing further cleavage. Zygotes derived were 'putatively haploid' in ~38% of cases with a limited number of chromosomes assessed. However, on karyotypic analysis, blastomeres isolated from cleaving embryos showed a chaotic distribution of chromosomes. Oocytes could induce 'putative haploidization' of transplanted somatic cell nuclei independently of donor cell gender. Fertilization of artificial oocytes was followed by embryonic cleavage despite blastocyst development and chromosomal content possibly being compromised.

Keywords: *fertilization, fluorescence in-situ hybridization, human oocytes, meiosis, nuclear transplantation*

Introduction

One problematic cause of infertility is the higher incidence of oocyte aneuploidy in older women (Hassold and Chiu, 1985; Munné *et al*., 1995; Dailey *et al*., 1996). Independent of the initial indication or the assisted reproduction technique used to treat it, the pregnancy rate follows a downward slope starting at 35 years (Tietze, 1957). Cytogenetic analyses of oocytes collected from women of varying ages, and their ability to support pregnancies in older women, indicate that the oocyte is the main source of the infertility seen in older women. Because a clear relationship exists between oocyte ageing and the non-disjunction of bivalent chromosomes during meiosis (Munné *et al*., 1995; Dailey *et al*., 1996), it is likely that such ageing compromises the meiotic apparatus (Battaglia *et al*., 1996; Volarcik *et al*., 1998), perhaps via suboptimal perifollicular circulation (Gaulden, 1992; Van Blerkom, 1996; Van Blerkom *et al*., 1997) that might compromise oocyte mitochondria (Beermann *et al*., 1988; Van Blerkom, 1994). Indeed, non-sequitur mutations in mitochondrial DNA have been observed in the oocytes of older women (Shigenaga *et al*., 1994; Keefe *et al*., 1995; Barritt *et al*., 2000).

Other than preimplantation genetic diagnosis (Gianaroli *et al*., 1997; 1999; Munné *et al*., 1999; Verlinski *et al*., 1999), two further logical ways of avoiding oocyte aneuploidy would be cryopreservation of metaphase II (MII) oocytes (van Uem *et al*., 1987; Chen, 1988; Porcu *et al*., 1997), or of the entire ovarian cortex retrieved at a younger age (Gosden *et al*., 1994; Newton *et al*., 1996; Oktay *et al*., 1998, 2004; Shaw *et al*., 2000; Kim

et al., 2002). Another quite novel approach has been the use of nuclear transplantation techniques (Takeuchi *et al*., 1999a, 2001a; Zhang *et al*., 1999).

Although nuclear transfer has been successful in producing some animal offspring, its overall efficiency remains extremely low (Takeuchi *et al*., 1999a, 2004, 2005; Li *et al*., 2001). The main bottleneck for germinal vesicle (GV) transfer, however, is the availability of oocytes. Oocytes of older women are not just 'compromised' but generally are available only in limited numbers. However, even when the technique is optimized, nuclear transplantation has to deal not only with an initially low number of eggs, but a limited efficiency of the in-vitro maturation process, both serious challenges for ageing women.

A more radical approach would be the haploidization of a somatic nucleus and its transformation into a pseudo-gamete ready to be inseminated (Takeuchi *et al*., 1999b; Tsai *et al*., 2000). This meiosis-like reduction division of a diploid somatic nucleus will take place within an enucleated donor oocyte (Takeuchi *et al*., 1999b; Tsai *et al*., 2000) and would provide a definitive treatment for age-related infertility. It has previously been shown that the mouse ooplast can 'haploidize' a somatic nucleus (Palermo *et al*., 2002a,b).

In order to assess such ooplasmic ability in humans, somatic cell nuclei were injected into enucleated MII oocytes and induced their haploidization by means of either activation or sperm injection. The efficiency of each micromanipulation step, as well as oocyte activation response, was evaluated according to pronuclei formation and cytogenetic assessment. Furthermore, development and genomic status of the resultant embryos were also evaluated.

Materials and methods

Source of gametes

Spare human GV oocytes were obtained from consenting patients undergoing intracytoplasmic sperm injection (ICSI). The procedures as well as the handling of patient material were performed in accordance with a research protocol approved by the Committee of Human Rights of New York Presbyterian Hospital-Weill Medical College of Cornell University (IRB number 0198–082). Oocytes were retrieved following treatment with a gonadotrophin-releasing hormone antagonist (GnRHa) and gonadotrophins (Palermo *et al*., 1995a). After cumulus cells were removed by enzymatic and/or mechanical treatment, the oocytes were examined under an inverted microscope to assess their condition and stage of nuclear maturation. GV oocytes were incubated in human tubal fluid medium (HTF, Irvine Scientific, Santa Ana, CA, USA) supplemented with 0.4% human serum albumin (HSA; Irvine Scientific) for 24 h to allow maturation to MII (Takeuchi *et al*., 2001a).

Nuclear transplantation and activation of human oocytes

All the micromanipulation and electrostimulation procedures were carried out in a shallow plastic Petri dish on a heated stage under an inverted microscope equipped with a hydraulic micromanipulator. The zona pellucida was breached with a glass needle, and after short exposure to 5 μg/ml of cytochalasin B (CCB, Sigma Chemical, St Louis, MO, USA), the metaphase chromosomes together with first polar body (PB) were removed with a glass micropipette of ~20 μm inner diameter (Takeuchi *et al*., 1999a, 2001a). A fluorescent DNA vital stain (0.5 μg/ml; Hoechst 33342, Sigma Chemical) or a polarizing light microscope (SpindleView™; Cambridge Research and Instrumentation, Inc., Woburn, MA, USA) was used to monitor this step (**Figure 1a**, **b**), thus avoiding exposure to ultraviolet radiation (Tsunoda *et al*., 1988). Enucleated oocytes were then cultured in CCB-free HTF medium for at least 30 min and then a cumulus cell was introduced into the oocyte through an ICSI needle (**Figure 2**). In a preliminary experiment, cell cycle analysis of cumulus cells was performed by flow cytometry according to their DNA content (Takeuchi *et al*., 2000). It showed that the proportion of cells at G_0/G_1 cell cycle stage was 84%. The reconstituted oocytes were then cultured in HTF medium for at least 1 h. Oocyte activation for initiation of meiosis-like division was performed by a brief exposure to 5 μmol/l of calcium ionophore A23187 (Sigma Chemical) for 5 min or to an electrical pulse (500 V/cm, for 50 μs). In order to establish the ideal time of activation, stimuli were applied at increasing time intervals.

Assessment of pronucleus formation and cytogenetic analysis of embryos

Activated oocytes were cultured for 16–20 h in HTF medium and scored for morphological indicators such as extrusion of a PB or the appearance of a pronucleus (PN). Because DNA replication occurs a few hours after pronucleus formation (Capmany *et al*., 1996) and is followed by syngamy, the assessment of the original chromosomal complement of each individual PN required the prompt isolation of a PN, therefore leaving a monopronucleated 'haploid' oocyte (**Figure 3**). For cytogenetic analysis, some of these monopronucleated oocytes were processed for two rounds of fluorescence in-situ hybridication (FISH) analysis with specific probes for chromosome 13, 14, 15, 16, 18, 21, 22, and X (**Figure 4a**, **b**) or incubated further for up to 24 h to enter metaphase and then stained for individual chromosomes (Takeuchi *et al*., 2001a).

After assessment of the first set of chromosomes [LSI probes 13 (Spectrum Green) and 21 (Spectrum Orange), and CEP probes X (Spectrum Green and Orange) and 18 (Spectrum Aqua)], the specimen was rinsed three times for 2 min in 2× standard saline citrate (SSC; Vysis Inc., Downers Grove, IL, USA). Then the slides were sequentially dehydrated in increasing concentrations of ethanol (70, 85, and 100%), rinsed and denatured in 70% formamide/2× SSC at 70°C for 5 min, then dehydrated in sequential strips in increasing concentrations of ethanol at 4°C for 1 min in each solution. Additional probes were applied [LSI probe 22 (Spectrum Green), CEP probes 15 (Spectrum Green and Orange) and 16 (Spectrum Aqua), and Telomere probe 14 (Spectrum Orange)] to analyse the corresponding chromosomes. All chromosome probes were purchased from the same manufacturer (Vysis Inc.).

Slides were viewed under a fluorescence microscope (Olympus BX 61®; New York/New Jersey Scientific Inc., NJ,

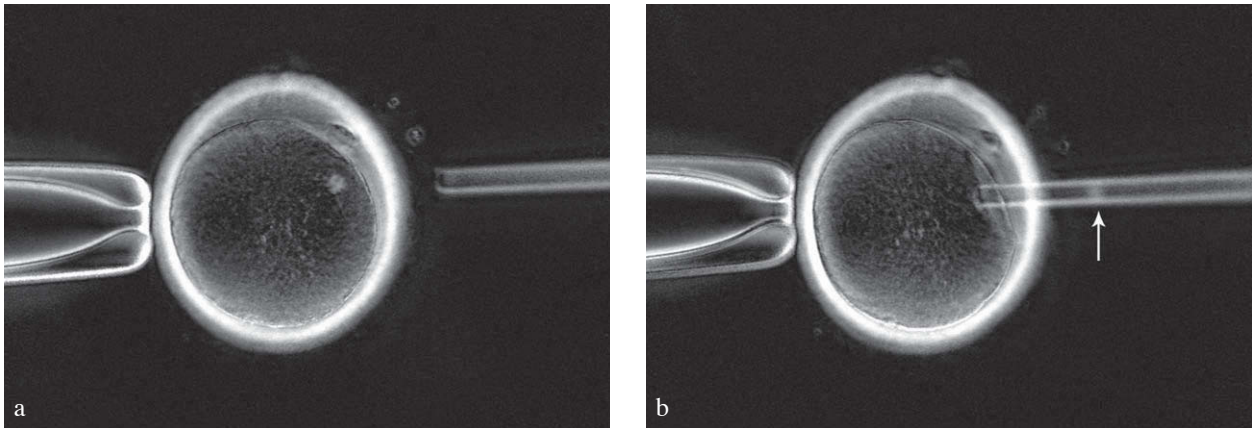

Figure 1. (**a**) Human metaphase II meiotic spindle. (**b**) Removal of a spindle (arrow). Visualized by polarizing microscope. Original magnification ×400.

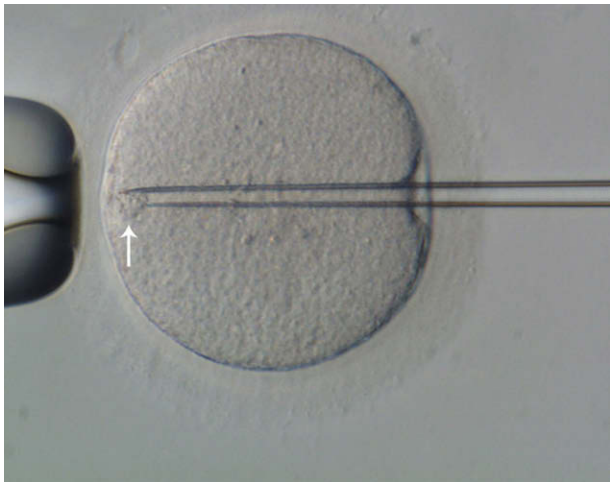

Figure 2. Injection of a somatic cell (arrow) into a human oocyte. Original magnification ×400.

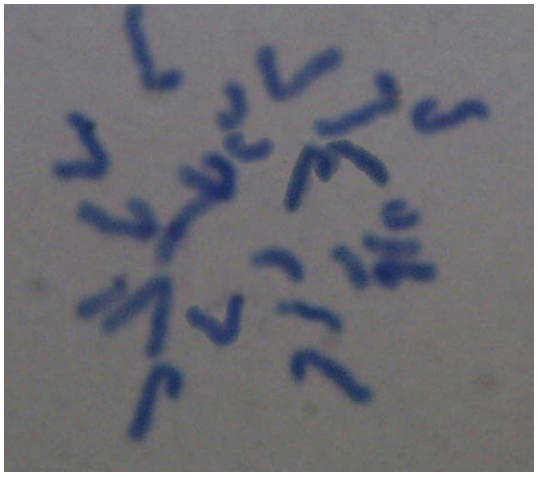

Figure 3. Twenty-three human metaphase chromosomes after fixation of a manipulated oocyte generated by injecting a human cumulus cell into a human ooplast (Giemsa). Original magnification ×600.

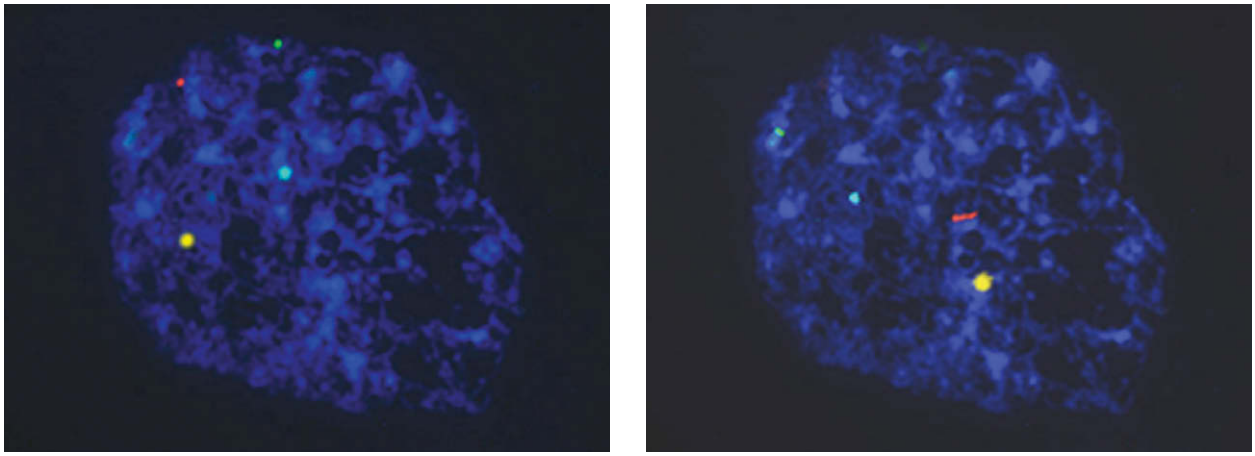

Figure 4. Cytogenetic analysis of a human haploidized pronucleus after somatic cell injection. (**a**) First round: red identifies chromosome 21 (1 signal), chromosome 18 appears in aqua (1 signal), green fluorochrome identifies chromosome 13 (1 signal), and yellow corresponds to chromosome X (1 signal). (**b**) Second round: green identifies chromosome 22 (1 signal), aqua fluorochrome identifies chromosome 16 (1 signal), red corresponds to chromosome 14 (1 signal), chromosome 15 appears in yellow (1 signal). Original magnification ×600.

USA) using single-bandpass filter sets including aqua (Vysis Inc.), rhodamine, fluorescein isothiocyanate (FITC) and DAPI (Olympus New York/New Jersey Scientific Inc.), and a triple bandpass filter set, DAPI/FITC/rhodamine (Vysis Inc.). After direct analysis, FISH images were captured and analysed using imaging software (Cytovision; Applied Imaging Corp., Santa Clara, CA, USA) Scoring criteria were as previously described (Munné *et al*., 1995; Dailey *et al*., 1996; Takeuchi *et al*., 2001a).

Sperm labelling, insemination, and fertilization assessment

In some oocytes, a spermatozoon injected 2 h after somatic cell transfer was tracked by midpiece mitochondrial labelling using MitoTracker Red® (Molecular Probes, Eugene, OR, USA) to identify the male PN (**Figure 5a**, **b**). Fertilization was assessed at 16–20 h after ICSI. Haploidization was considered successful according to the development of 3PN, two from the haploidized somatic nucleus and one from the male gamete (**Figure 6a**, **b**).

Experimental design

A total of 419 GV-stage oocytes were donated from 100 patients. These oocytes were allocated to three different experiments; in experiment I, optimal oocyte activation conditions were assessed. In order to establish the ideal time of activation, constructs were stimulated at 30-min intervals from 1 to 5 h after reconstitution and returned to culture. In experiment II, occurrence of 'haploidization' was assessed by cytogenetic analysis and in experiment III, reconstituted oocytes were inseminated with labelled spermatozoa and after ploidy correction cytogenetic analysis was carried out on the derived embryo. The dynamics of the experimental design is summarized in the flow chart (**Figure 7**).

Results

Experiment I: establishment of the optimal preincubation time for haploidization of human oocytes

From a total of 94 human GV oocytes, 65 reached the MII stage after 24 h in culture. Of these, 40 oocytes that survived somatic cell injection were subjected to activation. Oocytes were divided into two groups according to activation time (**Table 1**). A pre-incubation period of more than 2 h gave better cell survival, activation rates, and a higher 2PN rate. Although none of the surviving oocytes extruded a PB, cytogenetic evaluation showed that five out of nine oocytes with 2PN had undergone 'putative haploidization'.

Experiment II: efficiency of nuclear transplantation and pronucleus formation in human oocytes

Of a total of 295 GV oocytes retrieved, 192 had reached MII. Enucleation was successful in 159 oocytes and 119 ooplasts survived cumulus cell injection. However, while none of the activated survivors extruded a PB, 33 displayed 1PN, and 32 had 2PN (**Table 2**). The morphological characteristics of the pronuclei, i.e. size, appearance, and nucleolar number, were typical of those seen in normally fertilized or parthenogenetically activated oocytes after ICSI. FISH analysis of bipronuclear oocytes confirmed their initial 'haploid' state in 10 (38.5%) out of 26 analysed with eight chromosome-specific probes. Metaphase chromosomes were karyotyped in a total of six oocytes; one appeared to have 23 chromosomes while the others displayed different aneuploid combinations. The monopronuclear oocytes were diploid as expected, and the remaining non-activated oocytes presented with diploid nuclei arrested at interphase or metaphase.

Experiment III: restoration of diploidy and cytogenetics of human embryos generated from somatic cell haploidization

As in experiments I and II with human oocytes matured *in vitro*, haploidization of somatic cell nuclei resulted in the formation of 2PN, but without extrusion of a PB. The morphological characteriztics of the PN, i.e. size, appearance, and nucleolar number, were typical of normal fertilization. Among 25 MII oocytes, 24 were successfully enucleated and injected with cumulus cells. After ICSI, 15 (62.5%) of these oocytes were activated: six had 1PN, three had 2PN, four formed 3PN and the remainder displayed fragmented pronuclei. After identification of the male PN based on MitoTracker® Red labelling, a female PN was aspirated from each of the three 3PN zygotes, which later developed as far as the 6- to 8-cell stage. On day 3, cytogenetic analysis was performed on the blastomeres of the constructed embryos using specific probes for chromosome 13, 14, 15, 16, 18, 21, 22, X, and Y. Cytogenetic analysis of their blastomeres (total of 15) revealed chaotic mosaicism in all three embryos (**Figure 8**).

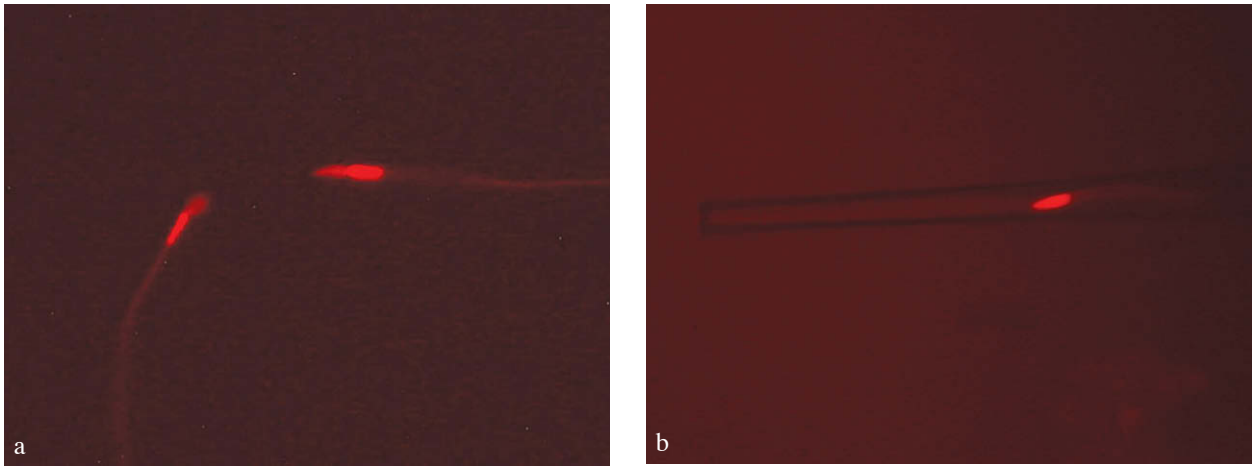

Figure 5. (**a**) Fluorescent labelling of the sperm mitochondrial sheath in free spermatozoa and (**b**) a spermatozoon in an injection pipette.

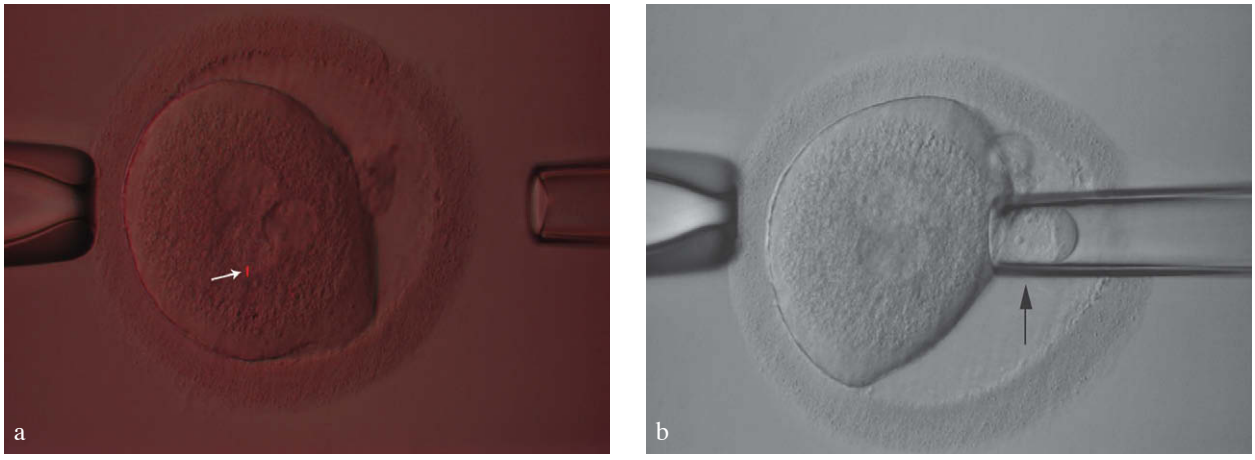

Figure 6. (**a**) The male pronucleus shows sperm mitochondrial labelling (white arrow) under fluorescence microscopy. (**b**) Removal of the additional female pronucleus (black arrow). Original magnification ×400.

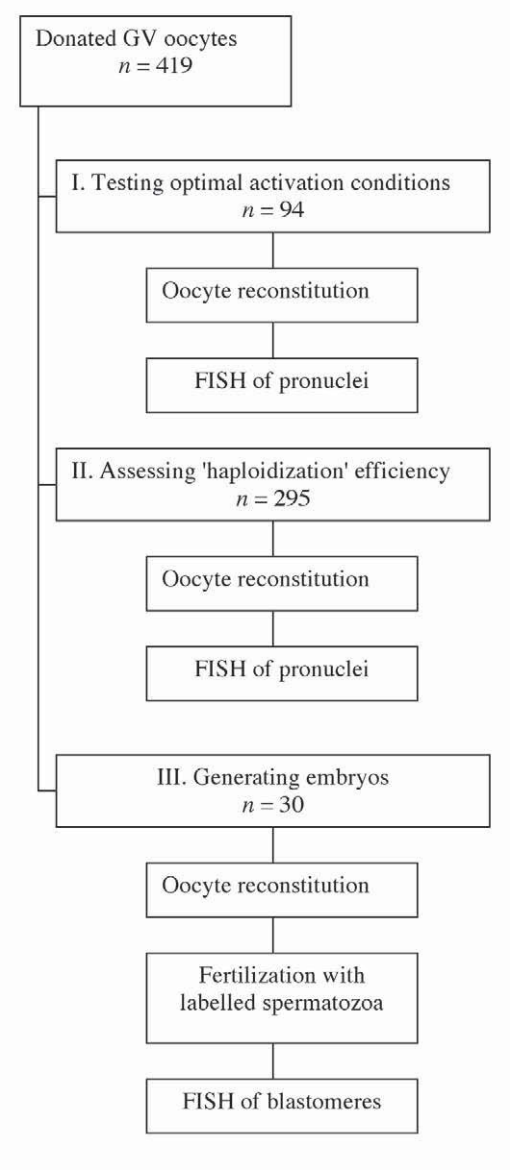

Figure 7. Experimental design. The total number of germinal vesicle (GV) stage oocytes donated for this study was allocated to (I) identify optimal activation conditions; (II) assess cytogenetics of 'haploidized' nuclei; (III) assess fertilization and embryo cleavage. FISH = fluorescence in-situ hybridization.

Table 1. Activation of human oocytes.

No. of oocytes (%)	*Post-transplantation time interval (min)*	
	60–90	*120–300*
Stimulated	18	22
Survived	11 (61.1)[a]	22 (100)[b]
Activated	4 (22.2)[c]	14 (63.6)[d]
With 1PN	4 (22.2)	5 (22.7)
With 2PN	0[e]	9 (40.9)[f]

[a versus b] χ^2, 2 × 2, 1 *df*, Effect of incubation time on survival, $P < 0.01$.
[c versus d] χ^2, 2 × 2, 1 *df*, Effect of incubation time on activation, $P < 0.01$.
[e versus f] χ^2, 2 × 2, 1 *df*, Effect of incubation time on 2PN, $P < 0.01$.
PN - pronucleus/pronuclei.

Table 2. Efficiency of 'haploidization' in human oocytes.

	No. of oocytes (%)
Intact MII	192
Enucleated	159 (82.8)
Reconstituted	119 (62.0)
Activated	68 (35.4)
With 1PN	33 (17.2)
With 2PN	32 (16.7)

MII = metaphase II, PN = pronucleus/pronuclei.

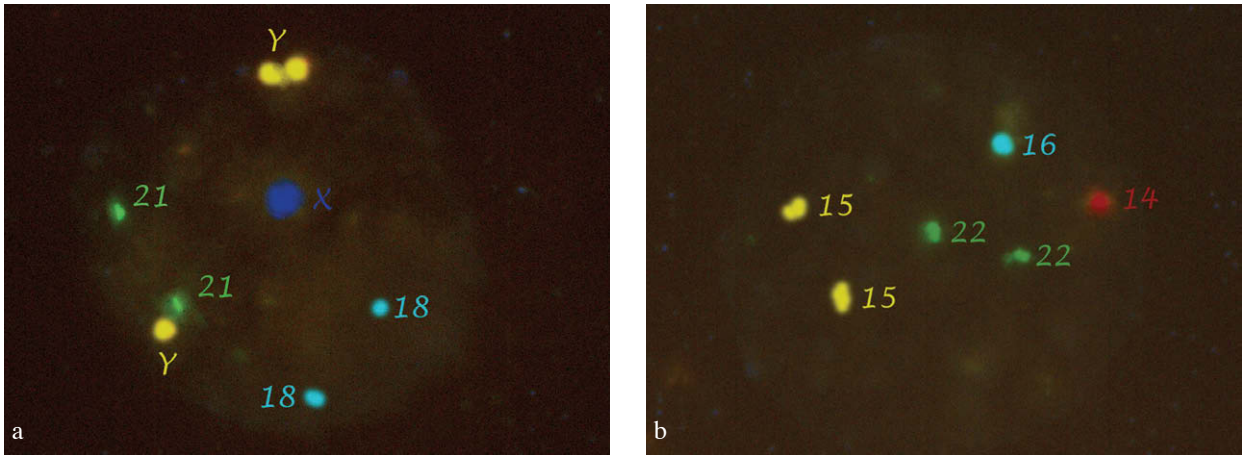

Figure 8. Cytogenetic analysis of an abnormal embryo obtained from the fertilization of a constructed human oocyte. Image generated by image analysing software for fluorescence in-situ hybridization (FISH) of a day 3 embryo. (**a**) First round FISH: yellow signal corresponds to chromosome Y (2 signals) and chromosome X appears blue (1 signal). Chromosome 21 appears green (2 signals), aqua fluorochrome identifies 18 (2 signals), and the red fluorochrome identifies chromosome 13 (no signal). (**b**) Second round FISH: aqua signal identifies chromosome 16 (1 signal) and green fluorochrome identifies chromosome 22 (2 signals). The red signal corresponds to chromosome 14 (1 signal) and the yellow identifies chromosome 15 (2 signals). Original magnification ×600.

Discussion

In this present study, haploidization of diploid somatic nuclei was induced by transferring somatic cells into enucleated human MII oocytes. After injecting somatic cell nuclei into enucleated oocytes, these were monitored for their ability to undergo normal fertilization and development to the blastocyst stage. An attempt was made also to identify the ideal period of incubation prior to oocyte activation. Haploidization of a somatic cell nucleus by a human ooplast consistently resulted in the formation of two distinct pronuclei, but no PB was extruded. Consequently, the injection of these constructs with a spermatozoon resulted in the appearance of three distinct pronuclei. Biparental diploidy was restored by removal of one of the somatic cell-derived 'haploid' nuclei, recognizable by the absence of mitochondrial labelling.

The morphological characteristics of the PN, PB, and blastomeres after 'manufacturing' human oocytes resembled those seen in normal oocytes inseminated by ICSI. Although most of the human 'manufactured' oocytes did not develop fully in the present study, some at least seemed to undergo early preimplantation development. The impaired blastocyst formation seen in many such cases may be attributed to unbalanced chromosomal segregation (Takeuchi *et al.*, 2002; Chen *et al.*, 2004; Heindryckx *et al.*, 2004).

Several reports suggest that the ability of the MII oocyte to haploidize cumulus cells and fibroblasts is retained by its ooplasm (Lacham-Kaplan *et al.*, 2001; Tesarik *et al.*, 2001). In contrast to GV ooplasm, however, mature oocytes require a spermatozoon or another stimulus to accomplish haploidization of the transplanted somatic nuclei, following which fertilization can occur with full development to the blastocyst stage (Lacham-Kaplan *et al.*, 2001; Tesarik *et al.*, 2001). It has previously been shown in the mouse that GV ooplasm supports haploidization-like reduction division of somatic cell nuclei (Palermo *et al.*, 2002a,b). However, GV oocytes need to undergo in-vitro maturation known to be a limiting factor for their further embryonic development and implantation (Takeuchi *et al.*, 2001a, 2004). In addition, in human oocytes, no extrusion of PB after transplantation of somatic cell nuclei has ever been observed in the authors' experience (unpublished data). Therefore, in this study, oocytes at the second meiotic division were used to examine the ability of the mature ooplasm to induce halving of somatic nuclei. On the other hand, the odds of obtaining a normal haploid complement of chromosomes in an oocyte undergoing its second meiotic division after somatic cell nuclear transplantation prove to be limited (Tateno *et al.*, 2003; Chen *et al.*, 2004; Heindryckx *et al.*, 2004; Galat *et al.*, 2005).

Regarding human oocytes, initial results indicated that lengthening the incubation time prior to oocyte activation after somatic cell injection improves cell survival and activation, and may lead to successful haploidization. Thus, an adequate interaction time (≥2 h) for the somatic genome with the host ooplast is considered critical for forcing spindle formation and transformation of transferred diploid nuclei to metaphase. Therefore, at least a 2-h incubation was used for later experiments. Mature MII stage ooplasm can force the somatic cell nucleus to undergo a premature M-phase, bypassing the S-phase, resulting in segregation of one set of chromatids displayed in a human oocyte by the formation of two pronuclei. Cytogenetic analysis of the pronuclei confirmed 'putative haploidization' of human cumulus cells injected into mature human ooplasts (Takeuchi *et al.*, 2001b; Tesarik *et al.*, 2001; Galat *et al.*, 2005). However, since many of the chromosomes were not tested, chances of true haploidization with a correct chromosome complement were probably lower than the observed rate.

Somatic cell nuclei can be putatively 'haploidized', forming distinct pronuclei in enucleated mature (MII) human oocytes (Kaneko *et al.*, 2001; Takeuchi *et al.*, 2001b), but somatic cell haploidization of these in-vitro matured human oocytes resulted in the formation of two PN, without PB extrusion (Takeuchi *et al.*, 2002). Because of the inability of some putative ooplasmic factors to interact with the cortical cytoskeleton, the

reconstructed oocytes were still not capable of extruding a PB even when a balanced chromosomal segregation had occurred (Leader *et al.*, 2002). Thus, the ability of GV and MII mouse oocytes to haploidize and therefore extrude a PB contrasts with the behaviour of human oocytes. In fact, a human GV stage oocyte, once enucleated and transplanted with a somatic nucleus, is unable to undergo ooplasmic maturation and consequent PB extrusion. On the other hand, the experiment carried out here with human MII ooplasm transplanted with a somatic nucleus generated two distinct PN and no PB following the activating stimulus. This is similar to the abnormal fertilization pattern (digynic three-pronuclei) occasionally observed after ICSI. These oocytes are normally triploid and their 2N ploidy can be rejuvenated by removal of the additional nucleus (Palermo *et al.*, 1994, 1996a,b).

Labelling of sperm mitochondria allowed identification of the male PN and enabled selective removal of one of the two female pronuclei. Although embryonic cleavage continued up to day 3 of culture, all these embryos were found to be karyotypic mosaics, explainable by the failed haploidization (uneven segregation of chromosomes) or even the presence of two competing centrosomes derived from the somatic cell and the spermatozoon respectively (Takeuchi *et al.*, 2002). The reason for the impairment of chromosomal number may find its explanation in the embryo karyotypes (data not shown), where a consistently lower number of chromosomes than expected was observed. This could be explained by an asynchrony between the two pronuclei in replicating their DNA content, or by a non-disjunction of the replicated chromosomes (Delhanty *et al.*, 1993). Moreover, the process may further be amplified in subsequent cleavage divisions (Palermo *et al.*, 1995b).

Correct chromosome segregation is crucial in artificial haploidization. Meiotic chromosomes in an oocyte display behaviour different from that of the mitotic chromosomes in the somatic cell (Fulka *et al.*, 2002a; Chang *et al.*, 2004). In meiosis, the MII chromosomes each consist of two chromatids which are physically attached to each other at their centromere, while G_0/G_1 cumulus cells contains monovalent chromosomes. The correct position and attachment of the chromosome on the spindle, as well as a distinctive regulation of the cohesion between sister chromatids, seems to be crucial for correct chromosome reduction (Fulka *et al.*, 2002a,b; Paliulis and Nicklas, 2003). When G_0/G_1 somatic chromosomes are transferred into MII ooplasm, there is no physical association between their homologous single chromatids. In the absence of any cohesion at all, reduction division may be totally random (Tesarik, 2002; Heindryckx *et al.*, 2004). The precise way in which these single chromatids in G_0/G_1 cells separate correctly remains to be determined. Considering that the estimate of the specific cell cycle was 84%, it is possible that some of the cell injected were out of the desired phase. Nevertheless, some artificially created oocytes are apparently chromosomally 'normal', as proven by FISH with a limited number of chromosome-specific probes (Takeuchi *et al.*, 2001b; Tesarik *et al.*, 2001; Galat *et al.*, 2005). This unexpected behaviour of presumably correct haploidization displayed by the ooplasm (Eichenlaub-Ritter, 2003), even in the absence of chromosomes, has the capacity to organize bipolar spindles, which requires expression of microtubules motor proteins, tubulin, and cell extracts with active maturation promoting factor, and cytosolic factor (Brunet *et al.*, 1998). Some back-up mechanisms underlie the segregation of pairs of non-exchange univalent chromosomes to opposite poles rather than the same pole during oogenesis in some species (Karpen *et al.*, 1996).

Another study has reported on the abnormal expression patterns for key developmental imprinted genes in the fetal tissue and adnexa generated through GV transplantation. The in-vitro maturation process, together with the nuclear transplantation itself, was possibly responsible for epigenetic alterations (Takeuchi *et al.*, 2005). Where the embryonic nuclear genome is created as a blend of the male gamete genome and that of a haploidized somatic cell, one major concern is the risk of imprinting abnormalities (Trounson, 2001; Nagy, 2004; Takeuchi and Palermo, 2004). However, the risk may be less in the present case than in cloning, since at least one allele of each chromosome originates directly from a gamete. Although both the epigender of the somatic nucleus and the status of the recipient cell would affect the outcome of the manufactured oocyte, the effect of artificial gamete production on imprinting is still unknown.

While the debate of reproductive cloning is still very heated (Sills *et al.*, 2001, 2004; Birnbacher, 2005; Gurdon, 2005; Strong, 2005), all related procedures involving nuclear transfer, such as the presently described aiming at generating gametes, are object of extreme controversy. Achievements in animal reproductive cloning, together with the recognition of therapeutic cloning in some countries, have provoked a revisit of the ethical position taken against those methods. From this, the public is looking at cloning as a method to shed light on scientific issues, therefore overcoming common prejudices. Although it has been stressed that somatic cell nuclear haploidization does not share the same ethical controversy as reproductive cloning, both procedures are technically similar. However, somatic cell haploidization aims at ensuring biparental genomic contribution, while reproductive cloning leads to asexual reproduction (Palermo *et al.*, 2002b; Tesarik, 2002; Nagy, 2003; Takeuchi and Palermo, 2004).

In conclusion, it has been demonstrated that the human ooplasm has the ability to induce meiosis-like reduction division of somatic nuclei. Labelling of sperm mitochondria allowed identification of the male pronucleus permitting restoration of diploidy in the so generated 'zygotes'. Although the entire chromosomal complement was not screened, some somatic nuclei seem to undergo a chromatinic halving process that resembles haploidization.

Acknowledgments

We thank the clinicians, embryologists, and scientific staff of The Centre for Reproductive Medicine and Infertility. We are most grateful to Professor J Michael Bedford for editing the manuscript, Dr Gayle Jones for her critical comment on the study design, and finally Professor Alan O Trounson for helping in the study planning and design.

References

Barritt JA, Cohen J, Brenner CA 2000 Mitochondrial DNA point mutation in human oocytes is associated with maternal age. *Reproductive BioMedicine Online* **1**, 96–100.

Battaglia DE, Goodwin P, Klein NA, Soules MR 1996 Influence of maternal age on meiotic spindle assembly in oocytes from

naturally cycling women. *Human Reproduction* **11**, 2217–2222.
Beermann F, Hummler E, Franke U, Hansmann I 1988 Maternal modulation of the inheritable meiosis I error Dipl I in mouse oocytes is associated with the type of mitochondrial DNA. *Human Genetics* **79**, 338–340.
Birnbacher D 2005 Human cloning and human dignity. *Reproductive BioMedicine Online* **10** (suppl. 1), 50–55.
Brunet S, Polanski Z, Verlhac MH *et al*. 1998 Bipolar meiotic spindle formation without chromatin. *Current Biology* **8**, 1231–1234.
Capmany G, Taylor A, Braude PR, Bolton VN 1996 The timing of pronuclear formation, DNA synthesis and cleavage in the human 1-cell embryo. *Molecular Human Reproduction* **2**, 299–306.
Chang CC, Nagy ZP, Abdelmassih R *et al*. 2004 Nuclear and microtubule dynamics of G2/M somatic nuclei during haploidization in germinal vesicle-stage mouse oocytes. *Biology of Reproduction* **70**, 752–758.
Chen C 1988 Pregnancies after human oocyte cryopreservation. *Annals of the New York Academy of Sciences* **541**, 541–549.
Chen SU, Chang CY, Lu CC *et al*. 2004 Microtubular spindle dynamics and chromosome complements from somatic cell nuclei haploidization in mature mouse oocytes and developmental potential of the derived embryos. *Human Reproduction* **19**, 1181–1188.
Dailey T, Dale B, Cohen J, Munné S 1996 Association between nondisjunction and maternal age in meiosis-II human oocytes. *American Journal of Human Genetics* **59**, 176–184.
Delhanty JD, Griffin DK, Handyside AH *et al*. (1993) Detection of aneuploidy and chromosomal mosaicism in human embryos during preimplantation sex determination by fluorescent in situ hybridisation (FISH). *Human Molecular Genetics* **2**, 1183–1185.
Eichenlaub-Ritter U 2003 Reproductive semi-cloning respecting biparental origin. Reconstitution of gametes for assisted reproduction. *Human Reproduction* **18**, 473–475.
Fulka J Jr, Martinez F, Templa O *et al*. 2002a Somatic and embryonic cell nucleus transfer into intact and enucleated immature mouse oocytes. *Human Reproduction* **17**, 2160–2164.
Fulka J Jr, Loi P, Fulka H *et al*. 2002b Nucleus replacement in mammalian oocytes. *Cloning Stem Cells* **4**, 181–187.
Galat V, Ozen S, Rchitsky S *et al*. 2005 Cytogenetic analysis of human somatic cell haploidization. *Reproductive BioMedicine Online* **10**, 199–204.
Gaulden ME 1992 Maternal age effect: the enigma of Down syndrome and other trisomic conditions. *Mutation Research* **296**, 69–88.
Gianaroli L, Magli MC, Ferraretti AP, Munné S 1999 Preimplantation diagnosis for aneuploidies in patients undergoing *in vitro* fertilization with a poor prognosis: identification of the categories for which it should be proposed. *Fertility and Sterility* **72**, 837–844.
Gianaroli L, Magli MC, Ferraretti AP *et al*. 1997 Preimplantation genetic diagnosis increases the implantation rate in human *in vitro* fertilization by avoiding the transfer of chromosomally abnormal embryos. *Fertility and Sterility* **68**, 1128–1131.
Gosden RG, Baird DT, Wade JC, Webb R 1994 Restoration of fertility to oophorectomized sheep by ovarian autografts stored at –196°C. *Human Reproduction* **9**, 597–603.
Gurdon JB 2005 Reproductive cloning: past, present and future. *Reproductive BioMedicine Online* **10** (suppl. 1), 43–44.
Hassold T, Chiu D 1985 Maternal age-specific rates of numerical chromosome abnormalities with special reference to trisomy. *Human Genetics* **70**, 11–17.
Heindryckx B, Lierman S, Van der Elst J, Dhont M 2004 Chromosome number and development of artificial mouse oocytes and zygotes. *Human Reproduction* **19**, 1189–1194.
Kaneko M, Takeuchi T, Veeck LL *et al*. (2001) Haploidization enhancement to manufacture human oocytes. *Human Reproduction* **16**, Abstract Book 1, 4.
Karpen GH, Le MH, Le H 1996 Centric heterochromatin and the efficiency of achiasmate disjunction in *Drosophila* female meiosis. *Science* **273**, 118–122.
Keefe DL, Niven-Fairchild T, Powell S, Buradagunta S 1995 Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women. *Fertility and Sterility* **64**, 577–583.
Kim SS, Soules MR, Battaglia DE 2002 Follicular development, ovulation, and corpus luteum formation in cryopreserved human ovarian tissue after xenotransplantation. *Fertility and Sterility* **78**, 77–82.
Lacham-Kaplan O, Daniels R, Trounson A 2001 Fertilization of mouse oocytes using somatic cell as male germ cells. *Reproductive BioMedicine Online* **3**, 205–211.
Leader B, Lim H, Carabatsos MJ *et al*. 2002 Formin-2, polyploidy, hypofertility and positioning of the meiotic spindle in mouse oocytes. *Nature Cell Biology* **4**, 921–928.
Li GP, Chen DY, Lian L *et al*. 2001 Viable rabbits derived from reconstructed oocytes by germinal vesicle transfer after intracytoplasmic sperm injection (ICSI). *Molecular Reproduction and Development* **58**, 180–185.
Munné S, Magli C, Cohen J *et al*. 1999 Positive outcome after preimplantation diagnosis of aneuploidy in human embryos. *Human Reproduction* **14**, 2191–2199.
Munné S, Alikani M, Tomkin G *et al*. 1995 Embryo morphology, developmental rates and maternal age are correlated with chromosome abnormalities. *Fertility and Sterility* **64**, 382–391.
Nagy ZP 2004 Haploidization to produce human embryos: a new frontier for micromanipulation *Reproductive BioMedicine Online* **8**, 492–495.
Nagy ZP 2003 Micromanipulation of the human oocyte. *Reproductive BioMedicine Online* **7**, 634–640.
Newton H, Aubard Y, Rutherford A *et al*. 1996 Low temperature storage and grafting of human ovarian tissue. *Human Reproduction* **11**, 1487–1491.
Oktay K, Buyuk E, Veeck L *et al*. 2004 Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. *Lancet* **363**, 837–840.
Oktay K, Newton H, Aubard Y *et al*. 1998 Cryopreservation of immature human oocytes and ovarian tissue: an emerging technology? *Fertility and Sterility* **69**, 1–7.
Palermo GD, Takeuchi T, Rosenwaks Z 2002a Technical approaches to correction of oocyte aneuploidy. *Human Reproduction* **17**, 2165–2173.
Palermo GD, Takeuchi T, Rosenwaks Z 2002b Oocyte-induced haploidization. *Reproductive BioMedicine Online* **4**, 237–242.
Palermo GD, Alikani M, Bertoli M *et al*. 1996a Oolemma characteristics in relation to survival and fertilization patterns of oocytes treated by intracytoplasmic sperm injection. *Human Reproduction* **11**, 172–176.
Palermo GD, Schlegel PN, Colombero LT *et al*. 1996b Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. *Human Reproduction* **11**, 1023–1029.
Palermo GD, Cohen J, Alikani M *et al*. 1995a Intracytoplasmic sperm injection: a novel treatment for all forms of male factor infertility. *Fertility and Sterility* **63**, 1231–1240.
Palermo GD, Munné S, Colombero LT *et al*. 1995b Genetics of abnormal fertilization. *Human Reproduction* **10** (suppl. 1), 120–127.
Palermo G, Munné S, Cohen J 1994 The human zygote inherits its mitotic potential from the male gamete. *Human Reproduction* **9**, 1220–1225.
Paliulis LV, Nicklas RB 2003 Topoisomerase II may be linked to the reduction of chromosome number in meiosis. *Bioessays* **25**, 309–312.
Porcu E, Fabbri R, Seracchioli R *et al*. 1997 Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertility and Sterility* **68**, 724–726.
Shaw JM, Cox SL, Trounson AO, Jenkin G 2000 Evaluation of the long-term function of cryopreserved ovarian grafts in the mouse, implications for human applications. *Molecular Cell Endocrinology* **161**, 103–110.
Shigenaga M, Hagen T, Ames B 1994 Oxidative damage and mitochondrial decay in aging. *Proceedings of the National Academy of Sciences of the USA* **91**, 10771–10778.
Sills ES, Takeuchi T, Tucker MJ, Palermo GD 2004 Genetic and

epigenetic modifications associated with human ooplasm donation and mitochondrial heteroplasmy – considerations for interpreting studies of heritability and reproductive outcome. *Medical Hypothesis* **62**, 612–617.

Sills ES, Takeuchi T, Rosenwaks Z, Palermo GD 2001 Reprogramming somatic cell differentiation and the Hayflick Limit: contrasting two modern molecular bioengineering aims and their impact on the future of mankind. *Journal of Assisted Reproduction and Genetics* **18**, 468–470.

Strong C 2005 The ethics of human reproductive cloning. *Reproductive BioMedicine Online* **10** (suppl. 1), 45–49.

Takeuchi T, Palermo GD 2004 Implications of cloning technique for reproductive medicine. *Reproductive BioMedicine Online* **8**, 509–515.

Takeuchi T, Neri QV, Katagiri Y *et al*. 2005 Effect of treating induced mitochondrial damage on embryonic development and epigenesis. *Biology of Reproduction* **72**, 584–592.

Takeuchi T, Rosenwaks Z, Palermo GD 2004 A successful model to assess embryo development after transplantation of prophase nuclei. *Human Reproduction* **19**, 975–981.

Takeuchi T, Katagiri Y, Veeck L *et al*. 2002 Identification of the male pronucleus during fertilization of artificial oocytes. *Fertility and Sterility* **78** (suppl. 1), S84.

Takeuchi T, Gong J, Veeck LL *et al*. 2001a Preliminary findings in germinal vesicle transplantation of immature human oocytes. *Human Reproduction* **16**, 730–736.

Takeuchi T, Kaneko M, Veeck LL *et al*. 2001b Creation of viable human oocytes using diploid somatic nuclei. Are we there yet? *Human Reproduction* **16** (suppl. 1), 5.

Takeuchi T, Akerman A, Raffaelli R *et al*. 2000 The efficiency of 'production' of mammalian haploid oocytes as a function of the cell cycle stage of the transferred somatic karyoplasts. *Fertility and Sterility* **74** (suppl. 1), S197–S198.

Takeuchi T, Ergün B, Huang TH *et al*. 1999a A reliable technique of nuclear transplantation for immature mammalian oocytes. *Human Reproduction* **14**, 1312–1317.

Takeuchi T, Tsai MC, Spandorfer SD *et al*. 1999b An alternative source of oocytes. *Human Reproduction* **14** (Abstract Book 1), 7.

Tateno H, Akutsu H, Kamiguchi Y *et al*. 2003 Inability of mature oocytes to create functional haploid genomes from somatic cell nuclei. *Fertility and Sterility* **79**, 216–218.

Tesarik J 2002 Reproductive semi-cloning respecting biparental origin: embryos from syngamy between a gamete and a haploidized somatic cell. *Human Reproduction* **17**, 1933–1937.

Tesarik J, Nagy ZP, Sousa M *et al*. 2001 Fertilizable oocytes reconstructed from patient's somatic cell nuclei and donor ooplasts. *Reproductive BioMedicine Online* **2**, 160–164.

Tietze C 1957 Reproductive span and rate of reproduction among Hutterite women. *Fertility and Sterility* **8**, 89–97.

Trounson A 2001 Nuclear transfer in human medicine and animal breeding. *Reproductive Fertility and Development* **13**, 31–39.

Tsai MC, Takeuchi T, Bedford JM *et al*. 2000 Alternative sources of gametes: reality or science fiction? *Human Reproduction* **15**, 988–998.

Tsunoda T, Shioda Y, Onodera M *et al*. 1988 Differential sensitivity of mouse pronuclei and zygote cytoplasm to Hoechst staining and ultraviolet irradiation. *Journal of Reproduction and Fertility* **82**, 173–178.

Van Blerkom J 1996 The influence of intrinsic and extrinsic factors on the developmental potential of chromosomal normality of the human oocyte. *Journal of the Society for Gynecologic Investigation* **3**, 3–11.

Van Blerkom J 1994 Developmental failure in human reproduction associated with chromosomal abnormalities and cytoplasmic pathologies in meiotically mature oocytes. In: Van Blerkom J (ed.) *The Biological Basis of Early Human Reproductive Failure*. Oxford University Press, New York, p. 283.

Van Blerkom J, Antczak M, Schrader R 1997 The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Human Reproduction* **12**, 1047–1055.

van Uem JF, Siebzehnrubl ER, Schuh B *et al*. 1987 Birth after cryopreservation of unfertilized oocytes. *Lancet* **8535**, 752–753.

Verlinski Y, Cieslak J, Ivakhnenko V *et al*. 1999 Prevention of age-related aneuploidies by polar body testing of oocytes. *Journal of Assisted Reproduction and Genetics* **16**, 165–169.

Volarcik K, Sheean L, Goldfarb J *et al*. 1998 The meiotic competence of *in vitro* matured human oocytes is influenced by donor age: evidence that folliculogenesis is compromised in the reproductively aged ovary. *Human Reproduction* **13**, 154–160.

Zhang J, Wang CW, Krey L *et al*. 1999 *In vitro* maturation of human preovulatory oocytes reconstructed by germinal vesicle transfer. *Fertility and Sterility* **71**, 726–731.

Received 11 March 2005; refereed 20 March 2005; accepted 24 May 2005.