No Restrictions.

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 12 April 2005.....
.....[redacted].....
Sec. Research Graduate School Committee

ERRATA

p 16 line 3-4, last sentence: Delete "As discussed below" and change to read "There are now several routes, real or theoretical, for the 'manufacture' of gametes that could meet that goal."

p 23 line 23: Change "is" to "as"

p 70 line 9: Delete "at"

p 165 Table 8-IV, Specimen, second line SC (metaphase), AcH4-K5 antibodies: Should read "-" for "+"

p 178, last paragraph, line 1: "suggests" for "suggest"

ADDENDA

p 16, at the end of the 1st paragraph add: "In fact, the generation of egg-like (Hübner et al., 2004) and sperm-like cells (Toyooka et al., 2003; Geijsen et al., 2004) directly from embryonic cells has been proposed.

p 178, at the end of the 2nd paragraph add: "An alternative approach would be to utilize an embryonic stem cell as a source of the somatic nucleus, to replace either the gamete of either gender (Takeuchi et al., unpublished data)."

p 191: Add the reference:

Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GO. (2004) Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 427, 148-54.

p 194: Add the reference:

Hübner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss III JF, Boiani M, Schöler HR. (2003) Derivation of oocytes from mouse embryonic stem cells. *Science* 300, 1251-1256.

p 216: Add the reference:

Toyooka Y, Tsunekawa N, Akasu R, Noce T. (2003) Embryonic stem cells can form germ cells in vitro. *Proceedings of the National Academy of Sciences USA* 100, 11457-11462.

MANIPULATION OF DEVELOPMENT BY NUCLEAR TRANSFER

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To my parents, Gino and Pia, with much love and gratitude.

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Synopsis

Micromanipulation of cells was first attempted more than a century ago in Germany, and was later refined as such by Chambers (1940) and de Fonbrune (1949). Since then, beginning with early injection of sperm into sea urchin eggs by Hiramoto (1962) and by Lin (1966) this technique has come to play a major part in contemporary gamete research, as well as in the correction of the gamete dysfunction related to infertility. Following its success in overcoming the inability of spermatozoa to fertilize in many infertile men, the present studies aimed to develop and to test methods that might provide a way through which to manipulate the oocyte genome in principle, and also to correct genetic defects, particularly those associated with oocyte aging in women. Chapter 3 describes the outcome of experiments aimed at the reciprocal transfer of germinal vesicles between fully grown mouse primary oocytes, and the potential of such reconstituted oocytes to resume meiosis and mature to metaphase II *in vitro*. Chapter 4 discusses experiments performed to elucidate the degree to which this process can be mimicked using human oocytes. Chapter 5 reports on the paradigm involving a novel means of selective damage to oocyte mitochondria as a model for oocyte aging, used to investigate whether normal ooplasm can 'rescue' the germinal vesicles of putatively 'aged' primary oocytes. Chapter 6 and 7, describe experiments aimed at the 'manufacture' of competent oocytes in mouse and man — comparing the ability of germinal vesicle and metaphase II oocytes to induce the haploidization of somatic nuclei — as another approach to overcoming the oocyte aneuploidy associated with aging in

women. Finally, Chapter 8 explores gene expression modulators such as DNA methylation and certain histone tail modifications in embryos and fetal tissues generated through nuclear transfer and/or extended *in vitro* culture.

General Declaration

Monash University

Monash Research Graduate School

Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with Monash University Doctorate Regulation 17/Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I, Gianpiero D. Palermo, hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes five original papers published in peer reviewed journals and three unpublished publications. The core theme of the thesis is nuclear transplantation. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Reproduction and Development under the supervision of Professor Alan O. Trounson.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. Detailed declaration accompanies each individual chapter.

Signed: ...[redacted].....

Date: .....10-29-2004.....

Chapter 1

Introduction

Declaration for Thesis Chapter 1

In the case of Chapter 1, contributions to the work involved the following:

	Name	% contribution	Nature of contribution
1	**Gianpiero D. Palermo**	100	Conception and design, collection, analysis, and writing of manuscript

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
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(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Weill Medical College of Cornell University, The Center for Reproductive Medicine, New York, NY, USA

Signature 1 [redacted] **Date** 10-29-2004

1.1 Background

The micromanipulation of cells and gametes was achieved more than 50 years ago (Chambers, 1940), but only recently has this been applied to the fields of molecular biology and molecular genetics. Some thirty years ago, micromanipulation was used to study fertilization in a variety of animal species (Hiramoto, 1962; Lin, 1966) and has been employed since then to evaluate the developmental potential of a single blastomere removed from an early stage embryo (Edwards and Gardner, 1967). More recently, nuclear transfer procedures have allowed the observation of structural changes in the nucleus and cytoplasm during cell differentiation (McGrath and Solter, 1983), and examination of the role of the nucleus in directing development of the early zygote (McLaren, 1984). Micromanipulation has also permitted the production of transgenic animals (Brinster et al., 1984), which, in expressing introduced genes or interference to existing genes, provides an opportunity for functional genomics and both forward and reverse genetic analysis in development and health.

Micromanipulation has been applied for the last 20 years to human gametes (Cohen et al., 1988), particularly to achieve fertilization where semen parameters are compromised. At the same time, its use has enhanced our understanding of human fertilization (Palermo and Rosenwaks, 1995) and has facilitated the diagnosis and treatment of human infertility. As a natural progression from the basic investigative work and the commercial use of micromanipulation, therapeutic application of this technology continues to develop.

Currently, micromanipulation of human gametes and embryos is used also in preimplantation genetic diagnosis (PGD), to diagnose and prevent chromosomal disorders as well as genetic diseases (Palermo and Bedford, 2000) and, in intracytoplasmic sperm injection (ICSI), to achieve fertilization in cases of severe oligozoospermia or when functional defects of the spermatozoa are present (Palermo et al., 1995a; 1995b). While *in vitro* fertilization (IVF) of human oocytes was used successfully to treat some cases of male infertility since the early 1980s (Cohen et al., 1984a), a poor fertilization rate or fertilization failure is often observed where the numbers are low or where the spermatozoa are sub-optimal (Palermo and Rosenwaks, 1995). Further improvement of fertilization rates was accomplished by (1) increasing the number of sperm cells in the fertilization medium (Cohen et al., 1984b); (2) inseminating oocytes in few microliters of medium (Fishel et al., 1995a); (3) sperm selection involving migration-gravity, sedimentation methods, or discontinuous density gradients (Guerin et al., 1989); (4) the use of motility enhancers (Yovich et al., 1990); and (5) removing the cumulus-corona cells (Lavy et al., 1988). Other treatments of sperm cells were directed towards destabilizing the acrosome (Palermo and Van Steirteghem, 1991). However, in cases of severe oligozoospermia, asthenozoospermia, and teratozoospermia, more invasive techniques based on micromanipulation were required to achieve fertilization (Palermo et al., 1992a; 1995a).

1.2 Micromanipulation of nuclei

Although, the techniques of nuclear transplantation was used nearly 40 years ago for cloning in vertebrates, it had broader applications than simply generating cohorts of identical animals. Nuclear transplantation experiments carried out on *Xenopus laevis* since 1958 (Fischberg et al., 1958) showed that the nuclei from the differentiating endoderm (Gurdon, 1960) or fully differentiated intestine cells (Gurdon, 1962) can support the formation of normal muscle and nerve cells. After transplantation into enucleated frog eggs, seven fertile adult male and a female were obtained after nuclear transplantation of differentiated intestinal epithelium from *Xenopus laevis* tadpoles (Gurdon and Uehlinger, 1966). The principal objective was to discover whether inactivation of nuclear genes in differentiated cells is irreversible or not — in other words whether, potentially, such nuclei retain their pluripotency. While this and the research that followed attracted much scientific interest, consideration of possible human applications was muted, mainly because of the distant evolutionary relationship of the frog and man, and the inability to generate adult frog clones from a differentiated adult cell nuclei (Gurdon and Laskey, 1970; Di Berardino, 1987; Kikyo and Wolffe, 2000). This view of cloning changed dramatically with the birth of 'Dolly' (Wilmut et al., 1997).

The success of Gurdon and colleagues with frogs, understandably, encouraged attempts at nuclear transfer in many other species including insects, ascidians, fish, and mammals (Gurdon and Uehlinger, 1966). In fact, pronuclei from mouse zygotes were able to support post-implantation development (McGrath and Solter, 1983). Reproducible successes in mammals were obtained in

mice also when nuclei from early cleavage stage blastomeres (Surani et al., 1984; 1986; 1990) were transferred into 2-cell embryos (Tsunoda et al., 1987a). Live births were also obtained when such experiments were performed on ruminants (Willadsen, 1986). Subsequently, three major biological factors emerged that seemed to bear on the success of nuclear transfer studies (Sun and Moor, 1995): the state of the recipient cytoplasm, the developmental status of the donor cell, and the cell-cycle stage of the donor nucleus relative to that of the recipient cytoplasm. The births of the sheep 'Megan' and 'Morag' in 1995 (Campbell et al., 1996a) and of 'Dolly' in 1996 (Wilmut et al., 1997) exploited these observations and provided final proof of the potential totipotency of the adult somatic cell nucleus. However, the achievement of 'Dolly' was more of a conceptual success than a utilitarian one. In fact, the overall efficiency rate of embryonic-derived cells and fetal fibroblasts, in terms of live offspring, were 4.6% and 10.0%, respectively, while adult epithelial cell nuclei produced a rate of 3.4%. More importantly, only 29 (11.4%) embryos reached the morula or the blastocyst stage from the 247 initial embryos.

An important development in the mid-1990s was the appreciation by several groups working in mammals that the cell cycle status of both the donor and recipient cell was crucial to the success of nuclear transfer experiments (Barnes et al., 1987; Cheong et al., 1993; Campbell et al., 1993). In particular, it became clear that the cytoplasmic environment of the metaphase II (MII) stage had a great influence on the fate and integrity of the donor cell chromosomes. As reviewed by Campbell et al. (1996b), dissolution of the nuclear membrane and condensation of chromosomes in the transplanted nucleus depend not least on the presence of high levels of oocyte maturation promoting factor (MPF). Upon subsequent activation of the oocyte, the nuclear membrane

reforms and DNA synthesis commences. If the incoming nucleus is diploid, only one round of replication takes place. However, if the transplanted nucleus has a greater ploidy, any ongoing replication will continue and new uncoordinated replication will lead to abnormal (i.e., > 4n) ploidy as well as chromosome pulverization (Harper and Delhanty, 1996) in certain S phase nuclei. Starting with blastomere nuclei from 4-cell embryos synchronized at metaphase and transferred into enucleated ooplasts mouse clones were produced by serial nuclear transfer. These transplanted oocytes formed two diploid nuclei following activation and suppression of polar body extrusion with cytochalasin B. Transfer of such nuclei into enucleated zygotes resulted in an 83% blastocyst development and 57% live young (Kwon and Kono, 1996). These restrictions on the cell cycle status of the donor apply to a lesser extent when activated eggs or fertilized embryos are used as recipients. However, most success has been reported with MII oocytes, and this may be attributable to the fact that the use of earlier developmental stages allow more time for reprogramming of the incoming genome.

Much of this approach bears on the issues currently being considered in regard to cloning. Several advanced cloning protocols have been developed using embryos of the mouse, in which studies of developmental and reproductive biology are greatly facilitated by a short gestation of 20 days and a generation period of approximately 3 months. Although transgenic and gene deletion ('knockout') models are of fundamental relevance, the mouse is not ideal for certain aspects of reproductive engineering because of technical problems involved in manipulating unfertilized oocytes. Microinsemination and nuclear transfer were used successfully in sheep, cows, and rabbits as early as the 1980s (First and Prather, 1991; Iritani, 1991). However, their application

in mice remained problematic until a reliable microinjection involving a piezo device was introduced by Kimura and Yanagimachi (1995a), who obtained normal pups after using primary spermatocytes with this technique (Kimura et al., 1998; Ogura et al., 1998). Mouse cloning is very difficult also because only 2-cell blastomere nuclei were completely reprogrammed after transfer into enucleated oocytes (Kono et al., 1991). Working independently and using slightly different serial nuclear transfer protocols, two Japanese groups discovered that nuclei from 4-cell or later stage embryos had to be transferred twice: first into enucleated oocytes and then into enucleated embryos, to achieve full term development of these reconstructed embryos (Kwon and Kono, 1996; Tsunoda and Kato, 1997; 1998). It is unclear why this serial transfer was needed, although it is commonly believed that "reprogramming factors" within mature oocytes, so far unidentified, are insufficient for full reprogramming of the donor nuclei within the short period of mouse preimplantation development. However, as somatic cell cloning in the mouse can now be accomplished with simpler techniques (Wakayama et al., 1998), blastomere cloning by serial nuclear transfer has limited practical applications. Somatic cell cloning in the mouse was first achieved by the transfer of cumulus cells (Wakayama et al., 1998) and more recently, microinsemination with either spermatozoa or spermatogenic cells has resulted in the production of transgenic mice and the propagation of infertile mouse strains (Ogura et al., 2001).

1.3 Micromanipulation of human gametes, zygotes, and embryos

Since the first human birth in 1978 (Steptoe and Edwards, 1978), IVF has been used extensively to treat infertility. However, micromanipulation techniques are critical where spermatozoa cannot fertilize in many cases of 'male factor' infertility (Palermo and Rosenwaks, 1995). These techniques have allowed fertilization in cases of severe oligozoospermia and even abnormal sperm morphology, and have provided a powerful tool also for investigation of oocyte maturation, fertilization, and early development. Partial dissection of the zona (hatching) at the 4- to 8-cell embryo stage is sometimes used in an effort to promote implantation but its value remains a matter of debate. Micromanipulation is also central to PGD performed on isolated blastomeres after removal from 4-8 cell embryos.

1.3.1 Micromanipulation techniques used to achieve fertilization

The practical use of micromanipulation as a way of bypassing the zona pellucida burst onto the scene in the mid 1980s with zona drilling (ZD) (Gordon and Talansky, 1986; Gordon et al., 1988), partial zona dissection (PZD) (Cohen et al., 1988), and sub-zonal injection (SUZI) (Metka et al., 1985; Laws-King et al., 1987; Mettler et al., 1988; Fishel et al., 1991). However, these early approaches to achieving fertilization have been abandoned in favor of intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992a; 1995a).

ICSI involves insertion of a selected spermatozoon directly into the oocyte. The technique was initially pioneered by Hiramoto in the sea urchin (1962), then by Lin (1966) in the mouse. Later,

Uehara and Yanagimachi (1976) observed relatively high rates of sperm nucleus decondensation after microinjection of human or golden hamster spermatozoa into hamster eggs, and subsequently ICSI was used to study the determinants of male pronucleus formation (Perreault et al., 1984; Naish et al., 1987).

Because the fusion step of fertilization is bypassed in ICSI, in most species tested male pronucleus development requires oocyte activation (Palermo and Bedford, 2000) — this can be provoked by energetic suction of some cytoplasm immediately before or during sperm nucleus insertion (Perreault and Zirkin, 1982). In the hamster (Hoshi et al., 1992) and the bovine (Younis et al., 1989; Keefer et al., 1990), the likelihood of oocyte activation is increased further by exposure of oocytes to a calcium ionophore (e.g. A23187). Although this practice may exclude parthenogenetic activation of the oocyte, the formation of a second polar body together with the decondensation of the male gamete ensures a biparental conceptus as proven by cytogenetic studies (Sultan et al., 1995).

The first live offspring using ICSI were obtained in rabbits, and soon after in the cow (Iritani, 1988; Goto et al., 1990). The first human pregnancies were obtained in 1992 (Palermo et al., 1992a) and since then thousands of babies have been born using this approach (Medical Research International, 2002; Nygren and Andersen, 2002).

1.3.2 The correction of fertilization abnormalities

Much of the genetic manipulation performed so far in animals has involved removal or transplantation of nuclei and pronuclei (Gurdon et al., 1975; Willadsen, 1986; Palermo et al., 1995c). In zygotes, this has been used either to eliminate an extra pronucleus, to avoid diandry and digyny or, conversely, to bring a haploid egg to a diploid state (Malter and Cohen, 1989; Rawlins et al., 1988; Tang et al., 1994; Levron et al., 1995a; 1995b; Palermo, 1999).

Tripronucleate zygotes may arise either from dispermy or from a failure of extrusion of the second polar body. In most cases, correction of triploidy by pronucleus removal has involved aspiration with a micropipette. However, Fulka and Moor (1993) introduced chemical enucleation by simple exposure to cycloheximide and etoposide following which the entire chromosome complex is expelled during extrusion of the polar body. Although this does not remove pronuclei selectively, it may be used to obtain oocyte or blastomere nuclei for transfer.

A central issue in the correction of dispermy or digyny by enucleation is the gender of the targeted pronucleus, not least because diandric human embryos may develop as hydatidiform moles. The parental origin of a pronucleus is relatively clear in rodents where its nuclear size, the presence of the tail remnant, and its position *vis a vis* the second polar body all relate to this. In humans, unfortunately, male and female pronuclei are of similar size and the fertilizing sperm tail cannot be identified by light microscopy (Malter and Cohen, 1989; Wiker et al., 1990).

Since centrioles are absent in unfertilized but are present in fertilized human oocytes, the zygote centrosome must be paternally inherited in man (Palermo et al., 1994; Sathananthan et al., 1991; 1996). Therefore, control of the centrosome duplication which directs spindle formation during fertilization, differs from that in somatic cells, and specific mechanisms must exist to control this. If centrosomes from both gametes remained functional, the zygote would possess two centrosomes and four centrioles, resulting in multipolar spindles and so aneuploidy and mosaicism at the first mitotic division (Sluder et al., 1989). In order to avoid this, the centrosome of one gamete (usually of the oocyte) fails to develop, and, with the exception of mu:d rodents, the spermatozoon introduces the functional centrosome (Schatten, 1994; Sathananthan et al., 1991; 1996; Palermo et al., 1997a). The fertilization of human oocytes with two spermatozoa and therefore, two centrioles, was recognized by the abnormal chromosomal distribution of the chromosomes in the resulting embryo derived from tripronucleated oocyte (Kola et al., 1987).

The way that the centrosome functions bears on the strategies used to correct polyploidy. For example, to assess whether the pronucleus farthest from the second polar body is consistently paternal, coamplification of X and Y probes using multiplex polymerase chain reaction (mPCR), or simultaneous fluorescent *in situ* hybridization (FISH), have been applied to dispermic embryos and to triploid embryos from which the distal pronucleus was removed (Munné et al., 1993). Unfortunately, such dispermic zygotes have often later expressed chromosomal mosaicism, probably because they retain multipolar spindles resulting from the extra sperm centrosome (Palermo et al., 1994). Fortunately, removal of one pronucleus from triploid zygotes restores the embryo to a diploid state compatible with normal development (Rawlins et al.,

1988), though further studies are needed to determine the extent to which a human female pronucleus can be selected with accuracy.

1.4 Alternative sources of gametes

During IVF, some 40% of couples fail to achieve optimal fertilization because of poor sperm quality (van Uem et al., 1985), but the establishment of ICSI has greatly enhanced the chance of fertilization success in such cases (Palermo et al., 1992a; 1992b; 1996a), even for some azoospermic patients (Schoysman et al., 1993). The rapid adoption of ICSI in clinical practice has been favored also by its ethical acceptability (American Society for Reproductive Medicine, 1994; 1998; Lamb and Schlegel, 1999; Lamb, 1999; Schlegel, 1999) and by the fact that it generally avoids the need to use donor spermatozoa. Nevertheless, establishing a pregnancy even by ICSI has not been problem-free, particularly for older women.

The decreased fertility of older women stems in large part from a decline in the frequency of intercourse, in the number of primordial follicles and, in particular, from a higher incidence of oocyte aneuploidy (Tietze, 1957; Dailey et al., 1996). That oocyte aneuploidy is indeed a major culprit in older women, is suggested by the higher pregnancy rate in this group where young donor oocytes are used (Sauer et al., 1990; Navot et al., 1991). While infertility due to relatively advanced female age can now be successfully treated with oocyte donation, most older couples are more interested in generating their own biological (genetic) child, and the advances in reproductive medicine have raised these expectations. An interest in finding a treatment for

azoospermia and for oocyte aneuploidy has stimulated many investigators to seek alternative sources of genetically normal spermatozoa and oocytes for infertile couples (Trounson, 1998; Tsai et al., 2000; Lacham-Kaplan et al., 2001; Tesarik et al., 2001). As discussed below, there are now several routes, real or theoretical, for the 'manufacture' of gametes that could meet that goal.

1.4.1 Sources of sperm precursors

Although ICSI has been effective with spermatozoa from the obstructed epididymis, the only option for patients with non-obstructive azoospermia is retrieval from the testis. Fortunately, it is often possible to collect spermatozoa directly from seminiferous tubules (Silber et al., 1997) although this is not possible in some cases of complete spermatogenic failure (e.g. Sertoli cell-only syndrome) (Devroey et al., 1995; Schlegel et al., 1997).

The failure to find testicular spermatozoa in some azoospermic men has stimulated an interest in utilizing more immature gamete stages, an approach first suggested from successful pregnancies achieved by microsurgical insertion of round spermatids into mouse oocytes (Ogura and Yanagimachi, 1993) or by electrofusion (Ogura et al., 1993; 1994). In the human, fertilization and early cleavage have occurred following microinjection of round spermatids into mature oocytes (Vanderzwalmen et al., 1995). A healthy girl was born after ICSI with elongated spermatids (Fishel et al., 1995b; 1997) and two normal infants were born after ICSI with round spermatids (ROSI) obtained from ejaculates of clinically azoospermic men (Tesarik et al., 1995; 1996). In general, however, the success rates of ROSI has been disappointing (Tesarik and Mendoza, 1996;

Amer et al., 1997; Antinori et al., 1997a; Vanderzwalmen et al., 1997; Yamanaka et al., 1997; Palermo et al., 1999). The inconsistent fertilization and extremely poor implantation following ROSI have been related to the higher incidence of chromosomal abnormalities (Benkhalifa et al., 2004) and imprinting defects (Manning et al., 2001).

Another problem in using immature germ cells concerns the lack of oocyte activation (Holstein, 1975), for which intracellular calcium release is the universal trigger (Vitullo and Ozil, 1992; Homa and Swann, 1994; Tesarik et al., 1994a; 1994b; Palermo et al., 1997b; Fissore et al., 1999a; 1999b). Normally, a factor present in the fertilizing spermatozoon initiates repetitive transient calcium fluxes in the oocyte (Vitullo and Ozil, 1992; Ozil and Swann, 1995). Lack of such a factor in round spermatids retrieved from men with complete failure of spermatogenesis appears to be at least partly responsible for the poor results of ROSI (Palermo et al., 1997b; Tesarik et al., 1998a). This is in contrast to round spermatids from men with continuing spermiogenesis, which can induce calcium signaling similar to that induced by mature spermatozoa (Sousa et al., 1996).

Human oocytes injected with mature spermatozoa can apparently develop a relatively high incidence of premature chromosome condensation (PCC) (Schmiady et al., 1996), even when spermatids are used (Tesarik et al., 1998a; 1998b). Unlike mature spermatozoa, whose condensed -S-S- stabilized protamines prevent sperm nuclei from being driven to metaphase prematurely by oocyte MPF, in round spermatids the histone to protamine substitution has hardly begun. Thus, the differing nuclear maturity could explain the slightly higher fertilization rate using elongated

rather than round spermatids (Antinori et al., 1997b). Interestingly, PCC may not always lead to developmental failure, as demonstrated by the birth of normal offspring after injection of mouse oocytes with round spermatids followed by delayed oocyte activation (Ogura et al., 1999). It is possible that PCC may even be helpful in nuclear reprogramming of immature germ cells, similar to the chromatin remodeling observed after nuclear transfer of somatic cell nuclei (Cibelli et al., 1998; Wells et al., 1999).

It might be assumed that only haploid cells can be used for ICSI. However, the nuclei of secondary spermatocytes have been shown to complete meiosis when injected into mouse oocytes (Kimura and Yanagimachi, 1995b); by 2 hours, some spermatocyte nuclei exhibited premature chromosome condensation and microtubule attachment. After electrical activation of such oocytes, however, both oocyte and spermatocyte chromosomes formed two pronuclei and extruded two distinct polar bodies (one of paternal and one of maternal origin), and 24% of the resulting 2-cell embryos transferred to foster mothers developed to full term. In the one example of this involving human gametes, a female second polar body and a male pseudo polar body were extruded, two pronuclei formed and an embryo developed with the later delivery of a healthy child (Sofikitis et al., 1998).

Taking an entirely different approach to the provision of injectable sperm nuclei, Brinster and Zimmermann (1994) have reported that donor male germ stem cells could partially repopulate sterile mouse testes when injected into seminiferous tubules, and could develop into fertile spermatozoa. Subsequently, more than 110 days after transplantation of rat spermatogonia to the

testes of immunodeficient mice, they produced rat spermatozoa of normal morphology (Clouthier et al., 1996). More recently, spermatogenesis was reinitiated within the seminiferous tubules 4 weeks after autologous spermatogonial transplantation in the cynomolgus monkey (Schlatt et al., 1999).

This work in animals suggests that spermatogenic stem cells of different species may be successfully transplanted (Clouthier et al., 1996), offering the possibility of xenogeneic spermatogenesis of human spermatogonia. Human male germ cell transplantation may be a valuable technique through which to obtain fully developed spermatozoa, and when used in conjunction with ICSI this may offer an alternative treatment for those patients with spermatogenic arrest. Furthermore, it would provide an innovative way to preserve fertility in oncological patients undergoing chemo-radiotherapy, with particular benefit for pre-pubertal cancer patients.

1.4.2 Attempts to identify sources of female pronuclei

It is believed that several specific ooplasmic factors are important for the continued development of the zygote, particularly during early cleavage when transcription of the embryonic genome is minimal (Van Blerkom, 1996; Liu et al., 1997). Recently, transfer of ooplasm into MII oocytes was used as an approach to restore normal growth in developmentally compromised embryos where some ooplasmic deficiency might exist (Cohen et al., 1997; Barritt et al., 2001a). However, this cannot correct any chromosomal error that may appear earlier in meiosis.

Nuclear transplantation studies have shown that chromatin remodeling during successful embryo reconstitution requires the temporary presence of the MPF which is one major cytoplasmic controller of the cell cycle (Czolowska et al., 1984; Willadsen, 1986; Stice and Robl, 1988; Campbell et al., 1993). MPF is a heterodimer consisting of cyclin B, a regulatory component, and a catalytic subunit, p34cdc2. During the cell cycle, the concentration of p34cdc2 remains unchanged, whereas MPF activity begins to increase just before germinal vesicle breakdown and is sustained at a high level throughout metaphase I (MI). MPF values decrease at anaphase I and telophase I, but increase again as the cell enters MII, only to decline rapidly upon fertilization or oocyte activation. During transplantation, all nuclei transferred into a cytoplast with high MPF activity undergo nuclear envelope breakdown and chromosome condensation. The cell cycle can be restarted artificially by electric pulses or exposure to a medium containing ethanol or strontium (Whittingham, 1980). In addition, the stage of the donor nucleus and of the recipient cytoplasm are important factors for the 'ploidy' of the reconstituted cells (Kono, 1997). Altering the timing of oocyte activation with respect to the fusion of the donor nucleus provides a number of possible approaches to synchronizing the respective cell cycles of the donor and recipient (Campbell et al., 1996b).

In the case of age-related human infertility, both nuclear transfer and ooplasmic transfer, have one major problem: older women (aged > 40 years) with premature ovarian failure or those considered as poor responders, produce too few oocytes. However, it has been shown that ooplasm from a GV-stage oocyte is able to initiate a meiosis-like reduction division in mitotic nuclei originating either from germ cell lines or from more differentiated somatic cells (Kubelka and Moor, 1997).

Thus, a sufficient number of viable oocytes might be created by transplantation of a patient's somatic cell nucleus into an ooplast obtained from a younger donor. The resulting haploid oocytes would obviously still need the genetic contribution of the paternal gamete to develop normally. Notably, the genomic imprint of the haploid somatic cell nucleus may not be equivalent to that of an oocyte which may leave imprinting errors in the embryo formed. In preliminary experiments, I have attempted to haploidize somatic cells by transplanting them into immature oocytes (Takeuchi et al., 1999a). However, further cytogenetic information is needed as to how chromosome pairing of haploid somatic and sperm chromatin occurs during fertilization and its further effect on embryo cleavage. There may also be errors related to the active centrosome present in the fresh 'manufactured oocyte'.

A high rate of embryonic and fetal death arises from epigenetic errors in animal cloning (Edwards and Beard, 1998), and this would probably also apply in the case of somatic cell haploidization used to produce a 'new' egg. Nevertheless, fertilization might modify this outcome, since this establishes a completely new system not found in a simple cloning situation. Recently, mouse clones were produced using metaphase nuclei derived from 4-cell stage mouse embryos (Kwon and Kono, 1996), and more efficiently than with somatic cell nuclei (83% of reconstituted embryos developed into blastocysts, of which 57% resulted in live young). This suggests that the ability of the cytoplasm to support embryonic development and reset genomic imprinting, differs markedly between zygotes and parthenogenetically activated oocytes. However, for the moment, any application of this using adult human nuclei should be precluded until the reasons for the

fetal losses are fully understood, and the use of the technique is proven to be safe in animal experiments.

1.5 Correction of abnormalities of meiosis

Although chromosomal aberrations can interfere with the development of the embryo, several that arise from gamete defects have been corrected or overcome by using micromanipulation techniques. Cytoplasmic donation in oocytes may avoid aneuploidy by normalizing spindle behavior and nuclear division in oogonia or GV oocytes, and coincidentally promote embryo growth as a function of the transfer of some mitochondria (Flood et al., 1990; Zhang et al., 1999; Palermo et al., 2002a). This donation may be performed either by fusion, or by transfer of ooplasm (Cohen et al., 1996; Takeuchi et al., 1999a).

Cytoplasmic control of the nucleus has been demonstrated by nuclear and cytoplasmic transfers in several animal species (Willadsen, 1986; 1989), and involves germinal vesicle breakdown (Masui and Markert, 1971; Kishimoto, 1986), chromosome decondensation (Thadani, 1979), and metaphase arrest (Meyerhof and Masui, 1979; Muggleton-Harris et al., 1982). Sperm nuclear decondensation also depends on the stage of oocyte maturation (Thibault and Gerard, 1970; Usui and Yanagimachi, 1976). Several other studies addressing the importance of cytoplasmic factors and organelles during meiosis and cleavage indicate that mitochondria are closely associated with the mitotic spindle in normally dividing blastomeres, but are absent in arrested ones. In this

situation, resumption of normal mitosis and mitochondrial distribution occurred after transfer of G_2 cytoplasm from normal blastomeres (Muggleton-Harris and Brown, 1988). Cytoplasmic factors essential for developmental competence are absent in primate GV oocytes, is suggested by their normal fertilization and development only after receiving mature oocyte cytoplasm (Flood et al., 1990).

Cytoplasmic transfer also introduces foreign mitochondrial DNA (mtDNA) into recipient oocytes/embryos. Such heteroplasmic mice with a phenotype encoded by the introduced mtDNA have been generated in this case to study mitochondrial segregation during development, and also the mechanisms underlying the onset of mitochondrial diseases (Jenuth et al., 1997; Inoue et al., 2000; Takeda et al., 2000).

Each mitochondrion contains 2 – 10 copies of mtDNA in all human tissues except platelets and oocytes, which contain only one copy per mitochondrion (Giles et al., 1980). Due to its location close to the site of reactive oxidative species formation, and a lack of protective histones (Tritschler and Medori, 1993), mtDNA is 20 times more susceptible to mutation than nuclear DNA (Kagawa and Hayashi, 1997). Nonetheless, and contrary to widely held notions, mitochondria can repair oxidative damage to their DNA quite efficiently (Bohr and Dianov, 1999). Moreover, the mutation rate is highly variable. Some genomic regions show nucleotide substitution rates similar to those of nuclear DNA, whereas synonymous sites and small rRNAs mutate about 20 times more rapidly, and tRNAs approximately 100 times more rapidly than their nuclear counterparts (Pesole et al., 1999). In most mitochondrial related diseases, patients'

cells carry a mixture of both mutant and wild-type (normal) mtDNA. This heterogeneous state is called heteroplasmy, the homogeneous state containing pure mutant or normal mtDNA being termed homoplasmy (Kagawa and Hayashi, 1997). The relative proportion of wild-type and mutant mtDNA appears to be important for the phenotypic expression of mitochondrial diseases (Newman et al., 1991; Boulet et al., 1992), and the 'dose' of mutant mtDNA also has an influence on the severity of phenotype abnormalities (Marchington et al., 1998). The level of mutant mtDNA varies in different tissues and changes with time (Poulton, 1996), probably related to the oxidative metabolism inherent in each organ.

mtDNA is always maternally inherited, and in recent years it has been the subject of increasing attention due both to the subtle role it may have in early development (Van Blerkom, 1989a; 1989b), and to its place in maternal age-related reduction of embryonic competence (Gaulden, 1992). Mitochondria are also a source of a variety of hereditary disorders. However, the mechanisms controlling the segregation and inheritance of mtDNA in mammals are poorly understood and are even controversial.

Although the mitochondrial genome is maternally inherited, the transmission of mitochondrial disorders is not always uniform (Wallace, 1999). Mutant mtDNA can arise *de novo* through a large-scale rearrangement without any familial history, or be maternally inherited as in the case of mtDNA point mutations (Egger and Wilson, 1983). Mitochondrial diseases can also follow an autosomal dominant pattern of inheritance that causes variable deletion of mtDNA or the expression of an autosomal recessive leading to profound cytochrome oxidase deficiency

(Dimauro et al., 1991). Finally, X-linked transmission is also seen as a possible inheritance mode (Poulton, 1996).

The proportion of mutant mtDNA transmitted from mother to offspring is variable because of a genetic bottleneck. For example, during germ-line development in early bovine embryogenesis, the number of mitochondria increases 100-fold, from about 1,000 per oogonium to over 100,000 per oocyte, while the mtDNA increases only about 10-fold, from 10,000 to 100,000 (Chen et al., 1995). As a result, each oocyte organelle harbors 1 mtDNA molecule, instead of the usual 5-10 observed in somatic cell mitochondria (Chen et al., 1995; Robin and Wong, 1988). Only a small number of mtDNA molecules replicate and give rise to the entire cytoplasmic genotype (100,000 mtDNA molecules) during the late stage of oogenesis (Hauswirth and Laipis, 1982). This single restriction/amplification event may be a necessary solution to the accumulation of mtDNA mutations during aging (Cortopassi et al., 1992), including that of oocytes as discussed earlier (Chen et al., 1995). During and after the mitotic divisions of cleavage, both mutant and normal mtDNA are distributed unevenly into the daughter cells that give origin to the fetus and its germ cells, thus enabling a complete switch within one or two generations (Meirelles and Smith, 1998).

Presently, there is no effective treatment for mitochondrial diseases. Following transfer by the cytoplasm fusion method of normal mtDNA into a cell containing mutated mtDNA (Kagawa and Hayashi, 1997), dilution of abnormal mtDNA to below the pathogenic level proved to be a potentially effective way of restoring both biochemical and morphological phenotypes of defective mitochondria. Pursuing the same logic, nuclear transplantation in human germ line cells

may offer an attractive therapeutic alternative in providing normal mitochondria, at least in patients compromised by a point mutation disorder. Theoretically, reconstitution of oocytes with healthy donor cytoplasm could diminish the transmission of defective mtDNA (Cohen et al., 1998a), but since the transplanted nuclei always carry a thin surrounding cytoplasm (Takeuchi et al., 1999a), a variable number of mitochondria may be transferred as well. However, the first polar body represents a karyoplast in which the mitochondria content is negligible (Tsai et al., 1999) and therefore its use may be a preferable way to minimizing mtDNA transmission. After injection of the first polar body, a mature enucleated mouse oocyte was later brought to a state of syngamy by ICSI and resulted in normal offspring (Wakayama and Yanagimachi, 1998a).

Nearly all the known activities required for replication of DNA in mitochondria and its expression are encoded by nuclear genomic products, necessitating communication between the two (Poyton and McEwen, 1996). It has been reported that perinuclear mitochondria are replicated preferentially at an earlier stage than those at the periphery of the cell/oocyte, and give rise to a higher ratio of karyoplast-derived mitochondrial genotypes in daughter cells (Davis and Clayton, 1996). The same observation was supported by an experiment in the mouse using pronuclear transplantation and later karyoplast-derived mtDNA assessment (Meirelles and Smith, 1998). Moreover, perinuclear mitochondria from avian and amphibian oocytes replicated more actively and appeared to segregate to the somatic cells of the fetus, while another subcortical group appeared to become localized in the primordial germ cells (D'Herde et al., 1995).

In a recent report, 20 of 21 cloned calves, showed a mtDNA genotype identical to that in the recipient cytoplasm, suggesting that the recipient mtDNA may become dominant after nuclear transfer (Takeda et al., 1999). What, therefore, will be the exact contribution of the karyoplast-derived mtDNA in offspring coming from nuclear transplantation? Can nuclear transplantation using a GV karyoplast actually minimize the transmission of maternal mutant mtDNA? As yet, the unpredictability of mtDNA segregation and a complex threshold variety involved in the phenotype expression preclude a firm conclusion.

If the transfer of cytoplasm and nuclei to oocytes becomes safe and effective, this would offer older women facing IVF failure a new option through which to conceive. In the case of nuclear transplantation, however, the biparental character of the reconstituted oocytes may have significant social, psychological, and legal implications. On the other hand, as a way of improving the developmental potential of the embryo, one could assume that transfer of ooplasm might have negligible impact on the genetic constitution of the offspring, since this involves so little mtDNA (Brenner et al., 1998; 2000; Barritt et al., 2001a; 2001b). Certainly, the significance of mitochondria transferred with a karyoplast, and the putative preferential replication of the perinuclear mtDNA, both need to be better understood to ascertain the exact contribution of the donor and recipient mitochondria in the children that result.

1.6 General introduction

The following chapters describe experiments which attempted to produce viable gametes with the desired parental genome. Consideration was given to techniques that relate to male germ cell isolation and transplantation, the procedures of nuclear transfer, somatic cell haploidization, and finally issues involved in cytoplasmic-heteroplasmic transfer. The efficiency of nuclear transplantation was evaluated using primary mouse oocytes and the outcomes assessed by their subsequent survival, their nuclear-cytoplasmic reconstitution, and their nuclear maturation. Studies focused on the optimal conditions for electrofusion, on the influence of karyoplast nuclear/cytoplasmic ratios, and the relative ability of GV- and MII-derived cytoplasts to support nuclear maturation. Possible genetic consequences of this procedure were assessed by oocyte karyotyping. Finally to assess the developmental ability *in vitro* of embryos generated after transplantation of nuclei into isolated cytoplasts, these were then transferred to pseudopregnant mice mated with vasectomized males.

In the case of human gametes, nuclear cytoplasm reconstitution and oocyte maturation were assessed, and the ability of the constructed oocytes to be fertilized and undergo early embryo development was investigated. Cytogenetic analysis was conducted on some of these conceptuses in order to assess the possibility of deleterious effects on the chromosome content of the oocytes and the resulting embryos. Since mitochondrial dysfunction can lead to oocyte aneuploidy (Keefe et al., 1995; Barritt et al., 2000), mitochondrial photosensitization was induced as a consistent means of producing dysfunctional ooplasm comparable to that

responsible for age-related effects (Barritt et al., 2000; Wilding et al., 2003). The effect of oocyte mitochondrial damage caused by a photosensitizing agent on oocyte maturation was examined. In addition, the possibility that such damage might be reversed by nuclear transplantation was assessed. Oocytes matured *in vitro* were examined after ICSI in regard to their embryonic development and whether GV oocytes with induced mitochondrial damage and then 'rescued' by nuclear transfer could develop normally. GVs were transferred into enucleated cytoplasts to assess the ability of diploid nuclei to undergo maturation in an allocytoplasmic environment as seen by oocyte survival, nuclear-cytoplasmic reconstitution, subsequent nuclear maturation, fertilization, and early embryonic development. In addition, some reconstituted oocytes were fixed and processed for cytogenetic analysis. Finally, in considering how to overcome the poor quality of oocytes from older women, whether or not somatic cell nuclei will undergo 'meiotic' reduction once transplanted into enucleated oocytes was explored.

In addition to heteroplasmy, the wide application of micromanipulation procedures, and particularly the exchange of nuclei and cytoplasm may induce certain changes not only at the DNA level, but also in the epigenome, and hence may be responsible for fluctuation in gene expression. These epigenetic alterations can be explored in different ways; by analyzing the methylation pattern at specific gene loci, or assessing modifications of the histone tails involved in sustaining gene complexes. Other approaches are the assessment of the transcripts, and therefore expression of the specific gene by reverse transcription and then real-time PCR, and it is even now possible by DNA chip technology to browse the entire genome.

Chapter 2

Instrumentation and Methodology

Declaration for Thesis Chapter 2

In the case of Chapter 2, contributions to the work involved the following:

	Name	% contribution	Nature of contribution
1	**Gianpiero D. Palermo**	100	Conception and design, collection, analysis, and writing of manuscript

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Weill Medical College of Cornell University, The Center for Reproductive Medicine, New York, NY, USA

Signature 1 [redacted] **Date** 10-29-2004

2.1 Equipment

Dissecting Scope

Description	Manufacturer	Model
Stereomicroscope	Olympus	SZX12

Micromanipulator Workstation

Description	Manufacturer	Model
Inverted microscope	Olympus	IX-70
Motor coarse controller	Narishige	MM-188
Hydraulic micromanipulators	Narishige	MO-188
Microinjectors including tool holders	Narishige	IM-6

Piezo-Electro-Actuator

Description	Manufacturer	Model
PiezoActuator	Burleigh	PiezoDrill
PiezoActuator	Primetech	PMM-150FU

Polarized microscope

Description	Manufacturer	Model
Spindle View System	CRI	SPV-001

Electroporation system

Description	Manufacturer	Model
ElectroCellManipulator	BTX	ECM2001

Imaging Systems

Description	Manufacturer	Model
Epifluorescent microscope	Olympus	BX-61
Imaging software and filter set	Applied Imaging	CytoVision Genetic

MICROTOOL EQUIPMENT

Description	Manufacturer	Model
Micropuller	Campden	753*i*
Microgrinder	Narishige	EG-40
Microforge	Narishige	MF-900

2.2 Microtool preparation

The micromanipulation pipettes are made from borosilicate glass capillary tubes (Drummond Scientific, Broomall, PA, USA) with an external diameter of 0.97 mm, 0.69 mm internal diameter and 78 mm length. Cleaning of the capillary tubes involves the following steps: (1) soaking

overnight in MilliQ water (Millipore Corporation, Bedford, MA, USA); (2) sonicating; (3) rinsing at least thirty times in MilliQ water before drying in a hot air oven; and (4) heat sterilization. The glass pipettes are made by drawing thin-walled glass capillary tubes using a horizontal microelectrode puller (Model 753, Campden Instruments Ltd., Loughborough, UK).

2.2.1 Microforge

The platinum filament of 100 μm thickness is bent into a triangular shape and placed on a horizontal plane positioned at 9 o'clock in relation to the center of the optical field of the microforge (Narishige Co. Ltd., Tokyo, Japan). On the tip of the filament a glass bead of approximately 50 μm diameter is made by melting the tip of a pulled pipette.

2.2.2 Holding pipette

tip of a pulled pipette is broken, maintaining a parallel orientation between the pipette and the filament in the opposite direction. The appropriate contact of the pipette with the glass bead is important in creating a regular break. The pulled pipette is lowered allowing it to touch the glass bead. The filament is heated slowly until the tool melts on the part touching the bead. The heating of the filament is suddenly stopped by releasing the foot switch and the pipette breaks sharply due to the cooling retraction of the platinum filament. If the margin of the break is irregular, the pipette has to be rebroken more proximally, provided the diameter is still within the desired range. After a break is made at approximately 70 μm diameter, the tip is fire-polished and appropriately shaped by overheating the filament, to obtain a final outer diameter of 60 μm and an inner diameter of 20 μm. The function of the holding pipette is to fix the oocyte in place

during the micromanipulation.

2.2.3 Injection pipette

A pulled pipette is lowered onto the turning tungsten carbide wheel of the microgrinder spinning at 60 rpm (Narishige Co. Ltd.). The process is monitored through a magnifying lens (approximately 6X). The wheel of the grinder is continuously moistened by a drop counter loaded with milliQ water. The drops create a wet track upon which the tip of the needle is lowered to an angle of 30°. The grinding time of approximately 15 – 18 seconds is calculated according to the pressure with which the tip touched the stone. The final tip has an outer diameter of approximately 7 µm, an inner diameter of approximately 5 µm and a bevel at 30 – 35°. The remaining water inside the pipette has to be dried by moving the pipette vertically up and down along the heated filament on the microforge. In order to create a spike at the correct position, the opening of the ground needle should face front, perpendicular to the filament position. This may require turning the needle on its axis in order to make certain that the opening is facing to the front. To make a spike, the glass bead is heated (the least necessary) and touched with the tip of the tool by lowering the pipette (with the micrometric screw of the forge for vertical motion) and then up rapidly to avoid overheating the fine tip. Overheating, may reduce the diameter or close the tip opening.

For mouse ICSI insemination, injection pipettes designed for a piezomicropipette driving unit (PiezoDrill, Burleigh Instruments, Inc. Fishers, NY, USA and PMM-150FU; PrimeTech, Ibaragi,

Japan) were prepared by cutting pulled micropipettes at 5 µm inner diameter. The pipettes are usually drawn just before use.

2.2.4 Bending the microtools

In order to be able to work horizontally in the Petri dish (Model 1006; Falcon) the tools should be angled at approximately 140°. The tool is brought vertically to a position perpendicular to the microfilament and then moved to the glass bead until approximately 800 µm from the tip. The tool will bend towards the left; heating should cease and the tool moved to the right. The injection pipette should be bent carefully and in several steps in order to avoid the narrowing of the inner diameter.

2.2.5 Nuclear transfer pipettes

Two kinds of glass microtools, besides the holding pipette, are used in the procedure of GV transplantation, a zona piercing needle and an enucleation pipette. The zona piercing needles are made by cutting the pulled micropipettes to a diameter of approximately 20 µm, followed by overheating the open end to allow the formation of a thick, and closed tip. The oocyte enucleation pipettes were made by cutting and fire polishing the pulled micropipettes with the diameter adjusted for each application, i.e., for mouse experiments 20 – 25 µm, while for work with human gametes, 30 – 35 µm inner diameters. Moreover, the pipettes utilized to enucleate MII oocytes have an inner diameter of 10 – 15 µm.

2.3 Culture media

Essentially, the culture medium is a buffered solution of amino acids and vitamins, able to satisfy the nutritive requirements of cells and tissues. The different types of media employed for the handling of gametes and embryos are as listed in the following table.

Medium	Manufacturer	Supplement (Concentration)
Human Tubal Fluid (HTF)	Irvine Scientific	10% SSS or 0.4% HSA
HTF-HEPES	Irvine Scientific	10% SSS or 0.4% HSA
Chatot Ziomek Bavister (CZB)	House made	0.5% BSA
CZB-HEPES	House made	0.5% BSA
Modified HTF-PVP	House made	10 % PVP
PVP solution	House made	0.4% HSA
M2	Sigma	0.4% BSA
M2-IBMX	Sigma	0.2mM IBMX
M2-CCB	Sigma	CCB (5 μg/ml, 25 μg/ml)
M2-Hyaluronidase	Sigma	Hyaluronidase (100 U/ml)
M199	Sigma	0.23 mM PA, 25 μg/ml GM, 0.3% BSA
M199-IBMX	Sigma	0.2mM IBMX
Waymouth's-M752/1	Gibco	10% FBS, 0.23mM PA, 50 μg/ml ST, 120 U/ml PCN-G

Waymouth-IBMX	Gibco	0.2mM IBMX
KSOM^{AA}	Special media	—
DMEM	Gibco	10% FBS
RPMI-125	Gibco	10% FBS

All manipulation were performed in air and therefore, HEPES-buffered media such as HTF-HEPES, CZB-HEPES, and M2 were used.

While spermatozoa may be manipulated in standard culture medium, slowing the spermatozoa motility and having greater control during their aspiration is preferable. Therefore, it is recommended that sperm cells be placed into a 10% solution of polyvinylpyrrolidone (PVP-K 90; Molecular weight 360,000, ICN, Biochemical, Cleveland, Ohio, USA).

2.4 Mouse strains

The mouse strain used can affect the overall efficiency of microinjection, with hybrid strains showing, an eightfold increase in efficiency over an inbred strain (Brinster et al., 1985). Therefore, a hybrid strain with a defined genetic background such as B6D2F1 was used for oocyte manipulation and embryo generation. While CD-1 albino mice have been utilized as foster mothers and carriers for the experimental embryos because their coat color is conveniently

different from the B6D2F1 strain. In addition, CD-1 vasectomized males were used for sterile mating of recipients.

2.5 Procedures

2.5.1 Oocyte collection

2.5.1.1 Mouse

Immature oocytes at the germinal vesicle (GV) stage were retrieved by puncturing large follicles of unstimulated ovaries dissected from B6D2F1 female mice, 7 – 11 weeks old. Cumulus-corona cells were removed mechanically by repeated aspiration through the tip of a hand-drawn pipette. In order to prevent spontaneous germinal vesicle breakdown (GVBD), cumulus denuded oocytes were cultured in a medium supplemented with a phosphodiesterase inhibitor (0.2 mM 3-isobutyl-1-methylxanthine; Sigma Chemical, St. Louis, MO, USA). Immature oocytes were kept in these conditions for approximately 2 hours until they exhibited a perivitelline space (Okada et al., 1986), therefore facilitating micromanipulation procedures.

Mature oocytes with first polar body (PB) extruded (MII stage), were collected 15 hours after hCG (10 IU i.p., Sigma Chemical Co.) administration to female mice given PMSG (10 IU i.p., Sigma Chemical) 48 hours earlier. The cumulus oophorus were removed by brief exposure to 100 IU/ml of hyaluronidase (Type VIII; Sigma Chemical Co.) and denuding completed with a glass pipette.

2.5.1.2 HUMAN

Immature human GV oocytes were obtained from consenting patients undergoing ICSI. Female partners were down-regulated with 1 mg subcutaneous luteal gonadotropin releasing hormone agonist (GnRHa) daily and subsequent ovarian superovulation was carried out by administering a combination of human menopausal gonadotropin (hMG) (Pergonal; Serono, Waltham, MA, USA) and/or pure follicular stimulating hormone (FSH) (Metrodin; Serono). Down-regulation by 0.5 mg subcutaneous low dose GnRHa daily was administered. In some cases direct ovarian superovulation, without GnRHa, was performed by administering FSH, hMG, or clomiphene citrate plus hMG. Human chorionic gonadotropin (hCG) was administered when the criteria for oocyte maturity were met and oocyte retrieval by vaginal ultrasound-guided puncture was performed 35 hours after hCG administration (Palermo et al., 1995a).

Under an inverted microscope at 100X magnification the cumulus-corona cell complexes were scored as mature (A), slightly immature (B), completely immature (C), or slightly hypermature (PM) (Table 2-I). Thereafter, the oocytes were further incubated for ≥ 4 hours. Immediately before micromanipulation, the cumulus-corona cells initially were removed by exposure to HTF-HEPES-buffered medium (21 mM HEPES concentration) containing 80 IU/ml of hyaluronidase (type VIII; Sigma Chemical Co.) (Palermo et al., 1995a). After cumulus-corona cells were removed by enzymatic and/or mechanical treatment, the denuded oocytes were examined under an inverted microscope to assess their condition and maturational stage.

Table 2-I. Classification of oocyte cumulus-corona cell complexes

Cumulus-corona cell complex grading	Description	Presumed nuclear status
A	Sunburst	MII
B	Unexpanded/dark	MI
C	Small, tight, atretic	GV
PM	Clumps	MII

2.5.2 Collection of spermatozoa

2.5.2.1 Mouse

The epididymides and vasa deferentes were excised from one 3- to 6-month-old F1 hybrid male mice (B6D2F1). The dissection of the distal part of the cauda allowed the collection of spermatozoa.

The spermatozoa were expressed from the cut cauda into ~ 500 µl of CZB medium. The medium droplets were covered with lightweight paraffin oil (BDH Ltd., Poole, UK) and the spermatozoa were allowed to disperse for ~ 15 mins. Subsequently, 200 µl of the sperm suspension was diluted in 200 µl of the medium and incubated for 1 – 2 hours at 37°C in 5% CO_2 in air until ICSI.

2.5.2.2 Human

Semen samples were collected by masturbation after at least 4 days of abstinence and were allowed to liquefy for at least 20 mins at 37°C prior to analysis. Semen concentration and

motility were assessed in a Makler counting chamber (Makler, 1978; Sefi-Medical Instruments, Ltd., Haifa, Israel) according to the World Health Organization (1999) criteria. Semen morphology was assessed by spreading 5 µl of the sperm suspension on prestained slides (Testsimplets®; Boehringer Mannheim GmbH, Mannheim, FRG) following the criteria of Kruger et al. (1986). Semen was considered abnormal when the sperm density was < 20 x 10^6/ml, or the progressive motility was < 40% (de Kretser et al., 1985), or when < 5% of the spermatozoa had a normal morphology (Kruger et al., 1986).

The sperm sample was washed by centrifugation at 500 *g* for 5 mins in HTF medium, supplemented with Plasmanate USP (Bayer Corporation, Elkhart, IN, USA). The pellet was resuspended and further processed by the swim-up procedure (Mahadevan et al., 1983). Semen samples with < 5 x 10^6/ml or < 30% motile spermatozoa were washed in medium by a single centrifugation at 500 *g* for 5 mins. Thereafter, the resuspended pellet was layered on a discontinuous density gradient (PURECEPTION™; Sage, Pasadina, CA, USA) and centrifuged at 300 *g* for 20 mins. The gradients had two layers (95 – 47.5%) (Dravland and Mortimer, 1988) or one (90%), depending on the volume of the semen pellet. The Percoll fraction containing the spermatozoa was washed by adding 1 ml of HTF medium and centrifuged at 500 *g* for 5 mins to remove the silica gel particles.

The concentration of the sperm suspension, assessed in a Neubauer hemocytometric chamber, was eventually adjusted to 1 – 1.5 x 10^6 spermatozoa/ml by addition of HTF medium and incubated at 37°C in a gas atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

2.5.3 ICSI

2.5.3.1 Mouse

A plastic dish (Falcon 1006; Becton and Dickinson, Franklin Lakest, NJ, USA) was used as a microinjection chamber. Two rows of three 5 µl droplets each were placed along the center line of the dish. The left three droplets were loaded with PVP medium. The first and the third droplet were for pipette washing. The second droplet contained the sperm suspension as well as PVP-medium. The right three droplets were CZB-HEPES medium for the oocytes. These droplets were covered with mineral oil (E.R. Squibb & Sons, Princeton, NJ, USA).

Sperm injection was performed by using a piezo micropipette-driving unit. This unit, based on the piezoelectric effect, is able to transmit sequential high frequency microvibrations to the injection tool (PMM-150FU; Prima, Primetech, Ibaraki, Japan). The piezo device allows a localized penetration of the zona pellucida with virtually no distortion of the cell (oocyte). A motile spermatozoon was sucked, tail first, into the injection pipette. When the junction between the midpiece and the principal piece (where the cytoplasmic droplet was commonly observed) was in the opening of the pipette, two to three piezo-pulses were applied to decapitate the spermatozoon. After the separation, each isolated head was injected into an oocyte.

The oocyte was positioned where the holding pipette was at 9 o'clock, and the metaphase II spindle was oriented at either 12 or 6 o'clock. The tip of the injection pipette was brought to an intimate contact with the zona at the 3 o'clock position, 2 – 3 piezo-pulses were given to advance

the pipette while a light negative pressure was applied to it. When the tip of the pipette had passed through the zona, a cylindrical piece of the zona in the pipette was expelled into the perivitelline space. After the sperm head was pushed forward until it was near the tip, the pipette was then advanced quickly into the ooplasm until its tip almost reached the opposite side of the oocyte's cortex. The sperm head was expelled into the ooplasm with the least amount of accompanying sperm suspension medium following a single piezo-pulse. After retrieval of as much as possible of the medium, the pipette was gently withdrawn, leaving the sperm head within the ooplasm. All injections were performed in CZB-HEPES at room temperature.

2.5.3.2 Human

Some of the reconstituted *in vitro* matured oocytes were subjected to ICSI in a standard fashion (Palermo et al., 1995a; 1996b), in most cases, 2 – 3 hours after observation of the first PB extrusion. The tip of the injection tool had a beveled angle of approximately 30° with a 2 μm spike. A 140° bend at 1 mm from the tip permitted the terminal end of both tools to operate almost parallel to the bottom of the injection dish.

To perform ICSI, the oocyte was held in place by the holding pipette via suction on the zona pellucida at 9 o'clock, maintaining the inferior pole of the egg in contact with the dish while injecting the pipette at 3 o'clock. Individual spermatozoa were aspirated by their tail or head and then positioned transversally to the tip of the injection tool after re-expulsion. The tip of the pipette was lowered gently, compressing or rubbing the sperm flagellum between the midpiece and tail. This action destabilized the sperm membrane, thereby impairing motility. A

spermatozoon with apparently normal morphology was chosen from the PVP drop and was aspirated tail first into the injection pipette. The injection pipette was introduced through the zona pellucida and oolemma, deep into the cytoplasm, in a single motion without hesitation. The spermatozoon was injected using the smallest possible amount of medium (Palermo et al., 1995a).

2.5.4 Fertilization assessments

2.5.4.1 Mouse

ICSI inseminated eggs were observed 6 hours after injection and the bipronucleate oocytes with a visible second polar body was recorded as normally fertilized. The oocytes with other pronuclear patterns such as one pronucleus and a second polar body, or three pronuclei and no second polar body, were considered abnormally fertilized.

2.5.4.2 Human

Approximately 16 – 20 hours after the injection, the oocytes were examined for the presence of two distinct pronuclei and two clear polar bodies, as criteria for normal fertilization.

2.5.5 Embryo culture

2.5.5.1 Mouse

Cleavage to 2-cell embryos were assessed 24 hours after the microinjection; thereafter further *in vitro* development was noted every 24 hours up to 96 hours.

2.5.5.2 HUMAN

Fertilized oocytes were then incubated further in HTF-HSA medium for up to 72 hours to evaluate their developmental capacity. The number and size of the blastomeres and the percentage of anucleate fragments were recorded for each embryo (Staessen et al., 1989).To assess embryo quality, the day 3 (D3) observation period was chosen according to our routine ICSI embryo transfer protocol (Palermo et al., 1995a; 1996c).

2.5.6 Embryo transfer

2.5.6.1 MOUSE

2.5.6.1.1 Oviduct transfer

Two-cell embryos were transferred into the oviduct of plugged pseudopregnant CD-1 females through the infundibulum. The embryos were transferred in buffered M2 medium prior to transfer. The recipient female was anaesthetized (using pentobarbital, 0.04 – 0.07 mg/g, i.p.) and placed on a dissecting microscope with the dorsal side up. A skin incision was made just below the rib cage (upper flank area) using a small pair of scissors exposing the abdominal muscles. With another pair of scissors, an opening was made into the peritoneum over the fat pad/ovary area. The oviduct was exposed by pulling the fat pad through the incision and held in place over the back of the mouse by a clamp. The infundibulum (the most anterior part of which was the natural opening of the oviduct) was visible through the bursa covering the ovary and part of the oviduct. A tear was made on the bursa directly above the infundibulum avoiding the capillaries. While keeping the tear open with fine tipped-forceps, the transfer pipette containing the

embryos was then inserted into the infundibulum and up to 2 mm deeper into the oviduct. The embryos were gently transferred along with a minimum amount of M2 medium. An air bubble drawn into the transfer pipette prior to the aspiration of the embryos function as an indicator to monitor that all embryos are successfully transferred. Up to 10 embryos were transferred into each oviduct. The fat pad was released from the clamp and grasped with a pair of forceps and carefully returned into the peritoneal cavity without touching the oviduct. The peritoneal and muscle incision was closed with absorbable suture (e.g. Polysorb 5-0; USSC, Tucker, GA, USA) and the skin with wound clips (Hogan et al., 1994).

2.5.7 Nuclear transfer

2.5.7.1 Mouse

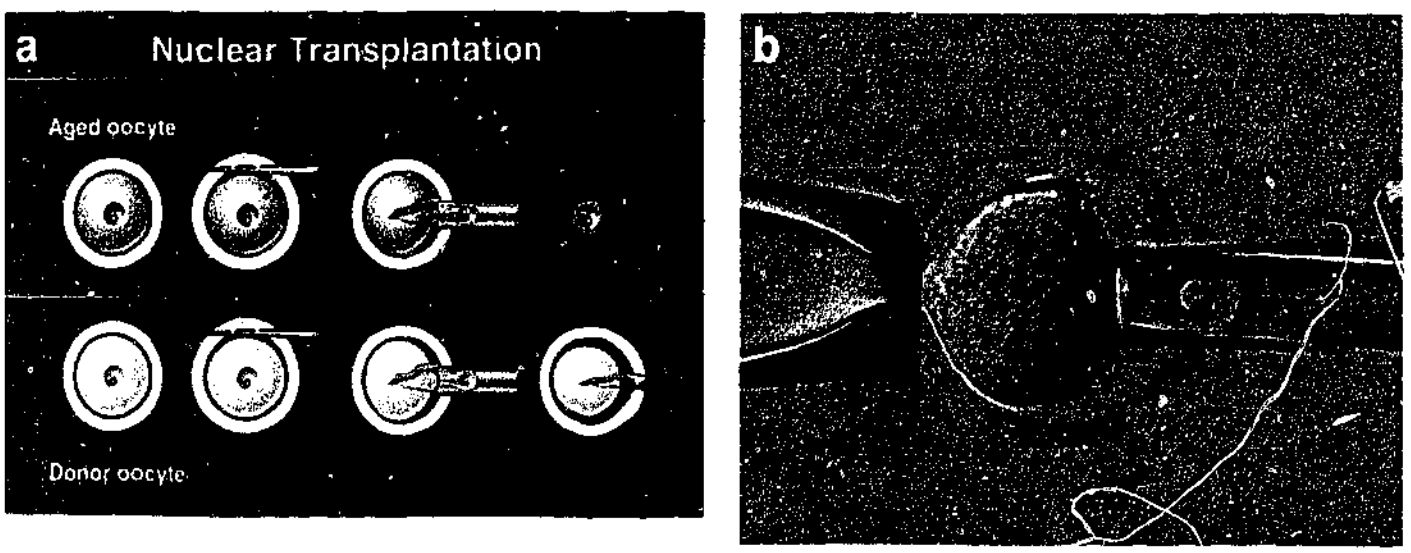

Figure 2.1 (a) Enucleation of immature oocytes — isolation of the karyoplast from the aged oocyte (green) and isolation of the cytoplast from a younger oocyte (pink). (b) Isolation of a karyoplast from a mouse oocyte.

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. The zona pellucida was breached by a glass microneedle, and then oocytes

were exposed to 25 µg/ml of cytochalasin B (CCB) (Sigma Chemical Co.). The GV nucleus surrounded by a small amount of cytoplasm (GV karyoplast) was removed by a micropipette with a 20 µm inner diameter. Following this, the isolated GV karyoplast was inserted into the perivitelline space of another previously enucleated oocyte (GV ooplast) (Figures 2.1a,b). Each grafted oocyte was aligned between two micro-electrodes perpendicular to their axes with a micromanipulator. To induce fusion, a single 1.0 kV/cm direct current fusion pulse was delivered for 100 µs in an electrolyte medium (M2) by an Electro Cell Manipulator (BTX 2001; Genetronics, Inc., San Diego, CA) (Figure 2.2a.b). Then, after washing and culture for 30 minutes in a CCB-free medium, these oocytes were examined to confirm cell survival and fusion, cultured further in IBMX-free *in vitro* maturation medium for 14 to 16 hours after the fusion treatment. Finally, their nuclear maturation was evaluated as evidenced by extrusion of the first polar body (PB).

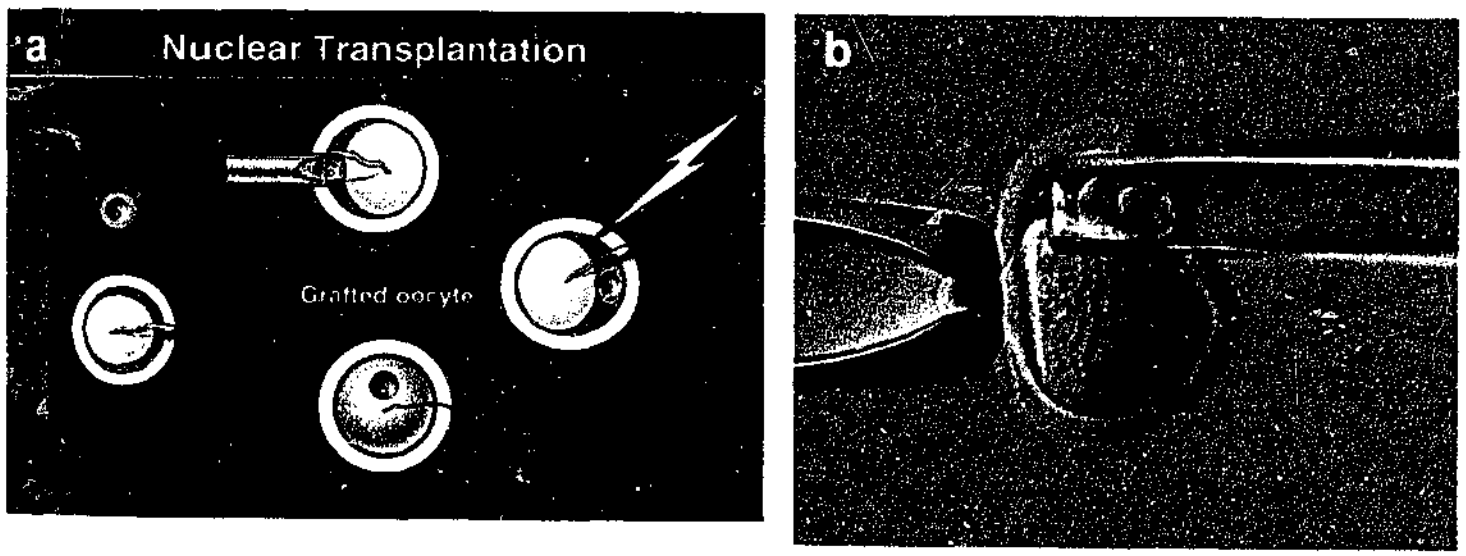

Figure 2.2 (a) Grafting, electrofusion, and reconstitution — the karyoplast is inserted into the perivitelline space of the young oocyte. The grafted oocyte is exposed to an electrical pulse to induce nucleus-cytoplasm fusion and reconstitute the immature oocyte (grey). **(b)** First stage of grafting a karyoplast into an enucleated oocyte (ooplast).

2.5.7.2 HUMAN

Germinal vesicles were removed from oocytes of older women by a glass tool similar to the one used for the production of cytoplasts before exposure to cytochalasin B. After mechanical breaching of the zona pellucida, the oocyte nucleus surrounded by a small amount of cytoplasm (karyoplast) was gently suctioned into a pipette of 30 μm inner diameter. At the same time the genomic material was removed from the young oocytes in the same fashion.

The GV karyoplast (from an aged oocyte; ≥ 38 years old) was then introduced into the perivitelline space of a previously enucleated young oocyte (< 35 years old). Subsequently, the assembled oocytes in a mannitol-containing medium (Levron et al., 1996) were aligned and subjected to AC pulses of 100 V/cm for 10 sec followed by DC single pulse 1.3 kV/cm for 70 μsec, delivered by a BTX ECM 2001 (Genetronics Inc., San Diego, CA, USA). Then the treated oocytes were then rinsed in fresh HTF medium and cultured for approximately 30 min to allow fusion to occur. The success of the procedure was confirmed by examination of the oocytes for extrusion of the polar body and chromosomal karyotyping, which demonstrates the haploidization of the immature reconstituted oocyte.

2.5.8 Somatic cell nuclei transfer

While better culture conditions might enhance the limited ability of immature human oocytes treated by nuclear transfer to mature *in vitro*, obtaining a sufficient number of oocytes from older women for such reconstitution remains another limiting factor. A sufficient number of oocytes

might be created by transplantation of a patient's somatic cell nucleus into an ooplast obtained from a younger donor (21 – 34 years old) (Figure 2.3a-c). The construction of viable oocytes from somatic cells would benefit older women with an exhausted ovarian reserve, women with premature ovarian failure, or those considered as "poor responders" to ovarian superovulation.

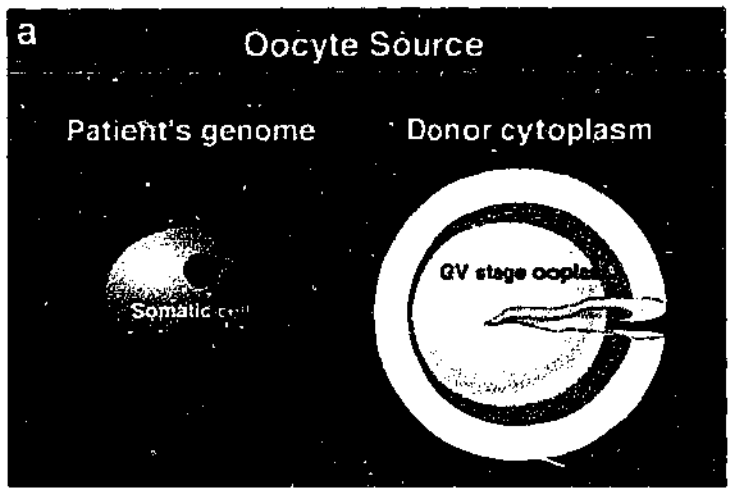

Figure 2.3 (a) Manufacturing oocytes — a somatic cell containing the patient's genome can be used to manufacture oocytes when transferred into an enucleated germinal vesicle (GV) ooplast.

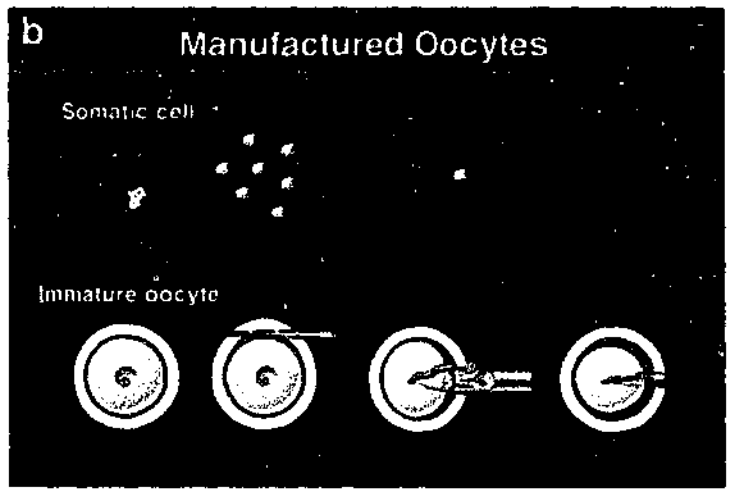

Figure 2.3 (b) Somatic cell (green) selected for injection in an enucleated immature oocyte (pink).

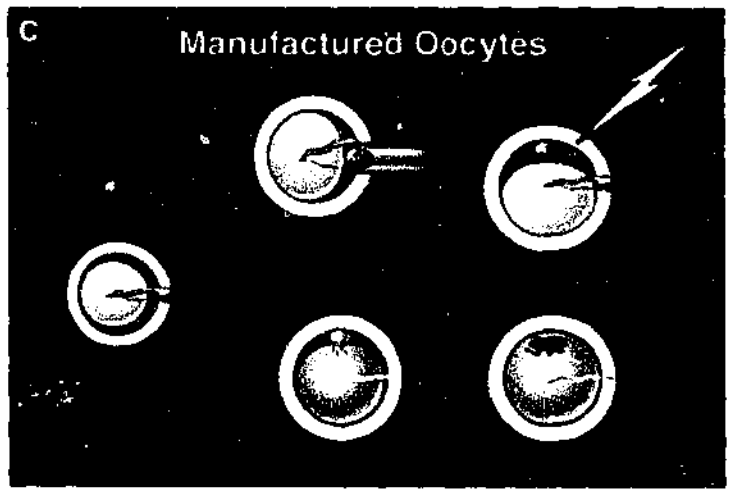

Figure 2.3 (c) Grafting, electrofusion, reconstitution, and haploidization — the somatic cell nucleus (extracted as the karyoplast, see Figure 2.1) is inserted into the perivitelline space of the young oocyte. The grafted oocyte is exposed to an electrical pulse to induce nucleus-cytoplasm fusion and form a metaphase I stage oocyte that subsequently extrudes a first polar body (grey).

Since GV oocytes can complete the first meiotic division spontaneously *in vitro*, it seemed possible that immature ooplasm might be able to induce 'haploidization' of diploid somatic nuclei (Kubelka and Moor, 1997). In accord with this it has been demonstrated that the immature ooplasm is capable of inducing 'meiosis-like reduction division' of transplanted somatic cell nuclei (Takeuchi et al., 2000a; Tsai et al., 2000).

2.5.8.1 Mouse

Mouse cumulus cells were obtained by brief exposure to hyaluronidase (Sigma Chemical Co.) (100 IU/ml) of oocyte cumulus complexes isolated from PMSG/hCG stimulated B6D2F1 strain mice. These cells were then cultured for up to 30 days with several passages in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Life Technologies).

2.5.8.2 Human

Human endometrial cells were obtained from biopsies of consenting patients undergoing endometrial cell-coculture during IVF (IRB Number 1195-099). Stromal and glandular cells were isolated by enzymatic digestion using 0.2% collagenase type II and separated by differential sedimentation (Barmat et al., 1998), then stromal cells were cultured in DMEM medium supplemented with 10% FBS for up to 30 days.

Human endometrium stromal cells were freed from the culture dish by a standard trypsin-EDTA procedure (Barmat et al., 1998). The isolated cell was then inserted subzonally into an enucleated oocyte, and subjected to electrofusion. In reconstituting eggs, the latter was preferred over direct injection because of the fragility of the GV stage ooplasts. The resultant reconstituted oocytes were cultured for 14 – 16 hours until extrusion of the first PB.

2.5.9 Cytogenetic assessments

2.5.9.1 CHROMOSOMAL STAINING AND FISH ANALYSIS

Mouse or human oocytes were fixed and stained with Giemsa to visualize and assess chromosome number and structure. To assess the distribution of chromatin, some oocytes were stained with 0.5 μg/ml Hoechst 33342 (Sigma Chemical Co.) solution and evaluated under a fluorescent microscope, while others were fixed beneath a coverslip with methanol/acetic acid (3:1; v/v), and stained with 1% aceto-orcein solution. Other oocytes were processed for karyotyping with Giemsa staining, or were subjected to FISH analysis as described in Section 4.5. Oocytes that had extruded the first PB were prepared for chromosome analysis by gradual fixation (Kamiguchi and Mikamo, 1986; Kamiguchi et al., 1993). Briefly, oocytes were treated in a hypotonic solution (0.068 M potassium chloride with 0.1% BSA) for 5 minutes, and swollen oocytes were plunged in fixative I (methanol:acetic acid:distilled water, 5:1:3, v/v/v) for 5 minutes. The oocyte was then placed on a clean slide and covered with fixative II (methanol:acetic acid, 3:1, v/v). The slide was kept in a Coplin jar containing fixative II for at least 5 minutes, then dipped into fixative III (methanol:acetic acid:distilled water, 3:3:1, v/v/v) for 1 minute and dried in moist warm air.

To evaluate as many chromosomes as possible, a sequential FISH was performed on the same oocyte with probes for chromosome X, 13, 14, 15, 16, 18, 21, and 22. The first step included direct label fluorescence probes: [LSI probes 13 (Spectrum Green) and 21 (Spectrum Orange), and CEP probes X (Spectrum Green and Orange) and 18 (Spectrum Aqua)]. The hybridization solution was prepared by mixing 5.4 μl Spectrum LSI Hybridization buffer; 0.4 μl of 18

SpectrumAqua; 0.4 µl of 21 SpectrumOrange; 0.4 µl of 13 SpectrumGreen; 0.2 µl of X SpectrumGreen and 0.2 µl of X SpectrumOrange. The mixture was vortexed thoroughly, centrifuged (6,000 *g*) for 1 – 3 seconds and left at room temperature for a short time. A total of 7 µl of the mixed solution was applied onto the specimen target area, and a coverslip was placed on the solution. Rubber cement was then applied to seal the coverslip on the slides. Denaturation was performed at 80°C for 3 minutes followed by DNA hybridization at 37°C in a moist dark chamber for at least 6 hours. After careful removal of the rubber cement, washing was performed by plunging the slides in a 0.3% Nonidet-P40 solution prepared in a diluted (15X) standard saline citrate (SSC) (Sigma Chemical Co.) at 70°C for 3 minutes followed by a 1 minute washing in 0.1% Nonidet-P40 with (2X) SCC at room temperature. Nuclei were counterstained and maintained with 14 µl of 4',6-diamino-2-phenylindole (DAPI) in antifade solution (0.5mg/ml; Vysis Inc.), covered with a coverslip, and observed at 600X with an epifluorescence microscope (Olympus BX 61; New York/New Jersey Scientific Inc.).

After assessment of the first set of chromosomes, the specimen was rinsed three times for two minutes in phosphate buffered detergent (PBD; Oncor, Gaithersburg, MD). The slides were then sequentially dehydrated in increasing concentrations of ethanol (70%, 85% and 100%), rinsed and denatured in 70% formamide with (2X) SCC at 70°C for 5 minutes and dehydrated in sequential steps in increasing concentrations of ethanol at 4°C for one minute 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 analyze the corresponding chromosomes.

Slides were viewed under a fluorescent microscope using single-bandpass filter sets including aqua and gold (Vysis Inc.), rhodamine, 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 analyzed with an imaging software (Cytovision, Applied Imaging Inc.). Scoring criteria have been described previously (Munné et al., 1994; 1995a; 1995b; Dailey et al., 1996; Palermo et al., 1995c). Normal haploid oocyte nuclei carried one signal for an X chromosome and one signal for an autosome. Oocytes missing one signal were nullisomic for the corresponding chromosome, and oocytes with an extra signal were disomic for the corresponding chromosome. The rate of technical FISH failure was estimated to be as high as 2% for intact oocyte nuclei and 1% for decondensed oocyte nuclei.

2.5.10 IRB protocol

2.5.10.1 Mouse

The mouse experimental protocols (No. 9707-415A, No. 0005-720A, No. 0305-125A) were fully approved by the Institutional Animal Care and Use Committee of Cornell University Medical College.

2.5.10.2 Human

Immature oocytes were obtained from consenting patients undergoing ICSI. The research 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).

2.5.11 Data analysis

The customary Pearson χ^2 test was utilized for discrete univariate and bivariate data, except where test assumptions were violated, necessitating the Fisher exact test. Statistical significance was stipulated as 0.05 for discrete and continuous analysis. The χ^2 test was utilized to identify differences in cell survival, reconstitution, and maturation rates observed between different groups. All statistical computations were conducted using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Statistical comparisons were reported only when they reached significance.

Chapter 3

Transplantation of Nuclei into Immature Mammalian Oocytes

Declaration for Thesis Chapter 3

In the case of Chapter 3, contributions to the work involved the following:

	Name	% contribution	Nature of contribution
1	**Gianpiero D. Palermo**	45	Conception and design, collection, analysis, and writing of manuscript
2	**Takumi Takeuchi**	25	Execution of work, collection and data analysis
3	**Tian Hua Huang**	15	Execution of work
4	**Queenie V. Neri**	15	Data analysis and manuscript editing

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Weill Medical College of Cornell University, The Center for Reproductive Medicine, New York, NY, USA

Signature 1 [redacted] **Date** 10-29-2004

Signature 2 **Date** 11-19-2004

Signature 3 **Date** 11-22-2004

Signature 4 **Date** 11-11-2004

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A reliable technique of nuclear transplantation for immature mammalian oocytes

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Transplanting a germinal vesicle (GV) to another enucleated oocyte provides a possible way to avoid age-related aneuploidy in metaphase II (MII) oocytes from older women. This study was conducted to examine the efficiency of each step of nuclear transplantation as reflected in the survival and maturation capacity of immature mouse oocytes subjected to this procedure. GV stage oocytes were retrieved from unstimulated ovaries. A GV removed with a small amount of cytoplasm (karyoplast) was transferred subzonally into a previously enucleated oocyte, which was then exposed to direct current to promote fusion. Such reconstituted oocytes were placed in culture to allow maturation, and some that had extruded a first polar body were fixed and processed for chromosome analysis. Each step of nuclear transplantation – survival, enucleation, grafting, and reconstitution – was successful in >90%, with the overall efficiency of reconstitution being 80%. The observation of normal karyotypes confirmed that the procedure did not increase chromosomal aneuploidy. An electrolytic medium, revealed to be superior for the reconstitution procedure, also allowed haploidization of the transplanted nucleus. These findings suggest that this technique can be applied to study the effects of a 'younger' woman's ooplasm on the disjunction of an 'older' woman's chromosomes during meiosis I.

Key words: aneuploidy/cell fusion/in-vitro maturation/nuclear transplantation/oocyte

Introduction

Advancing maternal age presents a clear inverse relationship with fertility, the decline in which is particularly evident by age 40 years (Tietze, 1957), and appears to be primarily related to an increased incidence of oocyte aneuploidy (Dailey *et al.*, 1996). Women of virtually any age can become pregnant by replacing embryos derived from young donor oocytes. The risk of conceiving an aneuploid fetus during in-vitro fertilization (IVF) increases from 6.8% for women 35–39 years old to ~50% in women 45 or older (Hassold and Chiu, 1985). The finding that 37.2% of morphologically normal 8-cell embryos in the 40–45 year maternal age group expressed chromosomal aberrations (Munne *et al.*, 1995) shows that aneuploidy impairs embryo implantation.

A clear relationship exists between ageing and abnormality of the chromosome/chromatid segregation, due to the non-disjunction of bivalents during meiosis (Dailey *et al.*, 1996). The meiotic spindle is generated by the ooplasm and it has been suggested that ageing of its organelles has an effect on the spindle's ability to undergo a balanced chromosome segregation/haploidization (Muggleton-Harris *et al.*, 1982; Pratt and Muggleton-Harris, 1988; Battaglia *et al.*, 1996). Why aneuploid oocytes are more common in aged women is still unknown, but it has been proposed that oxidative stress acting on primary oocytes, and/or surrounding ovarian cells may be a major factor (Tarin, 1995; Tarin *et al.*, 1996; Van Blerkom *et al.*, 1997). Other recent studies have demonstrated that the potential fertility of ovarian tissue can be maintained by cryopreservation of ovarian cortices (Gosden *et al.*, 1994; Newton *et al.*, 1996; Oktay and Gosden, 1996). This approach suggests that through ovarian tissue banking, it should be possible to preserve fertility in patients undergoing chemotherapy, and in women that opt to postpone their childbearing (Oktay *et al.*, 1998).

The problem remains for women that have already entered the 'downward slope' in regard to their ability to reproduce. Cytoplasmic transfusion into eggs or zygotes of non-human mammals has been shown to improve their developmental potential (Flood *et al.*, 1990; Levron *et al.*, 1996), and the transfer of cytosol from fertile donor oocytes has resulted in a successful delivery (Cohen *et al.*, 1997, 1998a). However, since this procedure was performed on mature oocytes, it cannot change any chromosomal imbalance that may appear during meiosis. This can be overcome only by transfer of the nucleus of a germinal vesicle (GV) stage oocyte to an enucleated immature oocyte from a younger woman (Zhang *et al.*, 1997).

In this study we have evaluated the efficacy of nuclear transplantation using mouse primary oocytes – as reflected in their subsequent survival, nuclear-cytoplasmic reconstitution, and nuclear maturation – with a focus on the optimal conditions for electrofusion, on the influence of karyoplast nuclear/cytoplasmic ratios and the relative ability of GV- and metaphase II (MII)-derived cytoplasts to support nuclear maturation. Finally, some oocytes were karyotyped in order to assess possible genetic consequences of this procedure.

Materials and methods

Animals

B6D2F1 female mice were purchased and housed in a temperature- and light-controlled room on a 12 h light:12 h dark photoperiod and

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A successful model to assess embryo development after transplantation of prophase nuclei

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BACKGROUND: Germinal vesicle transplantation (GVT) provides a means of investigating interactions between karyoplasts and cytoplasts isolated from different cells. Technically, GVT can be accomplished with a high degree of efficiency without compromising the maturation of either the human or mouse oocyte nucleus. Although maturation, fertilization and preimplantation development have been established using GVT, full-term development has been reported only after supplementation with fresh mature ooplasm. In this study, we assess the ability of immature oocytes collected from gonadotrophin-primed ovaries to mature *in vitro* after GVT and develop to full-term. METHODS: GV oocytes were retrieved from either non-stimulated or pregnant mare's serum gonadotrophin (PMSG)-primed female mice. Microsurgically isolated GV karyoplasts were transplanted into previously enucleated oocytes. Oocytes successfully reconstituted by electrofusion were cultured for 14 h to allow nuclear maturation. Metaphase II oocytes were subjected to Piezo-ICSI, and those fertilized normally were cultured to the blastocyst stage. Some such embryos were transferred to pseudopregnant female mice to examine their potential for normal development. Cumulus-denuded non-manipulated oocytes that were matured *in vitro* served as controls. RESULTS: The reconstitution and maturation rates were comparable in oocytes isolated from PMSG-primed and from unstimulated ovaries. The rate of normal fertilization in oocytes from primed ovaries was significantly higher than that of their non-primed counterparts (63.5 versus 39.6%; $P < 0.01$). This difference was also confirmed in terms of blastocyst development (31.8 versus 7.9%; $P < 0.01$). Of a total of 70 embryos transferred to the oviduct of five recipient mice, 21.4% developed to normal live offspring. All developed as normal adults and proved to be fertile. The live birth rate was comparable to that obtained using non-manipulated control oocytes (22.3%). CONCLUSIONS: Higher rates of fertilization and blastocyst formation were obtained after GVT of mouse oocytes isolated from PMSG-primed ovaries compared with their non-primed counterparts. These represent the first mouse offspring derived from *in vitro* matured, cumulus-denuded oocytes treated by allo-GVT and fertilized by ICSI. Thus, GVT appears not to impair oocyte maturation, fertilization and pre- and post-implantation development and, after gonadotrophin priming, allows generation of healthy mouse offspring without mature ooplasm supplementation.

Key words: embryo development/germinal vesicle transplantation/ICSI/*in vitro* maturation/nuclear transfer

Introduction

Oocyte aneuploidy is considered to be responsible for a lower implantation rate and increased pregnancy loss in women of advanced reproductive age. Currently, these women can be successfully treated by oocyte donation, but most couples express interest in utilizing their own gametes. Current clinical practice involves oocyte/embryo selection by preimplantation genetic diagnosis. This procedure, however, can only select among the available conceptuses. In women ≥40 years old, the higher incidence of aneuploidy would leave only a minimal number of normal embryos for replacement in such cases.

Aneuploidy in aged oocytes occurs at a specific maturational step, first meiosis, and is due to non-disjunction. This results from a dysfunction of the ooplasm which is unable to provide the molecular structural components necessary to generate a normal meiotic spindle. Specific mechanisms that underlie this may be related to mitochondrial DNA mutations (Keefe *et al.*, 1995; Barritt *et al.*, 2000; Schon *et al.*, 2000), oxidative stress (Tarin, 1995) or abnormalities in the ovarian microcirculation (Gaulden, 1992; Van Blerkom *et al.*, 1995; Van Blerkom, 1996). All of these age-related aberrations can lead to impaired mitochondrial metabolism and so incomplete chromosomal segregation. In fact, a lower mitochondrial membrane potential is associated with an age-related higher incidence of meiotic spindle abnormalities during oocyte maturation (Battaglia *et al.*, 1996; Volarcik *et al.*, 1998; Wilding *et al.*, 2003).

3.1 Summary

Transplanting a GV to an enucleated oocyte could provide a way to avoid age-related aneuploidy in MII oocytes from older women. This study was conducted to examine the efficiency of each step of nuclear transplantation as reflected in the survival and maturation capacity of immature mouse oocytes subjected to this procedure. GV stage oocytes were retrieved from unstimulated or stimulated ovaries. A GV with a small amount of cytoplasm (karyoplast) was placed subzonally into a previously enucleated oocyte, then exposed to direct current to promote their fusion. Such reconstituted oocytes were cultured to allow maturation, and some that extruded a first polar body were fixed and processed for chromosome analysis. Each step of nuclear transplantation — survival, enucleation, grafting, and reconstitution — was successful in > 90%, with the final efficiency of reconstitution being 80%. The observation of normal karyotypes confirmed that the procedure did not result in chromosomal aneuploidy. An electrolyte medium, revealed to be superior for the reconstitution procedure, also allowed haploidization of the transplanted nucleus. Fertilization ability of the GVT oocytes was evaluated by ICSI, *in vitro* embryo culture, and transferred to pseudopregnant female mice. These findings suggest that transplantation of nuclei will allow studies of the effects of a 'younger' woman's ooplasm on the disjunction of an 'older' woman's chromosomes during meiosis I.

3.2 Introduction

Advancing maternal age presents a clear inverse relationship with fertility, particularly evident by age 40 years (Tietze, 1957). This problem appears to be primarily related to an increased incidence of oocyte aneuploidy (Dailey et al., 1996), since women of virtually any age can become pregnant by replacing embryos derived from young donor oocytes (Hassold and Jacobs, 1984; Daily et al., 1996). The risk of conceiving an aneuploid fetus during *in vitro* fertilization (IVF) increases from 6.8% for women 35 – 39 years old to ~ 50% in women 45 or older (Hassold and Chiu, 1985).

A clear relationship exists between aging and the non-disjunction of bivalent chromosomes during meiosis (Dailey et al., 1996). It has been suggested that aging of the meiotic spindle compromises its ability to guide the chromosome segregation/haploidization in a balanced way (Battaglia et al., 1996; Volarcik et al., 1998). Why spindle defects occur as a correlation of aging is still unknown, it has been proposed that oxidative stress acting on primary oocytes and/or surrounding ovarian cells may be a major factor (Tarín, 1995; Tarín et al., 1996; Van Blerkom et al., 1997). One prospective approach that has been suggested would be ovarian tissue banking in women who opt to postpone their childbearing (Gosden et al., 1994; Newton et al., 1996; Oktay and Gosden, 1996; Oktay et al., 1998; 2004; Shaw et al., 2000; Kim et al., 2002; Gook et al., 2003).

In considering treatment of women already in their 40s, transfusion of ooplasm into eggs or zygotes of mice has been shown to improve their developmental potential (Flood et al., 1990;

Levron et al., 1996), and such transfer of human ooplasm has resulted in successful deliveries (Cohen et al., 1997; 1998a). However, since this procedure was performed on mature oocytes, it cannot correct any chromosomal imbalance that may appear during meiosis. This can be overcome only by transfer of a GV nucleus stage oocyte to an enucleated immature oocyte from a younger woman (Zhang et al., 1997).

In this study we have evaluated nuclear transplantation using mouse primary oocytes, as reflected in their subsequent survival, nuclear-cytoplasmic reconstitution, and maturation. Optimization of the technique was attempted by varying the conditions for electrofusion, the karyoplast nuclear/cytoplasmic ratios, and the relative ability of GV- and MII-derived cytoplasts to support nuclear maturation. Finally, some oocytes were karyotyped in order to assess possible genetic consequences of this procedure.

3.3 Source of mouse eggs

B6D2F1 female mice were housed in a temperature- and light-controlled room with an alternative 12 hour light/dark photocycle and were provided with food and water *ad libitum*.

GV oocytes were retrieved, according to Section 2.5.1.1, by puncturing follicles of unstimulated ovaries dissected from B6D2F1 female mice 7 – 11 weeks old. Cumulus-corona cells were removed mechanically by repeated aspiration through the tip of a hand-drawn pipette. In order to

prevent spontaneous germinal vesicle breakdown (GVBD), oocytes were cultured in medium (M199) supplemented with a phosphodiesterase inhibitor (0.2 mM 3-isobutyl-1-methylxanthine; Sigma, St Louis, MO, USA). Immature oocytes were kept in these conditions for 2 hour until they exhibited a perivitelline space.

MII oocytes with first PB extruded were collected 48 hour post PMSG injection and 15 hour after hCG injection according to Section 2.5.1.1. The cumulus oophorus was removed by brief exposure to 100 IU/ml of hyaluronidase (Type VIII; Sigma Chemical Co.) and completed with a glass pipette.

3.4 Enucleation and reconstitution of mouse eggs

Tools were controlled by two microinjectors (Model IM-6; Narishige, New York/New Jersey Scientific Inc.). Micromanipulation procedures were carried out on a heated stage (Eastech Laboratory, Centereach, NY, USA) placed on an inverted microscope (Olympus IX-70; New York/New Jersey Scientific Inc.) equipped with two electrical/hydraulic micromanipulators (Model MM-188 and MO-109; Narishige, New York/New Jersey Scientific Inc.).

Prior to enucleation, oocytes were exposed for at least 15 min to M2 medium containing 25 μg/ml of cytochalasin B (CCB; Sigma Chemical Co.) and 3 mg/ml BSA. Thus, the zona pellucida was penetrated by pressing a glass microneedle tangentially into the perivitelline space against the

holding pipette. The GV nucleus surrounded by a small amount of cytoplasm (GV karyoplast) was removed by a micropipette with a 20 μm inner diameter (Figure 3.1). In earlier reports, GV enucleation was performed 'indirectly' (Sun and Moor, 1991; Meng et al., 1996), by increasing the pressure inside a holding pipette to expel a GV karyoplast through a slit made in the zona. In the present study, the enucleation was performed 'directly' with a calibrated enucleation pipette, for speed and to determine the size of the karyoplast. Thereafter, the isolated GV karyoplast was inserted with the same tool into the perivitelline space of another previously enucleated oocyte at either the GV (GV cytoplast) or MII stage (MII cytoplast) to form a 'grafted oocyte'. Enucleation of MII mouse oocytes was performed by removing the metaphase spindle with a small amount of ooplasm, and the first PB (Wakayama et al., 1998b). In the mouse, the spindle is identifiable as a translucent region. Similar enucleation of the MII spindle from mature oocytes required a lower concentration of CCB (7.5 μg/ml) for the same length of time.

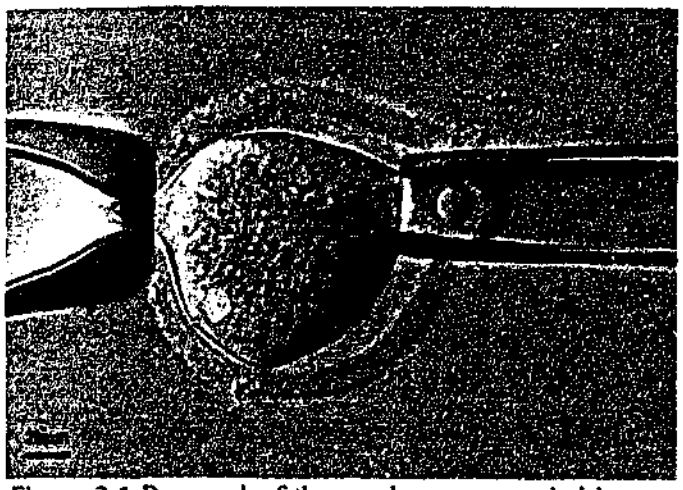

Figure 3.1 Removal of the nucleus surrounded by a small amount of ooplasm from a mouse germinal vesicle.

In order to define the influence of the cytoplasm surrounding the enucleated GV, a preliminary study on spontaneous nuclear maturation was performed using isolated karyoplasts of different sizes. Oocytes receiving karyoplasts first classified according to their diameter (small: < 30 μm;

medium: 30 – 40 μm; large: > 40 μm) (Figure 3.2) were then cultured individually and observed at 3, 12, and 24 hour intervals for GVBD and PB extrusion.

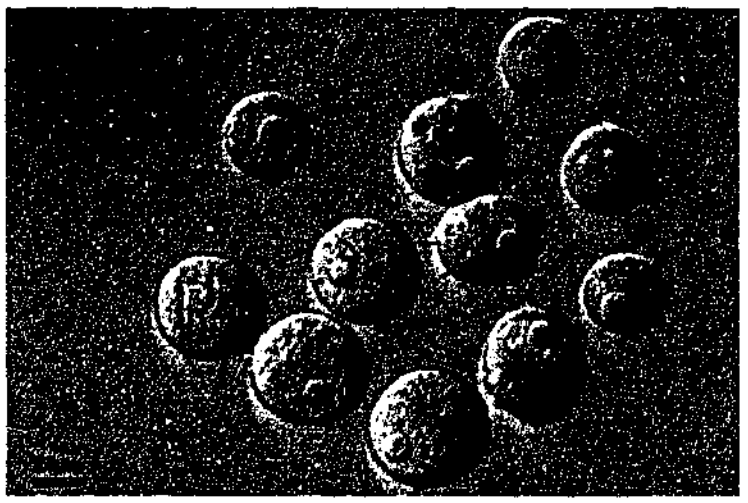

Figure 3.2 Isolated mouse karyoplasts of different sizes.

After insertion of the karyoplast, grafted oocytes were washed in fresh medium to remove CCB, and then cultured in M199 medium containing 25 μg/ml gentamycin, 0.22 mM pyruvic acid, and 3 mg/ml BSA for at least 15 min prior to electrofusion.

An Electro Cell Manipulator (BTX 200 and 2001; BTX Inc., San Diego, CA, USA) was used. Each grafted oocyte was aligned with a micromanipulator between two micro-electrodes of 100 μm diameter (ECF-100; Tokyo Rikakikai Co. Ltd, Tokyo, Japan) perpendicular to their axes (Figure 3.3). To induce fusion, a single or double 1.0 kV/cm direct current (DC) fusion pulse(s) was delivered for 50 – 99 μs in a non-electrolyte or electrolyte medium. In the non-electrolyte medium, pulses of alternating dielectrophoretic current (AC) (100 – 200 V/cm, 5 – 30 sec) were applied to ensure appropriate contact between karyoplast and cytoplast. Up to four electrical pulses were applied in total, each

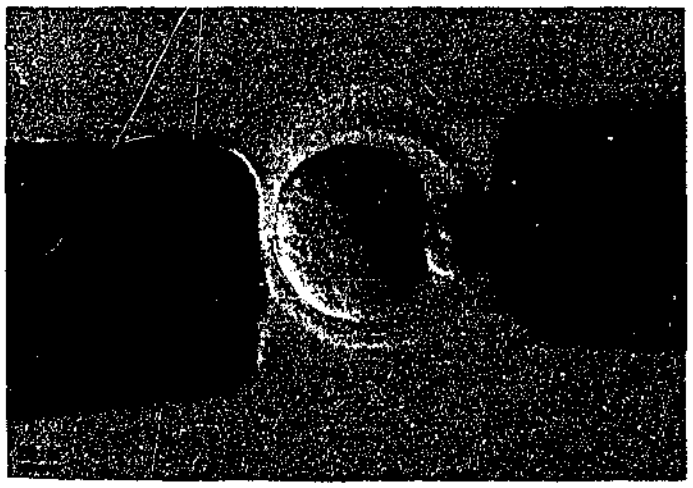

Figure 3.3 A grafted oocyte (couplet of a karyoplast and cytoplast) aligned between two microelectrodes.

at 30 min intervals. Then, after washing and culture for 30 min, fused oocytes were examined to confirm cell survival and fusion (Figure 3.4).

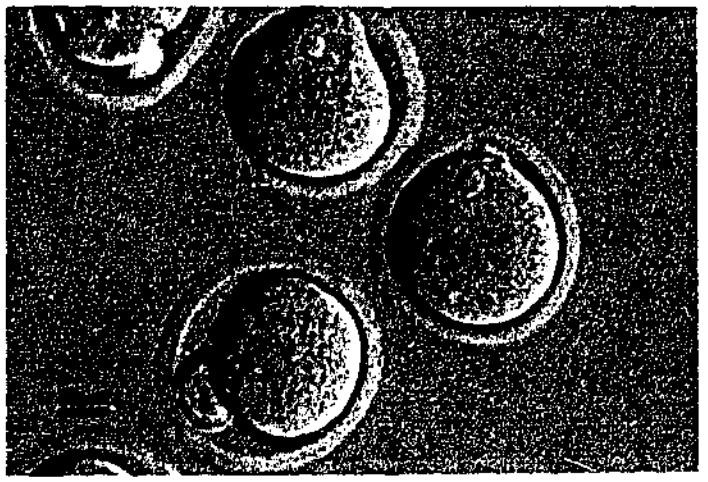

Figure 3.4 Grafted oocytes 30 min after electrofusion.

Oocytes were randomly assigned to three non-electrolytic and one electrolytic medium (M2 medium). Non-electrolytic media were: (i) 0.3 M concentration of mannitol supplemented with 0.1 mM $CaCl_2$, 0.05 mM $MgCl_2$ and 1 mg/ml BSA; (ii) 0.25 M concentration of sucrose; and (iii) Zimmermann's cell fusion medium [0.28 M sucrose, 0.5 mM Mg $(C_2H_3O_2)2H_2O$, 0.1 mM $Ca(C_2H_3O_2)_2$, 0.1 mM K_2HPO_4, 0.1 mM glutathione, 0.01 mg/ml BSA] (Wolfe and Kraemer, 1992). Each non-electrolytic medium had an osmolarity and pH of ~ 280 mOsm/l and a pH of approximately 7.4. Reconstituted oocytes were cultured in M199 medium and then examined at 12 to 16 hour after fusion treatment to evaluate nuclear maturation as evidenced by GVBD and a first PB.

3.5 Cytogenetic evaluation of reconstituted *in vitro* matured oocytes

Oocytes with a first PB were prepared for chromosome analysis after gradual fixation as described in Chapter 2 (Kamiguchi and Mikamo, 1986; Kamiguchi et al., 1993), by treatment in a hypotonic solution (0.068 M potassium chloride with 1 mg/ml BSA) for 5 min, following which swollen oocytes were immersed in fixative I (methanol:acetic acid:distilled water, 5:1:3, v/v/v) for

5 min. Then the oocyte was placed on a clean slide and covered with fixative II (methanol:acetic acid, 3:1, v/v), for at least 5 min, transferred to fixative III (methanol:acetic acid:distilled water, 3:3:1, v/v/v) for 1 min, dried, and stained with Giemsa.

3.6 Reconstitution of oocytes after GV transplantation

The results presented here were obtained from a set of 23 experiments performed successfully only after 53 preliminary attempts in which only 3.1% of 748 oocytes from 60 mice were reconstituted successfully. Of those 23 restored oocytes incubated in human tubal fluid (HTF) supplemented with 0.3% human serum albumin, only five (21.7%) extruded the first polar body. At this point, the electrofusion medium was removed and medium 199 culture was substituted, following which > 90% of surviving oocytes were successfully enucleated, grafted, and fused (Table 3-I). The overall efficiency of the technique from the enucleated immature oocyte to the reconstituted oocyte with an extruded PB was 80%.

Table 3-I. Individual steps in the nuclear transplantation process of immature mouse oocytes

	No. of oocytes (%)
A. Intact	542
B. Dissected and enucleated (% of A)	507 (93.5)
C. GV transferred and electrofused (% of B)	504 (99.4)
D. Reconstituted (% of C)	446 (88.5)
E. Matured (% of D)	399 (89.5)

GV = germinal vesicle

3.7 The influence of karyoplast size on nuclear maturation

In order to assess the influence of cytoplasmic volume on spontaneous nuclear maturation, isolated karyoplasts of three different sizes were maintained in culture and observed for up to 24 hour. GVBD was never higher than 5% at 3 hour regardless of karyoplast size, but at 12 hour only ~ 50% receiving medium size karyoplasts were at the GVBD stage compared to 100% for the large size karyoplasts (Table 3-II). These proportions had not changed after a further 24 hour in culture. In all karyoplasts, nuclear maturation proceeded no further than GVBD without any PB extrusion.

Table 3-II. Effect of cytoplasmic volume on maturation of isolated germinal vesicle karyoplasts

	Germinal vesicle breakdown (%)		
Karyoplast size (μm)	3 h	12 h	24 h
Small (< 30)	1/22 (4.5)	2/22 (9.1)[a]	2/22 (9.1)[d]
Medium (30 – 40)	1/19 (5.3)	9/19 (47.4)[b]	10/19 (52.6)[e]
Large (> 40)	1/23 (4.3)	23/23 (100)[c]	23/23 (100)[f]

[a vs b vs c] χ^2, 2 x 3, 2 *df*, Effect of cytoplasmic size on nuclear maturation at 12 h interval, $P = 0.0001$

[d vs e vs f] χ^2, 2 x 3, 2 *df*, Effect of cytoplasmic size on nuclear maturation at 24 h interval, $P = 0.0001$

3.8 Media composition and oocyte reconstitution

When transplanted karyoplasts were exposed to one of three non-electrolyte media or to an electrolyte medium (Table 3-III), the electrolyte medium was far superior as a supportive environment for electrofusion ($P = 0.0001$).

Table 3-III. Effect of media on reconstitution rate of mouse immature oocytes

	No. of oocytes (%)		
Fusion medium	Treated	Survived	Fused
Non-electrolytic (0.3 M mannitol)	139	122 (87.8)	11 (9.0)[a]
Non-electrolytic (0.25M mannitol)	123	91 (74.0)	2 (2.2)[b]
Non-electrolytic (Zimmerman's)	185	99 (53.5)	10 (10.1)[c]
Electrolytic (M2 medium)	109	93 (85.3)	75 (80.6)[d]

[d vs a+b+c]: χ^2, 2 x 2, 1 *df*, Effect of medium composition on cell fusion, $P = 0.0001$

3.9 The effect of cytoplast cell cycle stage on nuclear maturation

Ninety-three percent of 128 GV oocytes and all of the 51 MII oocytes were enucleated successfully. A GV was then introduced into each of 68 cytoplasts derived from GV oocytes, and into each of 51 MII oocytes. In contrast to the 93% success in the former, none of 41 reconstituted MII cytoplasts supported final maturation of the transplanted GV to MII. Of these, 20 (48.8%) remained arrested at the GV stage and 21 underwent GVBD but never extruded a first PB, even receiving an electrical pulse (Table 3-IV).

Table 3-IV. Ability of cytoplasts at different cell cycle stages to induce nuclear maturation

	Cytoplast stage	
Oocytes	GV	MII
A. GV transferred	68	51
B. Reconstituted by electrofusion	64 (94.1)[a]	41 (80.4)[b]
C. Maturation to MII stage (% B)	60 (93.7)[c]	0[d]

[a vs b] χ^2, 2 x 2, 1 *df*, Effect of cytoplasmic cell cycle stage on reconstitution rate, $P < 0.05$

[c vs d] χ^2, 2 x 2, 1 *df*, Effect of cytoplasmic cell cycle stage on nuclear maturation, $P < 0.001$

GV = germinal vesicle; MII = metaphase II

3.10 Cytogenetics of the reconstituted/matured oocytes

Of the 21 mature oocytes karyotyped successfully, 20 were normal (Figure 3.5), and one was hypohaploid for chromosome 19.

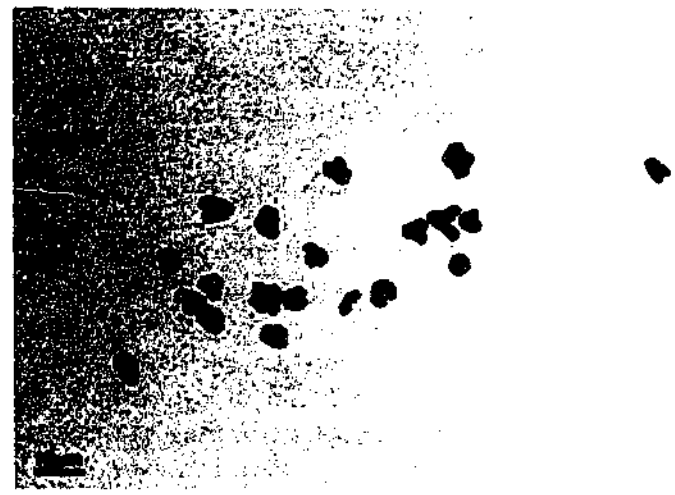

Figure 3.5 A normal haploid set of chromosomes obtained from a manipulated oocyte after extrusion of the first polar body.

3.11 Post-implantation development of the GV transplanted oocytes

When a total of 70 two-cell embryos derived from GVT oocytes were transferred to five recipients, 15 live offspring were born on day 20. This live-birth rate (ranging from 33.3 to 35.0%) was comparable with that of control non-manipulated GV oocytes (ranging from 29.4 to 41.7%) (Table 3-V). All the offspring developed as adults without any apparent abnormalities (Figure 3.6). The coat color of offspring was black ($n = 7$), grey ($n = 5$) or brown ($n = 3$) as expected (Wakayama and Yanagimachi, 1998a; 1998b). These young mice proved to be fertile after puberty, with normal litter sizes of 8 – 10. A total of 48 live offspring (51.1%) were obtained after oviductal transfer of 94 two-cell embryos derived

Figure 3.6 The foster mother (white) and four young pups (black) derived from germinal vesicle transplanted oocytes.

from *in vivo* matured oocytes. The live birth rate per transferred embryo was not significantly different among the three groups.

Table 3-V. Full term development of embryos derived from GV transplanted and intact oocytes

	In vitro matured oocytes	
No. of (%)	Non-manipulated	GV transplanted
ICSI inseminated oocytes	193	169
Fertilized oocytes	118 (61.1)	112 (66.3)
Replaced embryos (mean ± SD)	108 (15.4 ± 2)	99 (14.1 ± 4)
Recipients	7	7
Live offspring	25 (23.1)	22 (22.2)

GV = germinal vesicle

3.12 Discussion

This study in which a GV was transplanted with an overall efficiency of 80% demonstrated four key points. 1) The use of an electrolytic medium increased the fusion rate significantly, 2) recipient cytoplasts prepared from GV oocytes supported nuclear maturation, whereas those from MII oocytes did not, 3) transplantation is apparently more effective when the GV is surrounded by the smallest amount of cytoplasm, and 4) the rate of first PB extrusion was markedly enhanced by the adoption of a specific medium (M199) for *in vitro* maturation.

Although the technique of nuclear transplantation described here can be performed efficiently, it proved critical to define the size of the karyoplasts and the media used for cell fusion, as well as

the electrofusion settings. Cytoplasts prepared from primary oocytes were characterized by poor fusion rates, those from mature oocytes being more fusogenic with contact between the karyoplast and cytoplast being easier to establish. Cell fusion of immature oocytes was optimized by the adoption of an electrolyte medium. Moreover, it has been suggested that there is a need for a dielectrophoretic AC alignment pulse preceding the induction of cell fusion by DC (Zimmermann et al., 1984). Electrical alignment is generally performed for a large number of cells in an electrofusion chamber filled with a low-conductive, non-electrolyte solution made up of isotonic concentrations of sugars (Zimmermann et al., 1984), and is required to orient the axis along the adjacent cell membranes of the constructs perpendicular to the electric current vector. When an AC pulse passes through a conductive electrolyte medium, localized heating occurs with consequent cell distress. Since nuclear transfer usually involves a small number of cells, it does not require the formation of pearl chains of cells, and the alignment can be performed manually cell by cell, making non-electrolyte media redundant. Several authors (Kubiak and Tarkowski, 1985; Tsunoda et al., 1987b) reported a satisfactory fusion rate when DC fusion pulses were applied to 2-cell mouse embryos suspended in either 0.3 M mannitol or PBS without previous application of AC pulses. In these reports, however, cells were manually aligned, eliminating the need for an AC current. In view of the advantage suggested in placing the constructs in a physiologically balanced fusion medium (Rickords and White, 1992), we adopted manual alignment to avoid their exposure to the stress of AC. No detrimental effects of M2 as a fusion medium were observed on cell survival and subsequent nuclear maturation of the restored oocytes.

The observation of a direct positive relationship between the amount of perinuclear residual cytoplasm around an isolated karyoplast and its effect on the nuclear maturation rate (Fulka et al., 1998) draws attention to the nuclear/cytoplasmic ratio of the karyoplast, which often has been overlooked in transplantation experiments. GV karyoplasts with a very thin rim of cytoplasm seldom underwent GVBD, and none bearing less than one-third of the total oocyte cytoplasm extruded a PB. A possible explanation may be seen in the analogy with incompetent oocytes that acquire the ability to undergo GVBD only after their volume increases to at least 80% of the fully grown oocyte (Wassarman, 1988), perhaps because of an insufficient complement of cell cycle proteins (Motlik and Kubelka, 1990). While a total replacement with host ooplasm might be achieved by injecting nuclear material into the recipient ooplast, this is still not possible due to the large size of the nucleus. Therefore, during enucleation, the smallest amount of surrounding cytoplasm should be removed with the nucleus, in order to maximize the positive effect of the host ooplasm on its subsequent maturation.

MII oocytes seem in many respects ideal for cytoplast preparation because of their fusogenicity, their ability to support future development, and the ease with which their cumulus can be removed (Zhang et al., 1995; Cecconi et al., 1996). On the other hand, they cannot promote GVBD and extrusion of the first PB. In that last regard, several studies have identified maturation promoting factor (MPF) as responsible for inducing nuclear envelope breakdown and chromosome condensation (Masui and Markert, 1971; Meyerhof and Masui, 1979; Muggleton-Harris et al., 1982; Kishimoto, 1986; Muggleton-Harris and Brown, 1988). MPF activity appears shortly before GVBD, maintains a high level during MI, decreases prior to extrusion of the first

PB, and rises again throughout the MII stage (Campbell et al., 1996b; Fulka et al., 1992; 1996). When MII cytoplasts are used, the same electric current that induces fusion may activate the oocyte (Campbell et al., 1996a; Kono, 1997) and lead to a reduction of MPF, by cyclin degradation and a tapering off of the cytostatic factor (CSF) activity, allowing arrested MII oocytes to undergo cleavage division (Whitaker, 1996). In this study, electrical pulses applied to induce cell fusion, GVBD and eventual extrusion of the first PB, may have caused activation as well. Therefore, although oocytes derived from MII cytoplasts were not able to induce haploidization, they were able in 12% of the cases (5/41) to undergo first cleavage division. On the other hand, because immature oocytes are insensitive to electrical stimulation, the MPF activity is low throughout the fusion step and beyond (Kubelka and Moor, 1997).

To conclude, nuclear transplantation can be performed efficiently, and apparently does not bring chromosomal errors, and thus can be used to study the relationship between cytoplasmic aging and oocyte aneuploidy. Substitution of an old cytoplasm with a younger one appears promising as a way of reducing the incidence of aneuploidy in oocytes from older women. However, no one oocyte stage has all the ideal attributes for this. Though it is more fusogenic, is more easily manipulated, and has greater potential for development, the MII cytoplast cannot bring about maturation of the transplanted GV. Although cytoplasts prepared from GV oocytes support GV maturation, they are less fusogenic and may be functionally impaired by the attendant cumulus coronal cell removal. In spite of the fact that this impairment may be reversed by the addition of mature cytoplasm (Flood et al., 1990; Cohen et al., 1998a; 1998b), this remains a limiting factor. Another aspect that needs to be evaluated further is the role of maternal mitochondrial DNA and

its eventual influence on the original genome (Keefe et al., 1995; Houshmand et al., 1997; Brenner et al., 1998; Van Blerkom et al., 1998).

The ability to generate offspring from immature mouse oocytes in a direct manner provides a feasible model in which to study nuclear transplantation, and to examine whether this may offer a way of correcting oocyte aneuploidy. In addition, the occurrence of full term development without additional cytoplasmic infusions avoids the further complication of foreign mtDNA heteroplasmy (Cummins, 2001; 2002) and its transmission to future generations (Barritt et al., 2001a; Van Blerkom et al., 1998; St John, 2002). Furthermore, the epigenetic status of the reconstituted oocytes and derived conceptuses should be investigated, since altered DNA methylation patterns have been observed in offspring produced by nuclear transplantation (Reik et al., 1993; 2001; Humpherys et al., 2001; Hawes et al., 2002).

In conclusion, mouse GVT oocytes can undergo *in vitro* maturation, fertilization and full term development without supplementation with mature ooplasm. Oocyte survival after ICSI, fertilization and embryo development did not differ from that observed in non-manipulated, *in vitro* matured oocytes.

Chapter 4

Germinal Vesicle Transplantation into Immature Human Oocytes

Declaration for Thesis Chapter 4

In the case of Chapter 4, contributions to the work involved the following:

	Name	% contribution	Nature of contribution
1	**Gianpiero D. Palermo**	55	Conception and design, collection, analysis, and writing of manuscript
2	**Takumi Takeuchi**	20	Execution of work, collection and data analysis
3	**Jianli Gong**	15	Execution of work
4	**Ming C. Tsai**	10	Data analysis

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Weill Medical College of Cornell University, The Center for Reproductive Medicine, New York, NY, USA

Signature 1 **Date** 10-29-2004

Signature 2 **Date** 11-19-2004

Signature 3 **Date** 11-22-2004

Signature 4 **Date** 11/17/2004

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Preliminary findings in germinal vesicle transplantation of immature human oocytes

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Transplanting a germinal vesicle (GV) from an aged woman's oocyte into a younger ooplasm has been proposed as a possible way to reduce the incidence of oocyte aneuploidy which is considered to be responsible for age-related infertility. In this study, we have assessed the efficiency of each step involved in nuclear transplantation—specifically cell survival, nuclear-cytoplasmic reconstitution, and the capacity of the reconstituted oocytes for in-vitro maturation. In addition, we have evaluated the fertilizability and karyotypic status of the manipulated oocytes by intracytoplasmic sperm injection (ICSI) and fluorescent in-situ hybridization technique respectively. Nuclear transplantation was accomplished with an overall efficiency of 73%. Due to the limited availability of materials, most nuclear transplantation procedures were performed between sibling oocytes. The maturation rate of 62% following reconstitution was comparable with that of control oocytes, as was the incidence of aneuploidy among the reconstituted oocytes. The ICSI results of the reconstituted oocytes yielded a survival rate of 77%, a fertilization rate of 52%, and a satisfactory early embryonic cleavage. Furthermore, in a limited number of observations where the nucleus of an aged oocyte was transferred into a younger ooplasm, there was an appropriate chromosomal segregation. These findings demonstrate that human oocytes reconstituted with GV nuclei are able to undergo maturation, fertilization, and early embryo cleavage, and maintain a normal ploidy. Although in-vitro maturation seems to be a limiting step, this technique would allow us to investigate further the nuclear-ooplasmic relationship during meiotic maturation.

Key words: aneuploidy/human oocytes/ICSI/in-vitro maturation/nuclear transplantation

Introduction

During the last 20 years, assisted reproductive techniques have been evolving to the point that it is now possible to obtain an ~50% pregnancy rate for younger couples regardless of the aetiology of the infertility. Yet, in spite of this high success rate and the innovative techniques being utilized, maternal age remains a limiting factor in achieving a pregnancy for older couples. The effect of oocyte ageing on implantation is considered to be related to an increased incidence of aneuploidy (Munné *et al.*, 1995a, 1999; Dailey *et al.*, 1996; Gianaroli *et al.*, 1997, 1999), and in turn to an abnormal state of the meiotic spindle (Battaglia *et al.*, 1996; Volarcik *et al.*, 1998). These spindle abnormalities seem to arise primarily during meiosis I (Hassold and Chiu, 1985), but it is not totally clear that abnormal distribution of the chromosomes between the oocyte and the polar body (PB) always results from an error in spindle behaviour *per se*. Segregation of chromosomes appears to be controlled by the meiotic spindle, but its components are largely supplemented by the ooplasm. Therefore, it has been suggested that dysfunctional cytoplasmic factor(s) are responsible for structural abnormalities of meiotic spindles, which lead to eventual chromosomal malsegregation (Gaulden, 1992; Van Blerkom, 1994; Battaglia *et al.*, 1996).

Attempts to improve ongoing pregnancy/delivery rate in women with age-related infertility, who apparently are at increased risk for oocyte aneuploidy, have been made by selecting oocytes and embryos through preimplantation genetic diagnosis (PGD) (Gianaroli *et al.*, 1997, 1999; Munné *et al.*, 1999; Verlinski *et al.*, 1999). The positive results of those studies with increased implantation and reduced miscarriage rates following PGD prove that oocyte/embryo aneuploidy is responsible for the impaired implantation rate in aged women. However, although the selection approach might enhance the implantation rate by identifying oocytes/embryos with a normal chromosomal content, the drawback is the limited number of embryos that can be transferred.

In view of the key role played by cytoplasmic factors on embryonic development (Muggleton-Harris *et al.*, 1982; Prau and Muggleton-Harris, 1988; Van Blerkom *et al.*, 1995; Liu *et al.*, 1997), a different approach has been proposed, namely

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Technical approaches to correction of oocyte aneuploidy

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BACKGROUND: This study describes the technical approaches used in treatment of age-related oocyte aneuploidy, the efficiency of each step of nuclear transplantation into mouse and human oocytes, and the ability of germinal vesicle (GV) transplantation to restore artificially induced ooplasmic damage. Finally, it examines the possibility of constructing viable female gametes by transferring diploid somatic cell nuclei into enucleated oocytes. METHODS: GV stage mouse oocytes were collected from unstimulated ovaries, and human GV oocytes were donated from consenting patients undergoing ICSI. Stromal (somatic) cells were isolated from uterine biopsies of consenting patients. Mouse cumulus cells were obtained after ovarian stimulation. GV ooplasts prepared by removing nuclei were transplanted either with GV nuclei or with somatic cells by micromanipulation. Grafted oocytes were electrofused and cultured to allow maturation, following which they were inseminated or analysed cytogenetically. Ooplasmic dysfunction was induced by photosensitization with a mitochondria-specific fluorescent dye. RESULTS: GV transplantation had an overall efficiency of 87 and 73% in the mouse and humans respectively. Maturation rates of 95 (mouse) and 64% (human) following reconstitution were comparable with those in control oocytes, as was the incidence of aneuploidy for five chromosome-specific probes after aneuploidy among the reconstituted oocytes. Photosensitization of oocytes significantly reduced the maturation rate to 4.2%, whereas 61.9% of oocytes matured after transfer of photosensitized GV karyoplasts into healthy ooplasts, with 52% of these mature oocytes being successfully fertilized by ICSI. Enucleated immature oocytes receiving mouse cumulus or human endometrial cell nuclei extruded a polar body in >40% of cases. Five out of seven successfully transferred aged human nuclei exhibited the expected number of signals with five chromosome-specific probes suggesting an appropriate chromosome separation in young ooplasm. CONCLUSIONS: Nuclear transplantation itself does not appear to interfere with chromosome segregation and can possibly rescue oocytes with damaged mitochondria. Finally, immature mouse ooplasm supported separation of somatic chromosomes to expected numbers, implying that haploidization may be occurring. The roles of genetic imprinting and fidelity of chromosome segregation are unknown.

Key words: aneuploidy/cell fusion/in-vitro maturation/nuclear transplantation/oocyte micromanipulation

Introduction

The chances of conception decrease inversely with age, more or less reaching a nadir as early as 40 years of age (Tietze, 1957). The reason resides primarily in the status of the egg and the conceptus rather than in the endometrium (Munné *et al.*, 1995; Dailey *et al.*, 1996), as demonstrated by higher pregnancy rates in older women receiving donor embryos. Coincidentally, the chance of generating chromosomally abnormal fetuses increases from 6.8% for women aged 35–39 years to ~50% in 45 year old women (Hassold and Chiu, 1985).

A clear relationship exists between oocyte ageing and the non-disjunction of bivalent chromosomes during meiosis (Dailey *et al.*, 1996). It has been suggested that such ageing compromises the ability of the meiotic apparatus to direct a balanced chromosome segregation (Battaglia *et al.*, 1996; Volarcik *et al.*, 1998), and this effect has been linked indirectly to a 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, mutations in mitochondrial DNA (mtDNA) have been observed in the oocytes of older women (Shigenaga *et al.*, 1994; Keefe *et al.*, 1995; Barritt *et al.*, 2000). 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; Verlinski *et al.*, 1999). Two further logical ways of avoiding oocyte aneuploidy would be cryopreservation of younger mature oocytes (van Uem *et al.*, 1987; Chen, 1988; Porcu *et al.*, 1997) or of the entire ovarian cortex taken at a younger age (Gosden *et al.*, 1994; Newton *et al.*, 1996; Oktay and Gosden, 1996; Oktay *et al.*, 1998).

It has been suggested (Zhang *et al.*, 1999) that the transfer of a germinal vesicle (GV) from an aged oocyte into a younger

4.1 Summary

As noted in Chapter 3, transplanting a GV from an aged woman's oocyte into a younger ooplasm has been proposed as a possible way to reduce the incidence of oocyte aneuploidy which is considered to be responsible for much of age-related female infertility. In this study, we have assessed the efficiency of each step involved in nuclear transplantation — cell survival, nuclear-cytoplasmic reconstitution, and the capacity of the reconstituted oocytes for *in vitro* maturation. In addition, we have evaluated the fertilizability and karyotypic status of the manipulated oocytes by ICSI and fluorescent *in situ* hybridization, respectively. Due to the limited availability of materials, most nuclear transplantation procedures were performed between sibling oocytes. Nuclear transplantation was accomplished with an overall efficiency of 73%. The maturation rate of 62% following reconstitution was comparable with that of control oocytes, as was the incidence of aneuploidy among the reconstituted oocytes. After ICSI, 77% of the reconstituted oocytes survived and 52% fertilized with a satisfactory early embryonic cleavage. Furthermore, in a limited number of observations where the nucleus of an aged oocyte was transferred into a younger ooplasm, there was an appropriate chromosomal segregation. These findings demonstrate that some human oocytes reconstituted with GV nuclei are able in principle to undergo maturation, fertilization, and early embryo cleavage, and maintain a normal ploidy. Although *in vitro* maturation seems to be a limiting step, this approach may allow us to investigate further the nuclear-ooplasmic relationship during meiotic maturation.

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aged women. However, although the selection approach might identify oocytes/embryos with a normal chromosomal content, only a limited number of embryos are then available for transfer.

In view of the key role played by cytoplasmic factors on embryonic development (Muggleton-Harris et al., 1982; Pratt and Muggleton-Harris, 1988; Van Blerkom et al., 1995a; Liu et al., 1997), a different approach has been proposed, namely the transfer of ooplasm isolated from oocytes of known developmental capacity into a MII oocyte of a woman whose embryos had previously demonstrated poor cleavage. Pregnancies have occurred after using this approach (Cohen et al., 1998a), the injection of 'fresh' ooplasm appearing to restore cytoplasmic deficiencies. This technique, however, does not correct for chromosomal abnormalities occurring as a result of non-disjunction at meiosis I.

A recent, more aggressive, approach is represented by the replacement of the entire cytoplasm. To be successful in preventing aneuploidy, this technique needs to be performed at an earlier maturational stage (Zhang et al., 1999). Nuclear transplantation entails the transfer of an isolated GV into a younger enucleated oocyte. Replacement by younger cytoplasm has been suggested as a way of ensuring normal meiotic spindle formation (Zhang et al., 1999). In the mouse, it has been demonstrated that nuclear transplantation can be accomplished efficiently, and appears not to impair subsequent oocyte maturation or increase the incidence of chromosomal abnormalities (Takeuchi et al., 1999a; 1999b).

In this study, the efficiency of transplanting nuclei isolated from human GV oocytes was assessed, in terms of oocyte survival, nuclear-cytoplasmic reconstitution, subsequent nuclear maturation, fertilizability, and early embryonic development. In addition, some reconstituted oocytes were subjected to cytogenetic analysis.

4.3 Retrieving human eggs

Immature GV oocytes were obtained from consenting patients undergoing ICSI. Oocyte retrieval was performed after pituitary desensitization with GnRHa and ovulation induction with gonadotropins as described in Chapter 2.

Immediately prior to ICSI insemination, cumulus-corona cells were removed by enzymatic and mechanical treatment (Palermo et al., 1995b; 1996a; 1996b). The denuded oocytes were then examined under an inverted microscope to assess their integrity and stage of nuclear maturation. In this study, only excess GV stage oocytes of patients undergoing ICSI procedure for male factor infertility were used.

4.4 Transplantation of GV nuclei to human GV oocytes

The zona pellucida was breached by a glass microneedle tangentially inserted into the perivitelline space, against the holding pipette. Then, the GV surrounded by a small amount of cytoplasm (GV karyoplast) was directly removed by a cylindrical micropipette with a 30 μm inner diameter (Figure 4.1a). Oocytes were exposed to HEPES-HTF-HSA containing 5 μg/ml of cytochalasin B (CCB; Sigma Chemical, St Louis, MO, USA) for at least 5 min prior to and throughout the procedure. The isolated GV karyoplast was immediately inserted with the same tool into the perivitelline space of a previously enucleated oocyte at the same maturational stage (GV cytoplast), so forming a 'grafted oocyte' (Figure 4.1b).

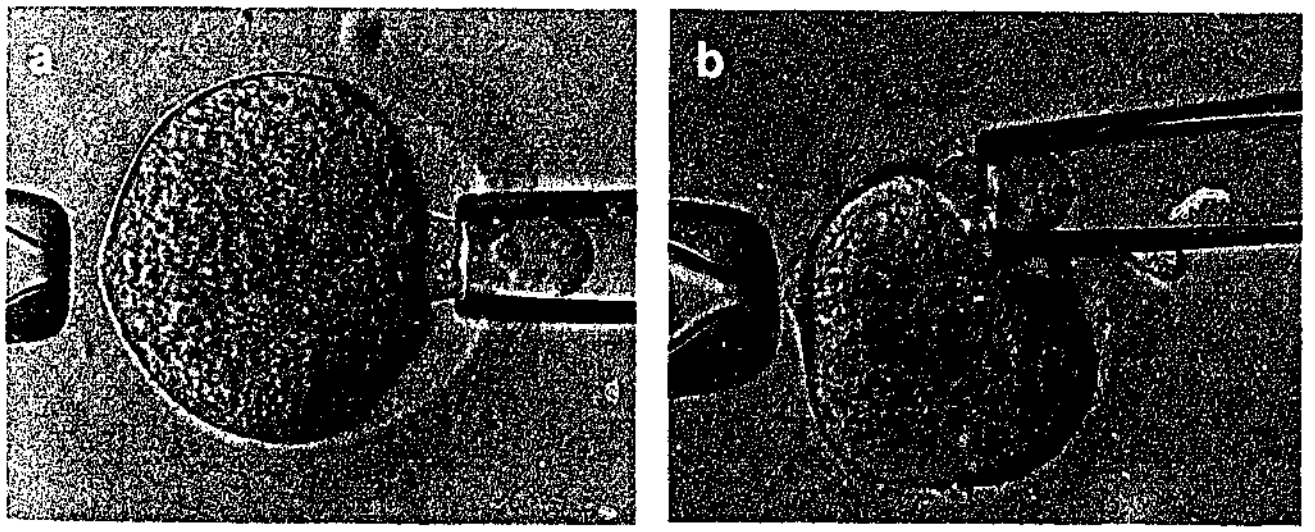


Figure 4.1 (a) Removal of the nucleus surrounded by a small amount of ooplasm from a human germinal vesicle stage oocyte (400X). **(b)** Transfer of an isolated germinal vesicle karyoplast into an enucleated oocyte (400X).

After insertion of the karyoplast, grafted oocytes (couplets of GV karyoplast and cytoplast) were washed in CCB-free medium, then incubated in HTF-HSA at 37°C in 5% CO_2 in air for 30 min to recover before further procedures.

An Electro Cell Manipulator (BTX 200 and 2001; Genetronics, Inc., San Diego, CA, USA) was used to deliver electrical pulses. Each grafted oocyte was placed between two micro-electrodes (ECF-100; Tokyo Rikakikai Co. Ltd, Tokyo, Japan) and manually aligned to place the karyoplast-cytoplast couplet perpendicular to the axis of the electrodes under micromanipulation control (Figure 4.2a). The diameter of the tip of each micro-electrode was 100 µm. To induce fusion, a single 1.0 – 1.5 kV/cm direct current fusion pulse in HEPES-HTF-HSA was delivered for 70 – 100 µs at 37°C. Fused oocytes were then washed and cultured in HTF-HSA, and observed 30 min later to confirm cell survival and signs of fusion (Figure 4.2b). Where fusion did not at occur, up to four electrical pulses were applied at 30 min intervals.

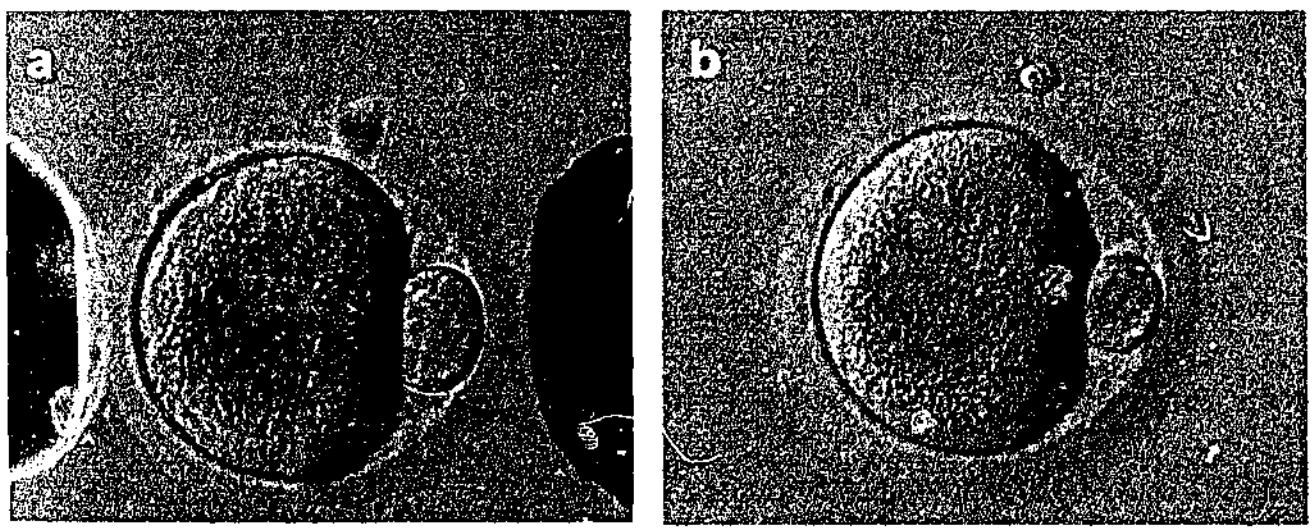


Figure 4.2 (a) A grafted oocyte (couplet of a karyoplast and a cytoplast) aligned between two microelectrodes (400X). (b) Beginning of fusion in a grafted oocyte 30 min after electrofusion (400X).

Immature oocytes were allowed to mature in culture (HTF-HSA), and were monitored at 24 and 48 hours after electrofusion to evaluate nuclear maturation, characterized by GVBD and subsequent extrusion of the first PB. Control GV oocytes that were not manipulated were cultured in the same conditions.

4.5 FISH analysis of reconstituted, *in vitro* matured, oocytes

Some oocytes that had extruded the first PB were prepared for chromosome analysis by gradual fixation as previously described in Section 2.5.10. Slides were then assessed immediately in a phase contrast microscope for presence of nuclei, dehydrated for 2 min each in increasing concentrations of ethanol (70, 85, and 100%) and either analyzed immediately or stored at -20°C.

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 phosphate-buffered detergent (PBD; Oncor, Gaithersburg, MD, USA). Then the slides were sequentially dehydrated in increasing concentrations of ethanol (70, 85, and 100%), rinsed and denatured in a solution of 70% formamide in (2X) SSC at 70°C for 5 min, then dehydrated in sequential steps of 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 analyze the corresponding chromosomes. All chromosome probes were purchased from the same manufacturer (Vysis Inc.).

Slides were viewed under a fluorescent microscope 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 analyzed with an imaging software (Cytovision; Applied Imaging Corp., Santa Clara, CA.) (Figure 4.3). Scoring criteria were as previously described (Munné et al., 1995b; Dailey et al., 1996; Palermo et al., 1997b).

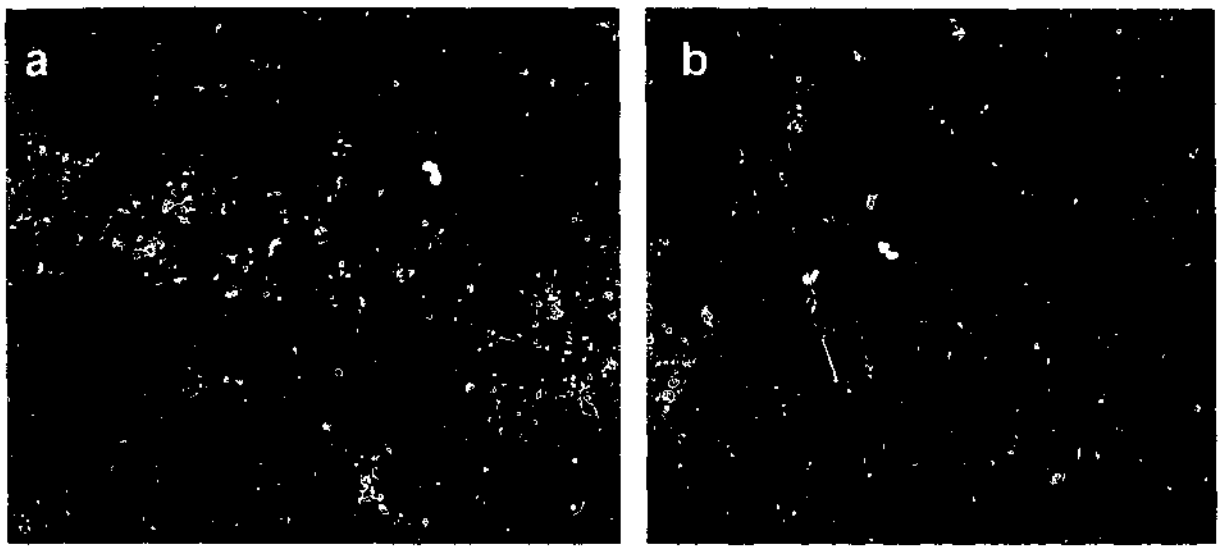


Figure 4.3 Fluorescent *in situ* hybridization (FISH) analysis of a metaphase II oocyte reconstituted by nuclear transplantation. **(a)** First FISH round – two red fluorescent signals representing the chromatids of chromosome 14, two green signals for chromatids of chromosome 22, and two aqua signals for chromatids of chromosome 16, and two yellow signals for chromatids of chromosome 15. **(b)** Second FISH round – two green fluorescent signals representing the chromatids of chromosome 13, two red signals for the chromatids of chromosome 21, two yellow signals for the chromatids of chromosome X, and two aqua signals for the chromatids of chromosome 18.

4.6 Fertilization assay

Some of the reconstituted *in vitro* matured oocytes were injected with a single spermatozoon (Palermo et al., 1995a; 1996b), in the majority of cases 2 – 3 hours after observation of the first PB extrusion. Approximately 16 – 20 hours after ICSI, the oocytes were examined for the presence of two distinct pronuclei and two PB. Fertilized oocytes were then incubated further in HTF-HSA medium, for up to 72 hours to evaluate their developmental capacity. The day 3 observation period was chosen according to our routine ICSI-embryo transfer protocol.

4.7 Reconstitution of oocytes after GV transplantation

A total of 197 GV human oocytes was obtained from 81 patients (mean age of 34.3 ± 4 years, range 25 – 43). Due to the limited availability of this human material, in most cases enucleated GVs were exchanged between sibling oocytes. In a preliminary experiment, nine GV oocytes which had arrested for at least 24 hour were subjected to nuclear transplantation. Seven of these nine were successfully enucleated (77.8%) and then their GVs grafted into five oocytes (71.4%). Although subsequent electrofusion generated five reconstituted oocytes (100%), as expected none extruded the first PB. This preliminary experiment led us to improve the enucleation technique by increasing the inner diameter of enucleation pipettes, from 20 to 30 μm, and by adopting a gentler aspiration to avoid rupture of the nuclear membrane. While we were aware of the importance of a control group, the majority of the available GV stage oocytes were used for nuclear transplantation, in order to maximize the information obtained from this aggressive technique. The results of nuclear transplantation of 158 freshly retrieved oocytes are shown in Table 4-I.

Table 4-I. Efficiency of nuclear transplantation of human germinal vesicle oocytes

	No. of oocytes (%)
a. Intact GV	158
b. Dissected and enucleated (% of a)	151 (95.6)
c. Grafted and electrofused (% of b)	141 (93.4)
d. Reconstituted (% of c)	115 (81.6)
e. Matured to metaphase II (% of d)	71 (61.7)

The overall incidence of reconstitution was 72.8% (115/158), and among 115 reconstituted oocytes, 71 matured spontaneously and extruded the first PB within 48 hour in HTF-HSA. The remaining 44 oocytes remained at the GV (13/115, 11.3%) or metaphase I stage (31/115, 27.0%). Among the GV stage control oocytes simply cultured *in vitro*, and not subject to either micromanipulation or electrostimulation, 19 of 30 (63.3%) matured as far as MII. Thus, the maturation rate of the reconstituted oocytes was not significantly different from the controls. After electrofusion, 26 out of 141 grafted oocytes failed to reconstitute.

4.8 Cytogenetic analysis of reconstituted oocytes

The average donor age for the oocytes analyzed cytogenetically was 32.4 ± 3 years (range 28 – 42). Among the 38 oocytes (21 reconstituted and 17 control oocytes) were processed for FISH. One (2.6%) was not analyzable because of a poor chromosomal spread, and FISH errors were evident in three slides (7.9%), as reflected in discrepancies of FISH signals between the oocyte

and the corresponding PB. Therefore, the overall efficiency of this FISH method was 89.5%. The karyotypes of the reconstituted oocytes generated by exchanging GVs between sibling oocytes are shown in Table 4-II. Normal complements of X, 13, 14, 15, 16, 18, 21, and 22 were observed in 11 of 14 oocytes, with three having abnormalities (21.4%). Non-disjunction of a bivalent chromosome 13 was observed in one oocyte and another showed unbalanced predivision of chromatid 18 and balanced predivision of chromatid X, while a third was diagnosed as unbalanced predivision of chromatid 16 and X, and balanced predivision of chromatid 21. Since only 11 out of 15 (73.3%) oocytes showed normal karyotypes in the control group (the average maternal age 33.7 ± 4, range 28 – 42 years), there was no significant difference in the chromosomal status of the two groups.

Table 4-II. Incidence of chromosomal abnormalities in sibling oocytes subjected to nuclear transplantation

	No. of oocytes (%)
A. Processed for FISH	16
B. Analyzed (% of A)	14 (87.5)
C. Normal karyotype (% of B)	11 (78.6)
D. Aneuploidy (% of B)	3 (21.4)
Predivision of monovalents (% of B)	2 (14.3)
Non-disjunction of univalents (% of B)	1 (7.1)

FISH = fluorescent *in situ* hybridization

In order to assess the contribution of the cytoplasmic replacement, 30 GV karyoplasts generated from 15 'aged' (37 – 43 years) and from 15 'young' (28 – 32 years) oocytes were reciprocally transplanted into cytoplasts of the opposite age (Figure 4.4a and b). A total of 24 out of the 30 oocytes (80.0%) successfully reconstituted and 19 (63.3%) extruded a first PB. Among 7 young

ooplasts (29.8 ± 2 years, mean ± SD) receiving an aged karyoplast (40.0 ± 2 years), 5 displayed a normal karyotype, whereas 9 of 12 young karyoplasts (31.4 ± 2 years) maturing in older ooplasm (38.1 ± 3 years) were abnormal.

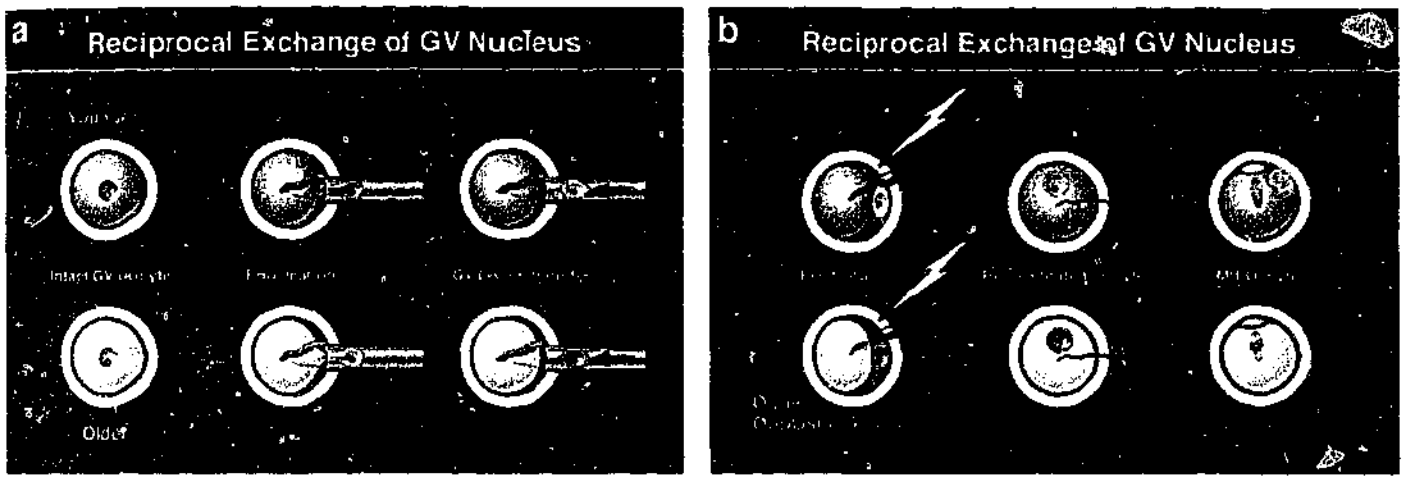


Figure 4.4 Cross-transplantation experiment: **(a)** Simultaneous isolation of germinal vesicle (GV) karyoplasts from primary oocytes obtained from women of different ages. An isolated karyoplast from an older oocyte (pink) was grafted into an enucleated younger oocyte (green), and vice versa. **(b)** Each grafted oocyte was then exposed to an electrical pulse to induce cell fusion and cultured to allow maturation. MII = metaphase II.

4.9 ICSI of reconstituted oocytes and their early embryonic development

Reconstituted MII oocytes (n = 31) subjected to ICSI were assessed for fertilization and subsequent development (Table 4-III). The average maternal age of this experimental group was 33.2 ± 4 years. In an initial study, the oolemma was pierced by an ICSI needle inserted through the area previously used for nuclear removal and cell fusion; only five of nine oocytes survived (56%) and only one was fertilized (11%). In a later series of 22 oocytes, the sperm injection avoided the area where cell fusion had probably occurred, and both survival and fertilization rates

were much higher, 86% (19/22) and 68% (15/22), respectively. The fertilized oocytes with 2PN were cultured until day 3 when 93.8% (15/16) had cleaved with a mean blastomere number of 4.9 ± 2 (mean ± SD) and an average fragmentation rate of 25.0 ± 15 (mean % ± SD). Of two embryos cultured until day 5, one arrested at 8-cells with the other reached the compacted morula stage.

Table 4-III. Fertilization outcome after intracytoplasmic sperm injection on reconstituted oocytes

	No. of oocytes (%)
a. Injected	31
b. Survived (% of a)	24 (77.4)
2PN (% of b)	16 (66.7)
1PN (% of b)	1 (4.2)
3PN (% of b)	1 (4.2)

PN = pronucleate

4.10 Discussion

Transplantation of nuclei into immature human oocytes can be accomplished efficiently, at a rate of > 80% for each step. Since the maturation rate of the manipulated oocytes (62%) was similar to that in the non-manipulated controls, *in vitro* maturation (IVM) appear to be the limiting step for a successful nuclear transplantation procedure. In previous investigations of IVM of cumulus-free GV oocytes isolated from stimulated ovaries, up to 38% matured during culture in simple defined media with or without serum (Janssenswillen et al., 1995; Goud et al., 1998).

No GV karyoplasts which had failed to reconstitute underwent GVBD during 24 hour incubation. This is in agreement with previous reports in the mouse that demonstrated an inverse relationship between spontaneous nuclear maturation and cytoplasmic volume (Kamikova et al., 1998; Takeuchi et al., 1999a; 1999c).

The incidence of chromosomal aneuploidy appears to be 25 – 35% in human oocytes matured *in vivo* (Plachot et al., 1988; Pellestor, 1991; Van Blerkom, 1994), and recently an incidence of aneuploidy of 25% was reported for cumulus-enclosed immature oocytes cultured *in vitro* in a specific medium supplemented with gonadotropins (Parl: et al., 1997). Our cytogenetic evaluation indicates that nuclear transplantation itself does not increase the incidence of aneuploidy after IVM compared to that in control oocytes. Although this observation includes only a limited number of cases, it indicates that younger ooplasm can support normal meiotic division of an older GV, and, conversely, that older ooplasm tends to induce an abnormal segregation of meiotic chromosomes. This finding agrees with an earlier report in which younger ooplasm supported normal meiotic divisions in 80% (four out of five) of the transplanted older GV nuclei (Zhang et al., 1999).

I have demonstrated that oocytes reconstituted by nuclear transplantation can mature *in vitro* and be normally fertilized after ICSI. Although their oocyte survival and the embryo quality may appear to be poor, nevertheless these endpoints and the patterns of fertilization were not different from those obtained using oocytes maturing *in vivo* (Spandorfer et al., 1998). The poor embryonic development observed in this study may have been due to suboptimal culture

conditions utilized for IVM. It should also be noted that all the oocytes were exposed to hCG administered *in vivo* before collection, and were denuded of corona cells prior to GVBD; therefore, they were not isolated and cultured in an ideal setting for IVM (Janssenswillen et al., 1995; Goud et al., 1998) and in fact, only occasional deliveries from such immature oocytes have been reported so far (Trounson et al., 1994; 2001; Nagy et al., 1996; Edirisinghe et al., 1997). On the other hand, isolation of immature oocytes prior to allowing GVBD with cumulus cells in place and avoiding exposure to LH has been associated with a more promising outcome (Lin et al., 2003; Chian et al., 2004). While IVM of oocytes shows promise, cumulus cells must be removed in order to perform nuclear transplantation. On the other hand, the cumulus has been shown to benefit oocyte maturation and even oocyte/cumulus co-culture does not overcome the effect of its premature removal (de Loos et al., 1991; Allworth and Albertini, 1993). Thus, IVM remains a challenge (Trounson et al., 1994; 1998) with more information needed as to the relationship between oocytes and their investing of cumulus during the resumption of meiosis.

The effect of the electrofusion procedure on further development of the reconstituted oocytes must be evaluated further, since it has been suggested that the developmental capacity of reconstituted oocytes is impaired by electrofusion (Cohen et al., 1998a). However, it has been recently reported that electrical stimulation had a beneficial effect in inducing oocyte activation and resulted in successful births in patients whose oocytes failed to fertilize after ICSI (Yanagida et al., 1999). In this study, microelectrodes controlled by a micromanipulator that allowed the avoidance of using alternating current for cell alignment, therefore, minimizing any unpredictable

and undesirable harmful effects during the electrofusion step (Rickords and White, 1992; Takeuchi et al., 1999a).

In summary, our findings provide further support for current hypotheses that ooplasmic factors are finally responsible for the equal distribution of bivalent chromosomes between the ooplasm and the first PB during the completion of meiosis I. An explanation for this may lie in damage to mitochondrial function by reactive oxygen species, with consequent reduction in ATP levels (Tarín, 1995; Tarín et al., 1998). Another possibility may be a compromised perifollicular microcirculation attributed to an age-related follicular hypoxia that would induce a lower intracellular pH (pHi) (Gaulden, 1992; Van Blerkom et al., 1997), compromising the meiotic spindle of maturing oocytes. The paucity of human oocytes, for inter-patient nuclear transplantations, makes it difficult to draw definite conclusions in this regard. Nonetheless, our nuclear transplantation technique is highly effective, reconstituting hybrid immature human oocytes at a rate of 73% with a subsequent maturation rate of ~ 62%. Even though the limiting maturational step might be improved by better culture conditions, again the small number of oocytes generally obtainable from women of > 40 years remains another limiting factor, as proven by the number available for cross-exchange in the present study. Other aspects of nuclear transplantation that also need to be evaluated include the role of mitochondrial DNA of donor/recipient oocytes for the development and phenotype of the resulting embryos (Tsai et al., 1999), as well as the availability and viability of donor ooplasm.

Chapter 5

Nuclear Transplantation Can Overcome The Consequences of Mitochondrial Damage in Mammalian Oocytes

Declaration for Thesis Chapter 5

In the case of Chapter 5, contributions to the work involved the following:

	Name	% contribution	Nature of contribution
1	**Gianpiero D. Palermo**	50	Conception and design, collection, analysis, and writing of manuscript
2	**Takumi Takeuchi**	20	Execution of work, collection and data analysis
3	**Ricciarda Rafaelli**	15	Execution of work
4	**E. Scott Sills**	15	Manuscript editing and data analysis

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Weill Medical College of Cornell University, The Center for Reproductive Medicine, New York, NY, USA

Signature 1 [redacted] **Date** 10-29-2004

Signature 2 **Date** 11-19-2004

Signature 3 **Date** 11-22-2004

Signature 4 **Date** 11-22-2004

5.1 Summary

Germinal vesicle transplantation (GVT) has been proposed as a possible treatment through which to correct age-related oocyte aneuploidy caused by dysfunctional ooplasm. How healthy ooplasm regulates normal meiosis and subsequent development has yet to be elucidated, but impaired mitochondrial metabolism may be attributable to incomplete segregation of the oocyte chromosomes. In this study, after ooplasmic mitochondrial damage by photoirradiating chloromethyl-X-rosamine, examination of the oocyte nuclei's ability to survive after transfer into healthy ooplasts was carried out. To assess their fertilizability and potential for development, GVT oocytes were fertilized by ICSI and transferred to foster mice.

Photosensitization consistently inhibited oocyte maturation. However, after GVT of photosensitized nuclei into healthy ooplasts, 67.2% were reconstituted and 76.2% of these matured normally, with an over all rate of 51.2%, much higher than that (6.0%) in the mitochondrially-injured oocytes. After ICSI, 65.8% of GVT oocytes were fertilized normally, and 21.1% eventually reached the blastocyst stage. The transfer of 132 two-cell GVT embryos into the oviducts of pseudopregnant females, resulted in 17 apparently healthy live offspring. Thus, one can induce in oocyte mitochondria a photosensitization-based type of damage which consistently inhibits GV breakdown, meiotic spindle formation, chromosomal segregation, and PB extrusion. Germinal vesicle transplanted and 'rescued' (GVTr) oocytes were able to undergo maturation, fertilization, embryonic cleavage, and ultimately develop to term. This approach may provide a model in which to study age-related ooplasmic dysfunction seen in human oocytes.

5.2 Introduction

As noted in Chapter 3, the fecundity of women is negatively affected by maternal age, due to a higher incidence of oocyte aneuploidy (Hassold and Chiu, 1985; Munné et al., 1995c; Dailey et al., 1996) which appears during the first meiotic division, as a result of predivision or non-disjunction of chromatids (Plachot, 2003). The segregation of meiotic chromosomes/chromatids depends on their correct binding to the fibers of the meiotic spindle (Eichenlaub-Ritter et al., 2003). While the dynamics of spindle function remain unclear, the polymerization of spindle microtubules appears to depend on ATP (Van Blerkom and Runner, 1984; Van Blerkom, 1991). The major organelles responsible for ATP production are mitochondria, which are distributed in particular patterns during the maturational stages and early zygote development (Van Blerkom and Runner, 1984; Calarco, 1995; Van Blerkom, 2000). Mitochondrial DNA may undergo point mutations and deletions, and may replicate abnormally or more importantly experience the effects of an underproduction of ATP, resulting in respiratory chain defects (Wallace, 1999). As in other cell types mutations in mitochondrial DNA accumulate with age (Corral-Debrinski et al., 1992; Michikawa et al., 1999; Shigenaga et al., 1994) and a similar situation has been encountered in aged oocytes and their surrounding granulosa cells (Keefe et al., 1995; Schon et al., 2000; Seifer et al., 2002). In accord with this 'mitochondrial aging' hypothesis (Schon et al., 2000), it has been demonstrated that a lower mitochondrial membrane potential is associated with an age-related

higher frequency of abnormal meiotic spindle formation in mature human oocytes (Battaglia et al., 1996; Volarcik et al., 1998; Wilding et al., 2003).

In aged ovaries, it is possible that cytoplasmic damage can be induced by abnormalities of the microcirculation (Van Blerkom et al., 1997). As serial ovulations and follicular atresia induce scarring of the ovarian cortex, this is progressively substituted by connective tissue, thereby creating a greater distance between the follicular content and the perifollicular capillary bed (Gaulden, 1992; Vaskivuo and Tapanainen, 2003). In this situation, both follicular hypoxia and a related production of oxygen free radicals have both been linked to the ooplasmic damage observed with age (Tarín, 1995; Tarín et al., 1996; Van Blerkom, 2000). GVT has been reported to prevent or treat the age-related cytoplasmic dysfunction responsible for oocyte aneuploidy (Zhang et al., 1999; Takeuchi et al., 1999a; 2001a), but there has been no experimental model of mitochondrial damage directly linked to chromosomal imbalance that is analogous to the situation in humans.

In addition to oocyte aging, oxidizing agents and, at high dosages, drugs such as chloramphenicol, (a potent inhibitor of mitochondrial peptidyl transferase) (Beermann and Hansmann, 1986), or diazepam (a tranquilizer, anticonvulsant) can disturb the spatio-temporal distribution of mitochondria during oocyte maturation. Binding of the latter drug to peripheral-type benzodiazepine receptors on the mitochondrial membrane may affect the availability of ATP and calcium homeostasis (Sun et al., 2001), ultimately leading to aneuploidy (Van Blerkom et al., 1995b; Van Blerkom, 2000; Tarín et al., 1996; 1998). Another drug, chloromethyl-X-rosamine

(CMXRos), a mitochondrion-specific fluorescent probe, has been recently shown to photosensitize living mitochondria after treated cells are excited by ionizing radiation (Minamikawa et al., 1999). In that connection, we have proposed mitochondrial photo-induced damage as a method through which to provoke ooplasmic dysfunction leading to aneuploidies similar to those observed with age (Palermo et al., 2002a).

In the present study, the effect of CMXRos-mediated mitochondrial photosensitization on the functional state of the ooplasm as monitored by the normal completion of the first meiotic division was investigated. To confirm mitochondrial damage, confocal imaging and ultrastructural analysis were performed on random oocytes. In an attempt to restore the ability to mature normally, GV nuclei from treated oocytes were transplanted into healthy enucleated cytoplasts. Karyotyping was performed to control for any possible effects of the micromanipulation procedures involved. Manipulated oocytes were then fertilized by ICSI, cultured to the blastocyst stage, and the 'rescued' embryos transferred to pseudopregnant mice.

5.3 Collection of gamete

Mouse oocytes and spermatozoa were obtained from 7 – 11 week B6D2F1 strain mice as described in Chapter 2. Oocytes were first cultured in Waymouth's medium (MB752/1; Invitrogen, Carlsbad, CA, USA) supplemented with a phosphodiesterase inhibitor (0.2 mM 3-

isobutyl-1-methylxanthine, IBMX; Sigma Chemical Co., St. Louis, MO, USA), 0.23 μM sodium pyruvic acid (Sigma Chemical Co.), and 5% fetal bovine serum (FBS, Invitrogen).

5.4 Induction of mitochondrial damage

Oocytes were cultured in Waymouth-IBMX containing 500 nM of CMXRos (MitoTracker® Red; Molecular Probes, Eugene, OR, USA) for approximately 30 min, rinsed (x3) in Waymouth-IBMX and exposed to epifluorescent illumination for 10 seconds. Excitation of the fluorochrome was performed on an inverted microscope (Olympus IX-70; New York/New Jersey Scientific Inc., Middlebush, NJ, USA) equipped with an epifluorescent attachment (100W mercury burner) with a Texas Red filter (excitation wave length 560/55 nm, emission wave length 645/75 nm) at 200X magnification. Control oocytes were exposed either to CMXRos alone or to photoirradiation alone.

5.5 Function, distribution patterns, and morphology of damaged mitochondria

To assess any implied changes in the membrane potential and distribution patterns of the mitochondria, fluorescent patterns in photosensitized oocytes were studied under an epifluorescent or a confocal laser scanning microscope (LSM510; Zeiss, Oberkochen, Germany).

Mitochondrial damage typical of the initial stage of cell apoptosis was characterized by more dilated, aggregated, and less homogeneously distributed fluorescent signals (Minamikawa et al., 1999).

In order to confirm the conclusions based on fluorescent signals, the morphology and distribution pattern of the mitochondria photosensitized in eggs were assessed by transmission electron microscopy (TEM). Two hours following their photosensitization, a group of GV oocytes was fixed for one hour in 2% glutaraldehyde in 0.1 M cacodylate buffer at room temperature and overnight at 4°C, rinsed in buffer, post-fixed in 2% osmium tetroxide for one hour, dehydrated through increasing concentrations of ethanol up to 100%, and embedded in Spurr's resin. Thick plastic sections were cut and stained with toluidine blue in borate buffer. Ultrathin sections stained with uranyl acetate and lead citrate, were studied in a JEOL 100S transmission electronmicroscope (Takeuchi et al., 2004a).

5.6 GV transplantation and oocyte maturation *in vitro*

The micromanipulation and electrofusion procedures were carried out in a plastic petri dish on the heated stage of an inverted microscope equipped with hydraulic micromanipulators (Model MM-188 and MO-109; Narishige USA Inc. East Meadow NY), and microinjectors (Model IM-6 and SRY-15; Narishige USA Inc.), in a dim light (Takeuchi et al., 1999a; 2001a). GV transplantation was performed after ~ 2 hours of photoirradiation. Then, GV karyoplasts were

transferred into enucleated oocytes in combinations where **A)** neither had been photosensitized (control), or **B)** only the karyoplast or **C)** only the ooplast had been photosensitized (Figure 5.1a,b and 5.2a,b) (see Table 5-II).

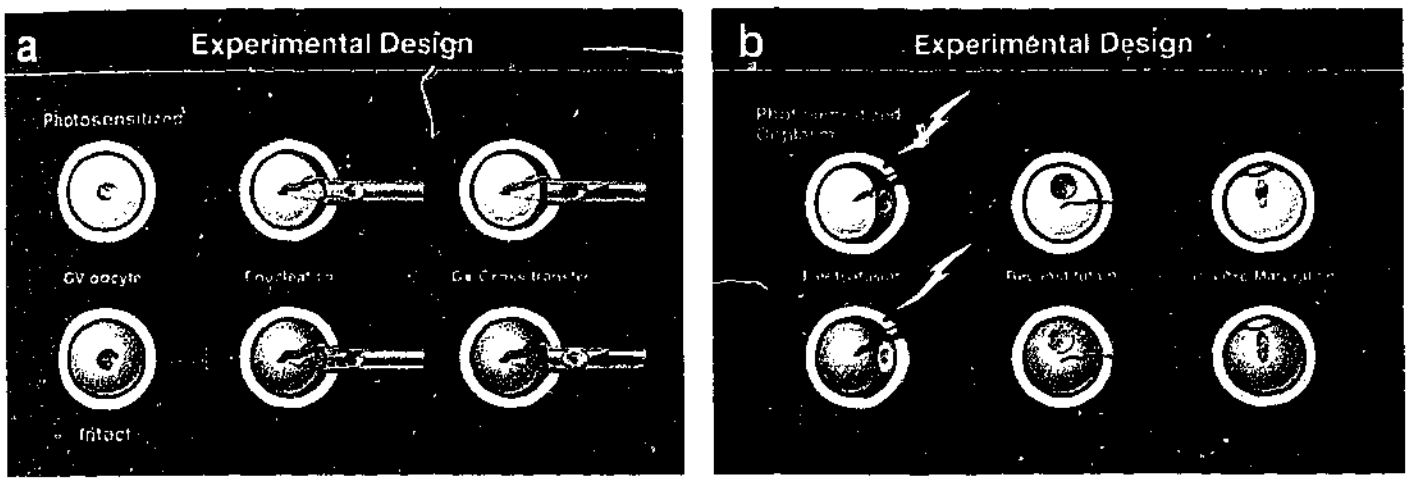


Figure 5.1 Reciprocal exchange of nuclei: **(a)** Germinal vesicle karyoplasts from photosensitized and intact oocytes were reciprocally cross-transferred to the corresponding ooplast. **(b)** The grafted oocyte during reconstitution and after 14-16 hours *in vitro* culture.

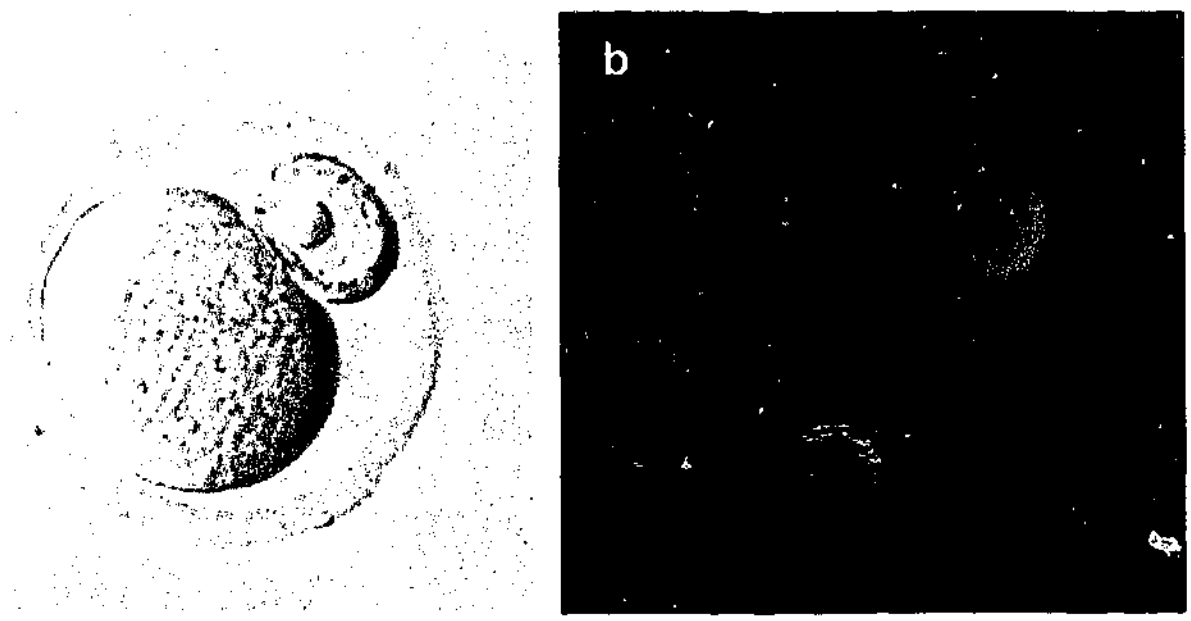


Figure 5.2 A grafted oocyte consisting of a photosensitized karyoplast and an intact cytoplast (a) bright field and (b) fluorescence microscopy (400X).

Successfully reconstituted oocytes were rinsed and cultured in IBMX-free Waymouth's medium for 14 – 16 hours to allow nuclear maturation, judged according to the extrusion of a defined polar body. Some of the oocytes that had matured were processed for karyotyping by staining chromosome spreads with Giemsa, as previously described (Takeuchi et al., 1999b).

5.7 Oocyte development and transfer

Oocytes matured *in vitro* with the different treatment combinations of karyoplast + cytoplast (see Table 5-II, **B** and **C**) were subjected to piezo-ICSI using isolated sperm heads, then cultured for 96 hours in KSOM medium in order to assess fertilization rates and embryo development (Takeuchi et al., 2004b). Oocytes that underwent *in vitro* maturation and non-manipulated after removal of surrounding cumulus cells served as controls. Two-cell stage embryos after rescue germinal vesicle transplantation (GVTr) were surgically transferred via a lateral back body wall incision into oviducts of CD-1 foster mothers mated with a vasectomized male of the same strain (Takeuchi et al., 2004b). In addition, some two-cell stage ICSI embryos derived from oocytes matured *in vitro* were transferred to pseudo-pregnant females, as controls.

5.8 Data analysis

The differences in oocyte survival, reconstitution, and maturation rates were assessed by the χ^2 test. A two-tailed test was used to assess significant levels, considered at 5% probability, and reported only when reached. All statistical computations were conducted using StatView 512+™ (BrainPower Inc., Calabasas, CA, USA).

5.9 Influence of photosensitization on membrane potential, morphology, and distribution patterns of oocyte mitochondria

As seen in the fluorescent and in the confocal scanning laser microscopes, two hours after photoirradiation, 49 of 51 photosensitized GV oocytes displayed clustering and swelling of their mitochondria, and a reduced intensity of fluorescent signals (Figure 5.3B2). These mitochondrial features were confirmed in ultrastructural analysis of photosensitized oocytes arrested at the GV stage, with more aggregated or dilated/swollen mitochondria in the photosensitized oocytes than in the controls (Figure 5.3C2). Some of the photosensitized oocytes displayed blebbing (Figure 5.3A2)of the plasma membrane or even degeneration (22% and 2.4%, respectively) during the 14 – 16 hours of the *in vitro* culture period.

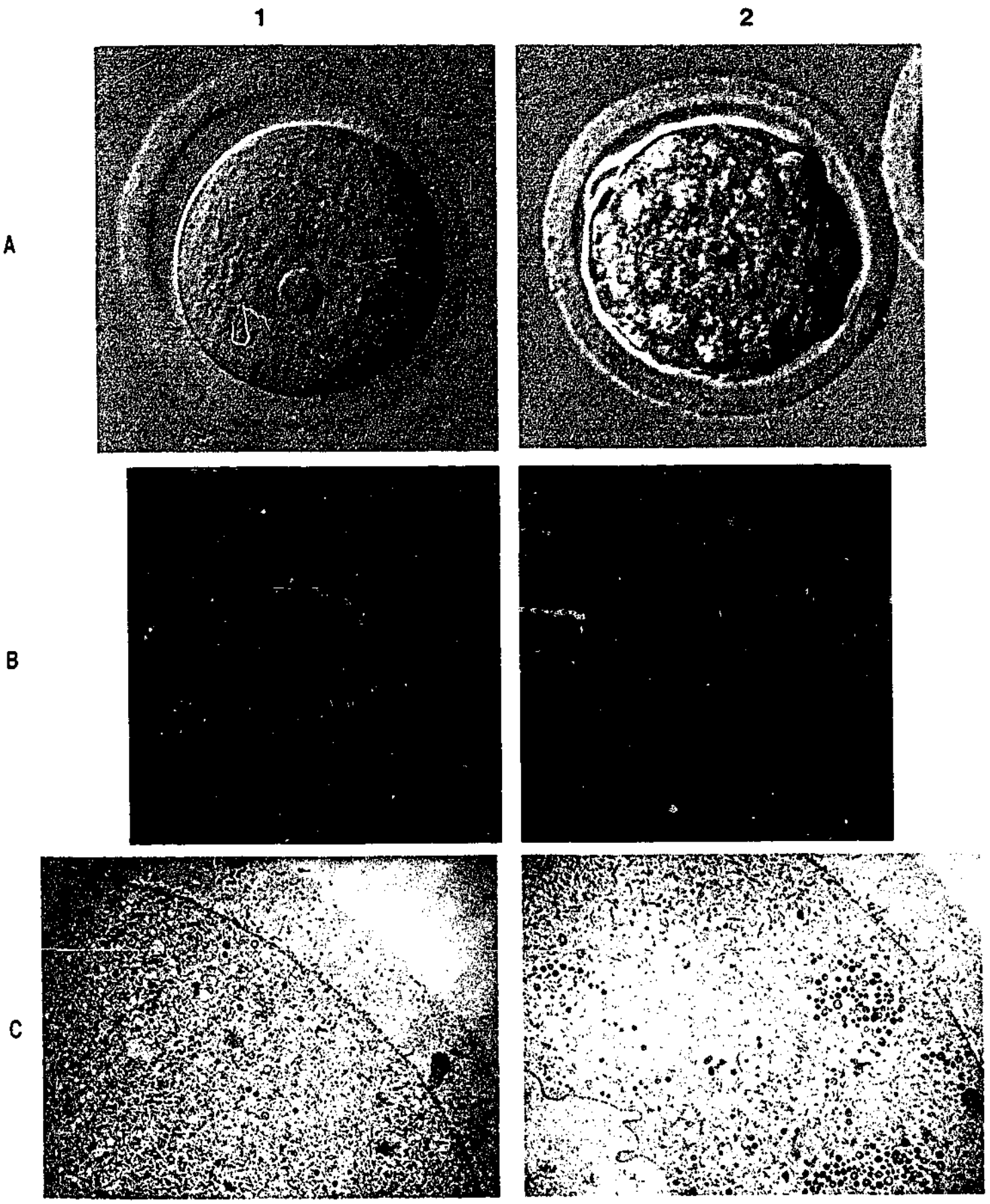
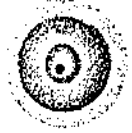


Figure 5.3 Intact germinal vesicle oocyte after MitoTracker loading (column **1**) and after photoirradiation (column **2**) depicted by different imaging techniques, bright field (row **A**), laser confocal (row **B**), and TEM (row **C**). The cellular damage can be demonstrated by the membrane blebbing (**A2**), while at the organelle level, swelling and clustering of mitochondria induced by photosensitization are visible (**B2, C2**).

5.10 The effect of mitochondrial photosensitization on oocytes maturation *in vitro*

Mitochondrial photosensitization interfered significantly ($P < 0.001$) with the oocyte maturation process. Neither exposure to CMXRos alone nor irradiation alone affected *in vitro* maturation rates, whereas photosensitization significantly inhibited oocyte maturation (Table 5-I). While 86% of intact controls matured to the MII stage, only 6% of the experimental group (CMXRos loaded and photoirradiated) did so after photosensitization.

Table 5-I. Effect of CMXRos loading and/or photoirradiation on oocyte maturation

	Treatments	No. of (%) GV oocytes	No. of (%) Oocytes matured to MII
	Intact GV	132	113 (85.6)[a]
	CMXRos loading	88	74 (84.1)[a]
	Photoirradiation	94	80 (85.1)[a]
	CMXRos loaded and photoirradiated	199	12 (6.0)[b]

[a vs b] χ^2, 2 x 4, 3 *df*, Effect of photosensitization on nuclear maturation, $P = 0.0001$

5.11 *In vitro* maturation of the reconstituted oocytes following GV transplantation

As described in Table 5-II, of the oocytes receiving non-photosensitized GVs **(A)**, 85% (45/53) survived with 95% (43/45) of those maturing normally. By contrast, when oocyte reconstitution involved a photosensitized karyoplast **(B)** or a photosensitized ooplast **(C)**, most showed extensive cell lysis after electrofusion ($P < 0.01$) (Table 5-II). Oocytes successfully reconstituted by transfer of a photosensitized karyoplast to an intact ooplast (GVTr) **(B)** underwent maturation to MII, as reflected by extrusion of the first polar body, and at a significantly higher rate than that of oocytes reconstituted with photosensitized ooplasts **(C)** (76.2% of 299 and 20.9% of 43 of the reconstituted oocytes, respectively). When photosensitized karyoplasts were transplanted into non-irradiated ooplasts the maturation rate was significantly greater than that in intact photosensitized oocytes ($P < 0.001$) (Table 5-II). Cytogenetic analysis was performed successfully in 21 of 33 reconstituted ('rescued') oocytes that had matured *in vitro*. All but one (20 of 21) had a normal haploid chromosomal complement (Figure 5.4).

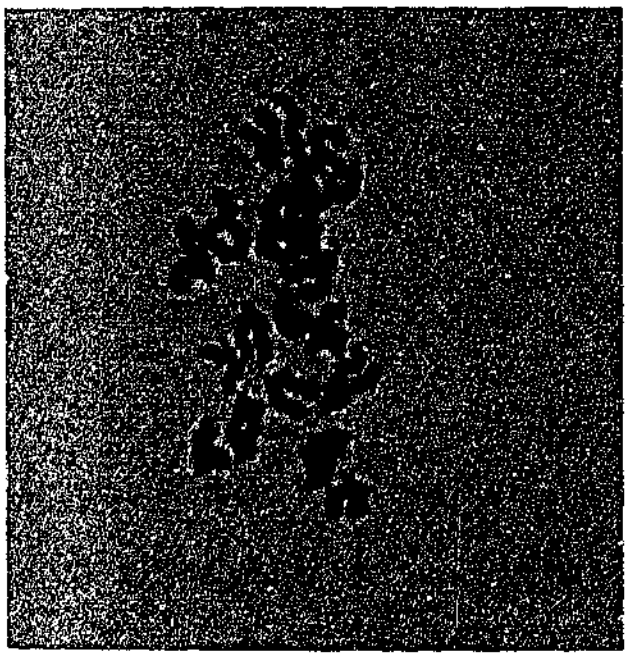


Figure 5.4 A normal haploid set of chromosomes from a 'rescued' *in vitro* matured oocyte (original magnification 1000X).

Table 5-II. Ability of ooplasm with functional/dysfunctional mitochondria to support nuclear maturation *in vitro*

Combinations of Karyoplast + Cytoplast	No. of (%) GV transplanted	Reconstituted	Matured to MII
A 	53	45 (84.9)[a]	43 (81.1)[c]
B 	445	299 (67.2)[b]	228 (51.2)[d]
C 	123	43 (34.5)[b]	9 (7.3)[e]

[a vs b] χ^2, 2 x 3, 2 *df*, Effect of photosensitization on reconstitution rates, $P < 0.001$

[c vs d vs e] χ^2, 2 x 3, 2 *df*, Effect of photosensitization on nuclear maturation rates, $P < 0.001$

 non-photosensitized photosensitized

5.12 Fertilization and embryonic cleavage of the reconstituted oocytes

The rates of fertilization after ICSI, and the *in vitro* development of mitochondrially-impaired oocytes up to 96 hours — intact, photosensitized, and after GVTr — are shown in Table 5-III. In the GVTr group, 65.8% were fertilized normally by ICSI and 13.9% developed as blastocysts. The rescued oocytes developed at a rate similar to the non-treated oocytes matured *in vitro*, while the few zygotes derived from non-manipulated *in vitro* matured photosensitized oocytes, did not develop.

Table 5-III. Embryo development of cumulus-denuded *in vitro* matured, photosensitized, and germinal vesicle transplanted 'rescued' oocytes

	Origin of oocytes		
No. of oocytes (%)	Intact *in vitro* matured	Photosensitized	GVTr
Inseminated	62	12	79
Fertilized	40 (64.5)[a]	2 (16.7)[b]	52 (65.8)[c]
2-cell stage	37 (59.7)[d]	2 (16.7)[e]	51 (64.6)[f]
Blastocyst	14 (22.6)	0	11 (13.9)

[b vs a,c] χ^2, 2 x 3, 2 *df*, Effect of photosensitization on fertilization, $P < 0.01$

[e vs d,f] χ^2, 2 x 3, 2 *df*, Effect of photosensitization on cleavage, $P < 0.01$

GVTr = rescue germinal vesicle transplantation ('rescued' oocytes)

5.13 Post-implantation development of the GV transplanted oocytes

When a total of 132 two-cell stage embryos derived from the 'rescued' oocytes was transferred to 10 recipients, a total of 17 live offspring was obtained at day 20 of pregnancy either by spontaneous delivery or by caesarean section (Figure 5.5). This live birth rate was comparable to that of control non-manipulated GV oocytes (Table 5-IV).

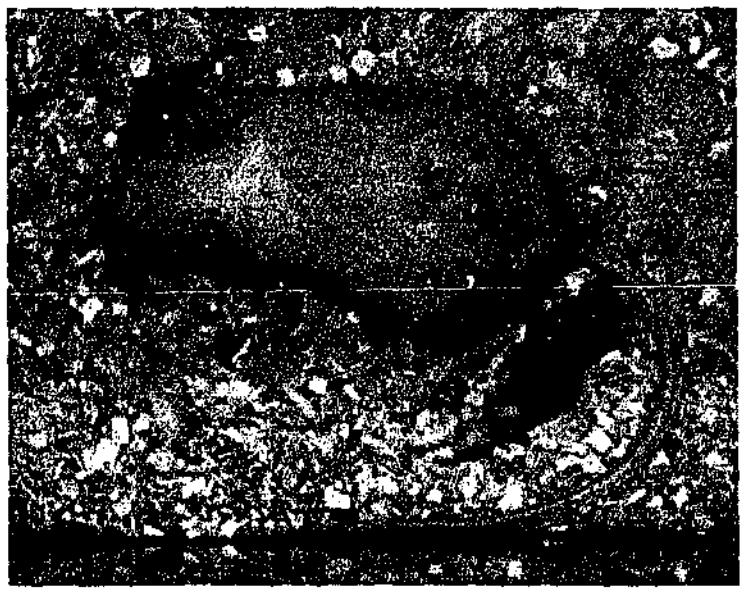


Figure 5.5 A foster mother and two offspring generated from 'rescued' oocytes.

Table 5-IV. Post-implantation development of conceptuses derived from rescue germinal vesicle transplantation

	Origin of oocytes	
No. of (%)	Intact *in vitro* matured	GVTr
Inseminated oocytes	207	232
Fertilized oocytes	118 (57.0)	139 (59.9)
Transferred 2-cell embryos	109	132
Recipients	8	10
Live offspring/Transferred embryos	21 (19.3)	17 (12.9)

GVTr = rescue germinal vesicle transplantation ('rescued' oocytes)

5.14 Discussion

This study demonstrates that photosensitization-induced damage of mouse oocyte mitochondria consistently inhibits GV breakdown, meiotic spindle formation, chromosomal segregation, and PB extrusion. Maturational arrest induced by such mitochondrial damage, which I believe may serve as a model for age-related ooplasmic dysfunction, has been reversed successfully by nuclear transplantation as evidenced not only by oocyte nuclear maturation but also by *in vitro* and *in vivo* embryo development. This study also demonstrates that normal meiosis can still take place where a nucleus isolated from a mitochondrially-damaged oocyte is exposed to healthy ooplasm, in that GVTr oocytes are able to undergo maturation, fertilization, and in some cases development to term.

The reciprocal transplantation of individual karyo- and cyto-plasts (Takeuchi et al., 1999c; 2001b; 2004b) has shown that the procedure itself does not impair the rate of maturation when GV nuclei were transferred to non-irradiated oocytes. On the other hand, meiosis was usually compromised where a normal nucleus was transferred to a photosensitized ooplast. Whenever maturation did occur it was at an extremely low rate (~ 6%), with fertilization also being low (17%). Yet although some cleavage occurred, none of the treated oocytes reached the blastocyst stage. On the other hand, situated in a healthy ooplasm, a photoirradiated nucleus was able to complete its maturation. However, this was somewhat less efficient than in the controls ($P < 0.001$) — perhaps due to the sensitized mitochondria or to mitochondrial leakage of factors normally confined to the matrix carried over with the residual ooplast (Takeuchi et al., 2000b; Fulka, 2004). This indicates that this procedure cannot be utilized for the treatment of mitochondria-related diseases until karyoplasts can be prepared without any residual cytoplasm and so some mitochondria (Fulka, 2004).

CMXRos used as a photosensitizer was able to produce selective damage in ooplasmic mitochondria in a dose-related manner. The damage entails mitochondrial swelling due to an altered permeability of the inner membrane, its electrical depolarization, and consequent cytochrome C leakage into the cytosol together with the production of reactive oxygen species, leading to cell apoptosis (Salet and Moreno, 1990; Minamikawa et al., 1999). In my experience, the insult of photosensitization was reflected in a maturational blockage, similar to that observed during aging of human oocytes (Tarin et al., 1998; Schon et al., 2000; Van Blerkom, 2000).

Recently, it has been postulated that, in oocytes, oxidative stress induces aneuploidy implicated in maternal age-related infertility (Tarín et al., 1996). Obviously the mitochondrial damage induced in mouse oocytes could only be monitored by the effect on the nuclear maturation. In fact, it has not been possible in rodents to demonstrate a spontaneous occurrence of oocyte aneuploidy in relation to aging, even in selected CBA/CA or SAM (senescence accelerated mouse) mouse strains (Eichanlaub-Ritter et al., 1988; Liu and Keefe, 2002). The incidence of aneuploidy is very limited, and at any meaningful level oocyte production drops exponentially to a point such that any consistent quantitative evaluation of aneuploidy is not possible. Therefore, as an indicator of cytoplasmic damage, a mitochondrial toxin at a dosage capable of inhibiting nuclear maturation was adopted.

Importantly, the maturational arrest was reversed once the nucleus from oocytes with photosensitized cytoplasm was transplanted to an untreated ooplast. As a corollary of this, it has been demonstrated that mitochondrial infusion can increase the ATP content of the recipient mouse MII oocytes (Van Blerkom et al., 1998), and that transfused mitochondria can survive throughout embryonic development and undergo replication (Barritt et al., 2001b). The residual irradiated peri-nuclear cytoplasm apparently did not influence the efficacy of nuclear transplantation (Takeuchi et al., 1999a). Thus, it seems possible that GV transfer could prevent oocyte aneuploidy — if indeed mitochondrial dysfunction is the sole cause of this — in the case of human oocytes as well (Palermo et al., 2002a).

The insult brought by CMXRos was confined to the mitochondria, leaving nuclear DNA unmodified, as demonstrated by the ability to obtain normal offspring. Moreover, full term development from GV oocytes following *in vitro* maturation, thus avoiding sequential transfer of mature cytoplast (Kono et al., 1996; Bao et al., 2000), accords with previous experiments (Takeuchi et al., 2004b). In conclusion, since GV transfer can counteract ooplasmic damage, this may provide a way of avoiding that seen in age-related aneuploidy (Palermo et al., 2002a). This procedure, however, is compromised by the limited availability of eggs in aging women, therefore, a definitive option would be to construct female gametes in an alternative manner, for example, from a somatic cell (Tsai et al., 2000; Palermo et al., 2002a; 2002b).

Chapter 6

Haploidization of Somatic Cell Nuclei and Fertilization of Reconstituted Mouse Oocytes

Declaration for Thesis Chapter 6

In the case of Chapter 6, contributions to the work involved the following:

	Name	% contribution	Nature of contribution
1	**Gianpiero D. Palermo**	60	Conception and design, collection, analysis, and writing of manuscript
2	**Takumi Takeuchi**	15	Execution of work, collection and data analysis
3	**Tian Hua Huang**	13	Execution of work
4	**Queenie V. Neri**	12	Data analysis and manuscript editing

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Weill Medical College of Cornell University, The Center for Reproductive Medicine, New York, NY, USA

Signature 1 [redacted] **Date** 10-29-2004

Signature 2 _____ **Date** 11-19-2004

Signature 3 _____ **Date** 11-22-2004

Signature 4 _____ **Date** 11-11-2004

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Symposium: Clinical prospects of nuclear transfer and somatic cell haploidization

Implications of cloning technique for reproductive medicine

Dr Gianpiero D Palermo

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. Dr Palermo completed his clinical training in Obstetrics and Gynaecology at the University of Bari in Italy and at the New York Presbyterian Hospital-Weill Medical College of Cornell (NYPH-WMC) in New York, attended the Masters and PhD programmes at the Brussels Free University and is currently completing additional post-doctoral training at Weill Medical College of Cornell University and Monash University. Dr Palermo has won many prestigious prizes and awards for his pioneer work in Reproductive Biology and has delivered hundreds of lectures before international audiences on topics of mammalian fertilization and genetics. 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. 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

The birth of Dolly following the transfer of mammary gland nuclei into enucleated eggs established cloning as a feasible technique in mammals, but the moral implications and high incidence of developmental abnormalities associated with cloning have induced the majority of countries to legislate against its use with human gametes. Because of such negative connotations, restrictive political reactions could jeopardize the therapeutic and scientific promise that certain types of cloning may present. For example, in addition to its proposed use as a way of generating stem cells, the basic technique of nuclear transplantation has proven useful in other ways, including its application to immature eggs as a new approach to the prevention of the aneuploidy common in older women, and for some recent advances in preimplantation genetic diagnosis. Thus, while attempts at reproductive cloning in man would seem premature and even dangerous at present, this field will require rational rather than emotional reactions as a basis for legislation if the therapeutic promise of stem cell research and the experimental potential of nuclear transplantation techniques are to be fully realized.

Keywords: nuclear transfer, reproductive cloning, somatic cell haploidization, therapeutic cloning.

Background

The word clone, coming originally from the Greek κλων meaning twig or slip, defines a method of replication as it occurs in some plants, and thus represents an asexual means of reproduction. For many plants, and some invertebrates and even a few vertebrates (some lower animals such as the hydra, the sea anemone, and others such as planarians and annelids), cloning is the natural way to reproduce. Among mammals, a form of cloning was achieved years ago by Willadsen (1979) by a method that simply involves 'embryo splitting'; it is simply performed by mechanically dividing an early embryo into two parts (Baker and Shea, 1985; Hall *et al*., 1993), but obviously this generates a limited number of copies of the original embryo.

Forty years ago, successful cloning of an adult frog was achieved by transferring isolated endothelial cells from tadpoles into an enucleated egg (Gurdon, 1962). Since then, cloning experiments have involved mainly nuclear transplantation and the two terms have often been used synonymously, though nuclear transplantation is also used in other procedures, where cytoplasmic exchange is required. In the public arena, however, cloning has been viewed with some suspicion, and there is a need to distinguish the generation of identical cells of potential use in regenerative medicine from the 'ethically unacceptable cloning' aimed at creating an identical copy of an individual. In fact, the most recent tendency is to avoid the term cloning altogether and to adopt nuclear transplantation as a term for all forms of cloning that are not aimed at reproduction.

6.1 Summary

Although one solution to age-related oocyte aneuploidy can be oocyte/embryo selection, another could be nuclear transplantation. The transfer of diploid somatic nuclei into enucleated oocytes may offer the possibility for aging oocytes to be reconstituted in a way that allows for normal embryonic development. Somatic nuclei were grafted into GV ooplasts by electrofusion, and the reconstituted oocytes were cultured to allow maturation, following which they were selected at random for fertilization by ICSI or cytogenetic analysis. Of 247 mouse GV oocytes, 96% were successfully enucleated and 85% survived somatic cell transfer. Of 192 subjected to ICSI, 149 displayed 2PN with a single PB. Their subsequent culture resulted in 6% forming blastocysts. Comparable results were obtained after a similar haploidization of male fibroblast. Cytogenetic analysis of the mouse ICSI embryos generated from somatic cell haploidized (SCH)-derived oocytes revealed that only 14.3% were diploid. Although further evaluation of their genetic status is needed, this procedure may allow the production of normal oocytes in cases of aged-related infertility. While technically similar to cloning, this would however generate a unique individual coming from both parental genomes.

6.2 Introduction

In a large majority of infertile couples, oocytes may harbor 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 metabolic defects, some of which may be treated by supplementation with ooplasm aspirated from a fertile donor oocyte (Cohen et al., 1998a). Nevertheless, some oocytes that are genetically abnormal can develop into embryos that, morphologically, would be considered suitable for uterine transfer (Munné et al., 1995b). 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 abnormal embryos probably do not implant, however, and if they do so the fetus generally does not reach term.

As noted in previous chapters, oocyte aneuploidy appears to be the primary factor inducing the decline in maternal fertility that is evident by 40 years (Tietze, 1957; Munné et al., 1995c; Dailey et al., 1996). As a corollary, older women have become pregnant using oocytes donated by younger women, eliminating the uterine environment as a possible factor (Rosenwaks, 1987; Legro et al., 1995).

The oocyte abnormalities associated with aging relate primarily to abnormal segregation during meiosis I (Eichenlaub-Ritter, 1998; Hassold and Hunt, 2001), and are due to defects on the meiotic spindle (Battaglia et al., 1996; Volarcik et al., 1998). Why the effect of aging on spindle function is still unknown, 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). Attempts to improve the chance for a pregnancy in women who are at increased risk for oocyte aneuploidy have involved selection of oocytes and embryos by PGD (Gianaroli et al.,

1997; 1999; Munné et al., 1999; Verlinski et al., 1999). On the other hand, 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, while maintaining identity, for aneuploidy prevention would be to transfer aged GV oocytes into younger (donor) ooplasts (Zhang et al., 1999). Fortunately, nuclear transplantation to the mammalian oocyte can be accomplished efficiently in principle 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. Not only are many from women of ≥ 40 years 'compromised', but 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., 1999c; 2000a). The availability of such 'manufactured' oocytes would be dictated by the number of donated eggs and not by the efficiency of the technique. In addition, somatic nucleus haploidization may be employed in generating male gametes and so treating azoospermic men. ICSI is successful only with spermatozoa or spermatids, but is ineffective with earlier stages of human male germ cells. In non-obstructive azoospermic patients, other approaches have been proposed ranging from *in vitro* maturation of early spermatogenic cells to transplantation of spermatogonia (Reis et al., 2000), but only for the cases where at least some sperm precursor cells exist.

In this study, haploidization of somatic nuclei was attempted by their incorporation into mouse ooplasts. These reconstituted oocytes were then evaluated for their ability to undergo normal fertilization and development to the blastocyst stage. Secondly, the ability of mouse male somatic cells to generate gametes was explored.

6.3 An alternative source of oocytes

As described in Chapters 3, 4, and 5, transplantation of a GV nucleus into a younger ooplast can correct age-induced aneuploidy. Although further improvements need to be made, especially in regard to the *in vitro* maturation process, GVT technique may open the way to eliminate chromosomal defects in 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 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). 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*, GV-stage ooplasm might be able to haploidize 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 endometrial stromal cells were isolated from consenting patients undergoing endometrial cell coculture during IVF, by enzymatic digestion using 0.2% collagenase type II with differential sedimentation (Barmat et al., 1998), and were cultured in RPMI supplemented with 10% FBS utilized for long-term culture. In the case of the mouse (B6D2F1 strain), 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 denuded by mechanical removal of cumulus cells.

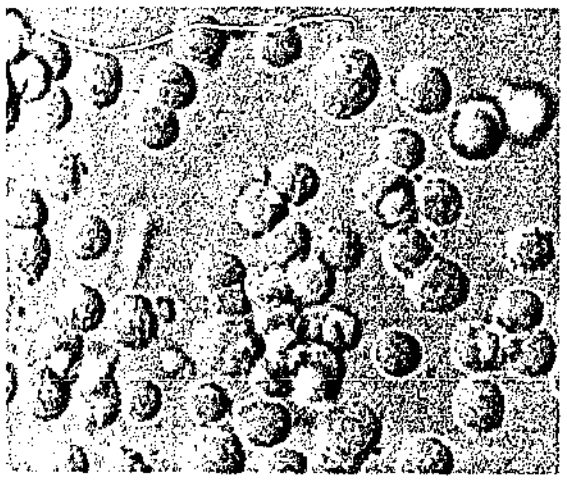


Figure 6.1 Cultured mouse cumulus cells (400X).

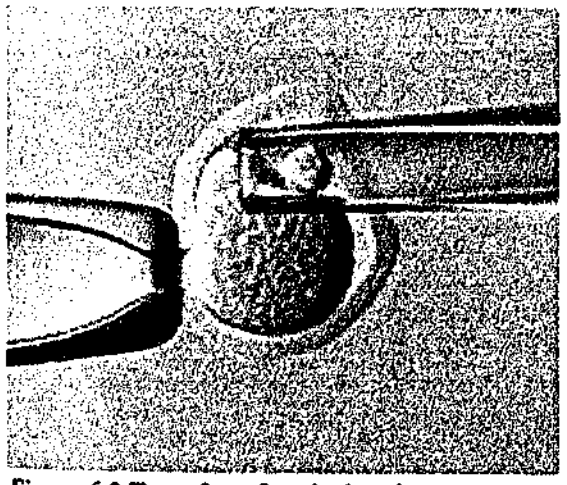


Figure 6.2 Transfer of an isolated mouse cumulus cell into an ooplast (400X).

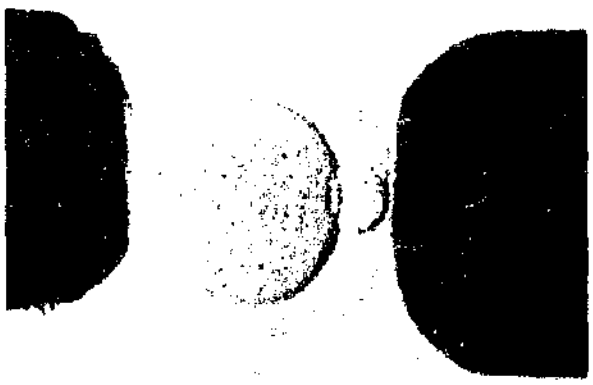


Figure 6.3 Grafted oocyte with a cumulus cell aligned between two microelectrodes.

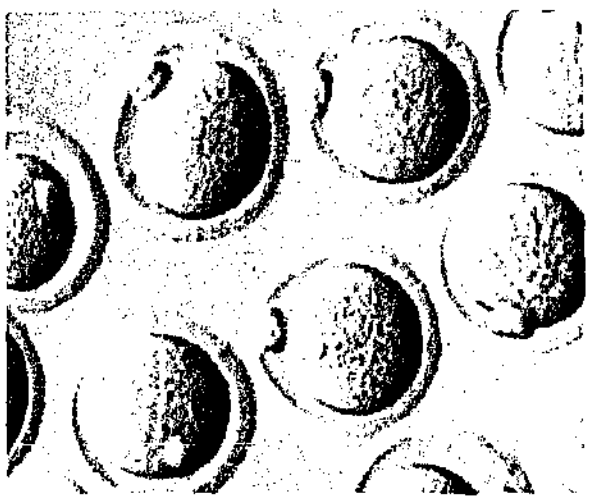


Figure 6.4 Mouse oocytes grafted with cumulus cells.

All the micromanipulation and electrofusion procedures were carried out as described in Chapter 2. Cultured human endometrial stromal and mouse cumulus cells (Figure 6.1) were released from the culture dish with trypsin-EDTA. After GV enucleation (see Figure 3.1), somatic cells were then inserted subzonally into an enucleated mouse GV oocyte (Figure 6.2). Each grafted oocyte was then manually aligned between two microelectrodes for electrofusion (Figures 6.3 and 6.4). To allow maturation, reconstituted oocytes (Figure 6.5) were incubated for 14 – 16 hour until extrusion of the first PB.

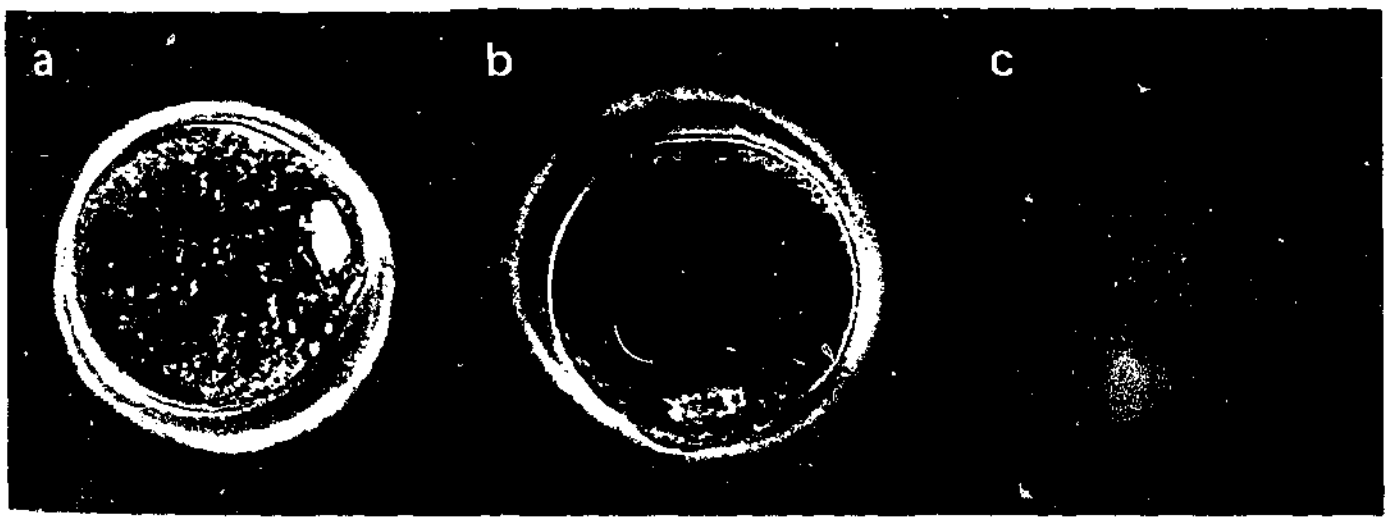


Figure 6.5 (a) Metaphase spindle of a mouse MII oocyte and (b) a mouse oocyte injected with a somatic cell visualized by polscope (a,b) and fluorescence microscopy (c).

To evaluate the distribution of nuclear chromatin, some mature oocytes were stained with a DNA-specific stain (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 6.6) was 39.1% for endometrial cells and 39.0% for cumulus cells, respectively (Table 6-I). Further *in vitro* culture for up to 24 hours did not improve these results.

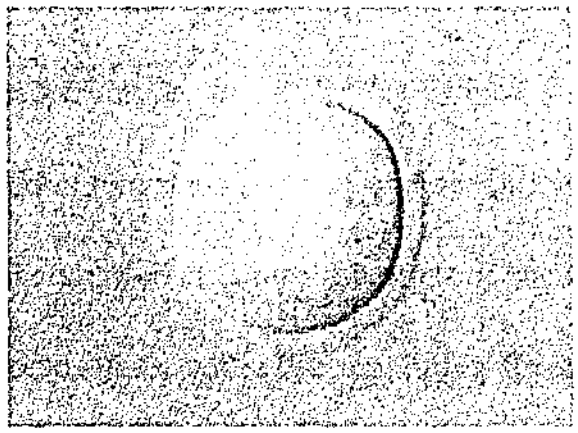


Figure 6.6 Mouse reconstituted oocyte with human endometrial cell showing segregated chromosomes in both the oocyte and the polar body.

Table 6-I. The ability of reconstituted mouse oocytes to extrude the first polar body

	Origin of the somatic cell nucleus	
No. of oocytes	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)

GV = germinal vesicle
IPB = first polar body

Further studies were performed to evaluate the karyotypes of reconstituted mature oocytes by gradual fixation and subsequent Giemsa staining (Takeuchi et al., 1999a). In all, 77 enucleated GV oocytes were grafted with a single cumulus cell, 72% (n = 56) being reconstituted successfully by

Figure 6.7 A normal haploid set of chromosomes in a reconstituted mouse oocyte after extrusion of the first polar body (Giemsa stain).

electrofusion, and 29 of the reconstituted oocytes (51.8%) extruded their first PB. Among those 13 that had an analyzable karyotype, five (38.5%) proved to have normal haploid chromosomes in the oocyte. In the PB (Figure 6.7), five showed structural aberrations such as pulverized chromosomes, while the remaining three were diploid with telophase-like structures.

6.4 Female and male mouse somatic cell isolation and MII oocyte retrieval

Mouse cumulus cells were isolated from oocyte-cumulus complexes of oocyte donors. Male fibroblasts were isolated by mincing skin tissue into small pieces (5 x 5 mm) followed by *in vitro* culture for approximately one week in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies). Once initial growth of fibroblasts was confirmed, the residual skin specimen was removed and the attached fibroblasts were then cultured for up to 30 days with several passages until use.

MII oocytes were retrieved from B6D2F1 strain mice after PMSG and hCG stimulation. Oocytes were freed from cumulus cells by brief exposure to hyaluronidase.

6.5 ICSI and 'haploidized' mouse oocytes

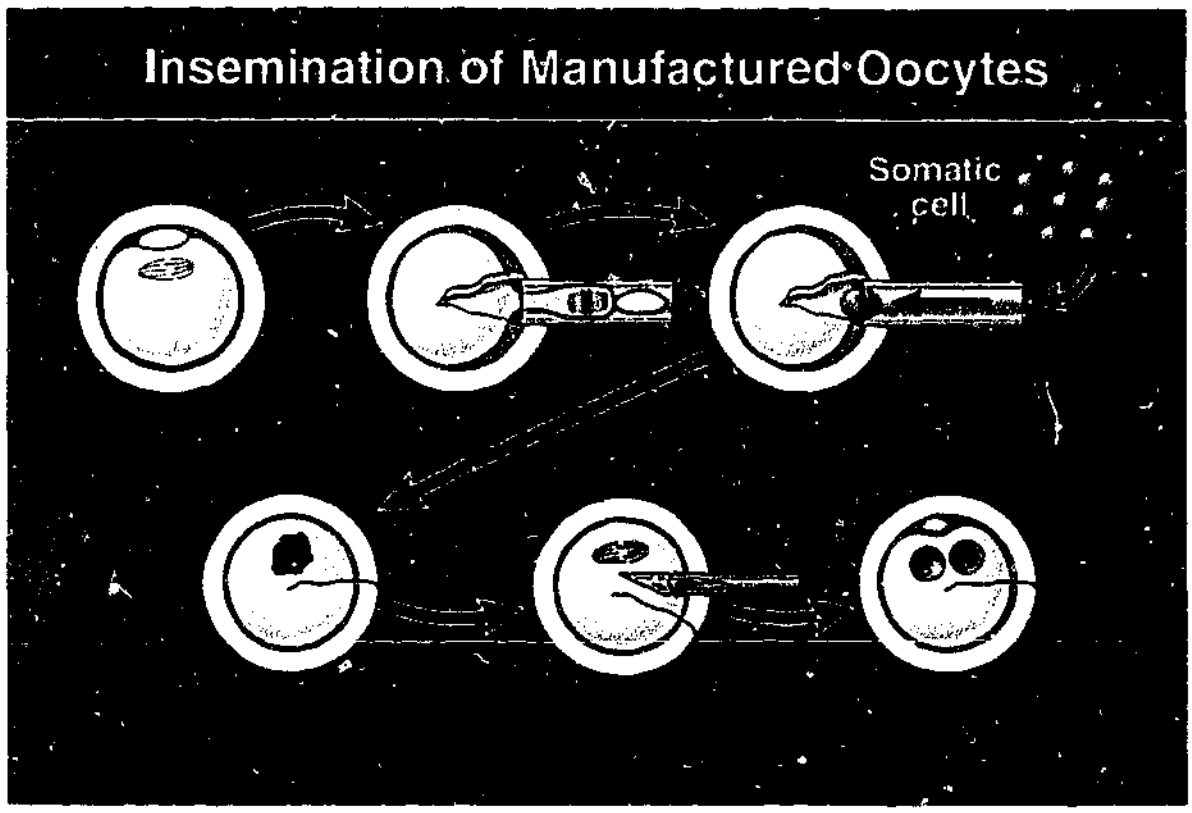


Figure 6.8 Sequential steps involved in somatic cell injection and fertilization.

Manufacturing an oocyte involves the removal of the meiotic spindle and usually the first PB, followed by the injection of a somatic nucleus that will be haploidized by the insemination of a spermatozoon (Figure 6.8).

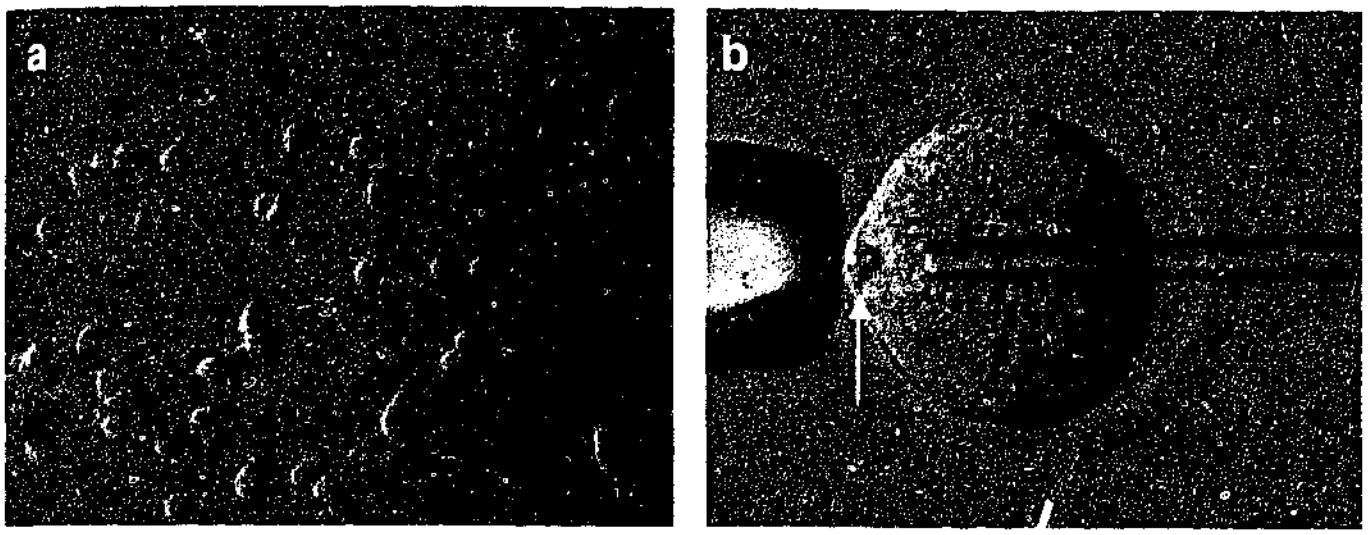


Figure 6.9 (a) Culture of mouse cumulus cells. **(b)** A cumulus cell (arrow) injected into an enucleated mouse MII oocyte (400X).

After cytochalasin B treatment, the MII spindle was extracted with a glass micropipette, and its removal confirmed by vital DNA staining with DAPI. Enucleated oocytes were then injected with a cumulus cell (Figure 6.9a,b). To allow transformation of the cumulus cell nucleus into a metaphase spindle, oocytes were incubated for at least one hour, at which point a single dissected sperm head was inserted by piezo ICSI (Takeuchi et al., 2004a).

6.6 Injection of a male somatic cell

Formation of a haploid male pronucleus after the injection of a somatic nucleus in a mouse MII oocyte (Figure 6.10).

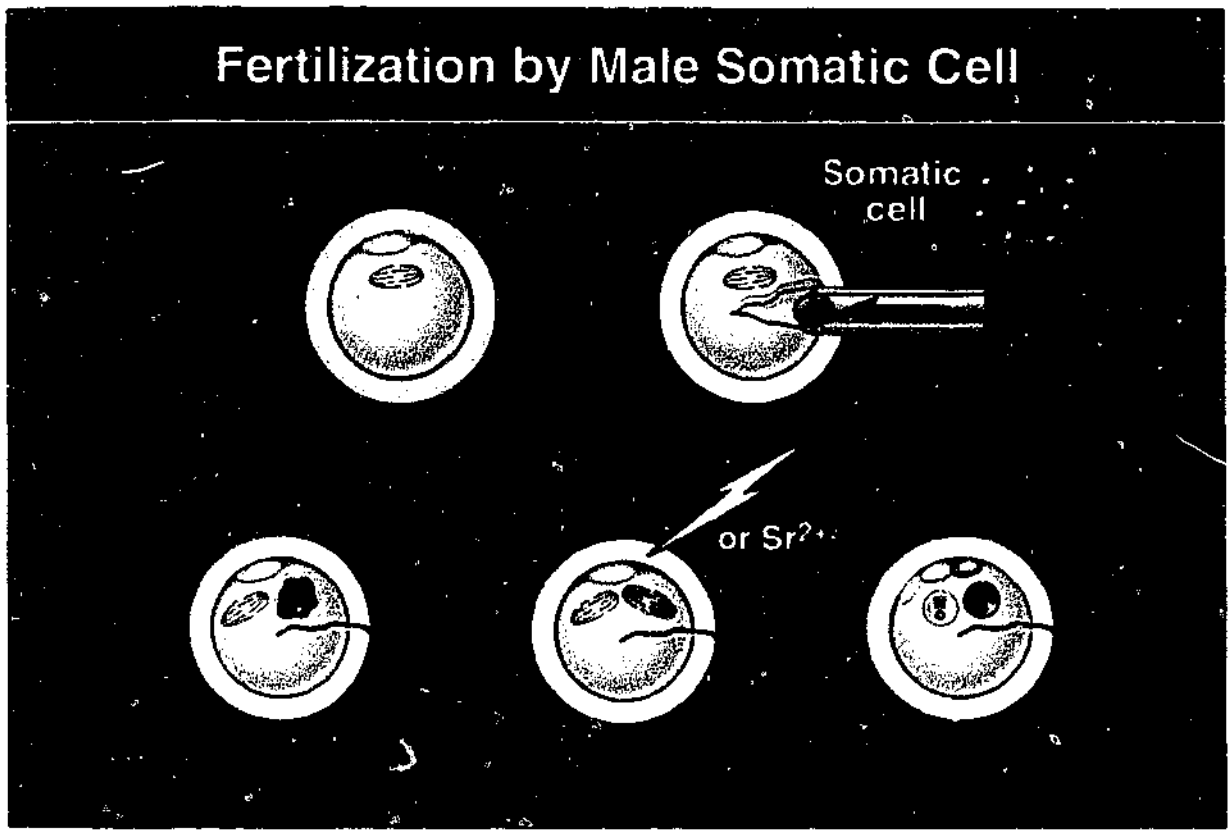


Figure 6.10 Schematic representation of male somatic cell injection and fertilization.

A single male fibroblast was introduced into each intact MII oocyte by direct injection with a piezo device (Figure 6.11a,b). Oocytes were then incubated for at least 2 hours and subsequently exposed to 10 mM $SrCl_2$ for 6 hours to induce activation and concurrent haploidization.

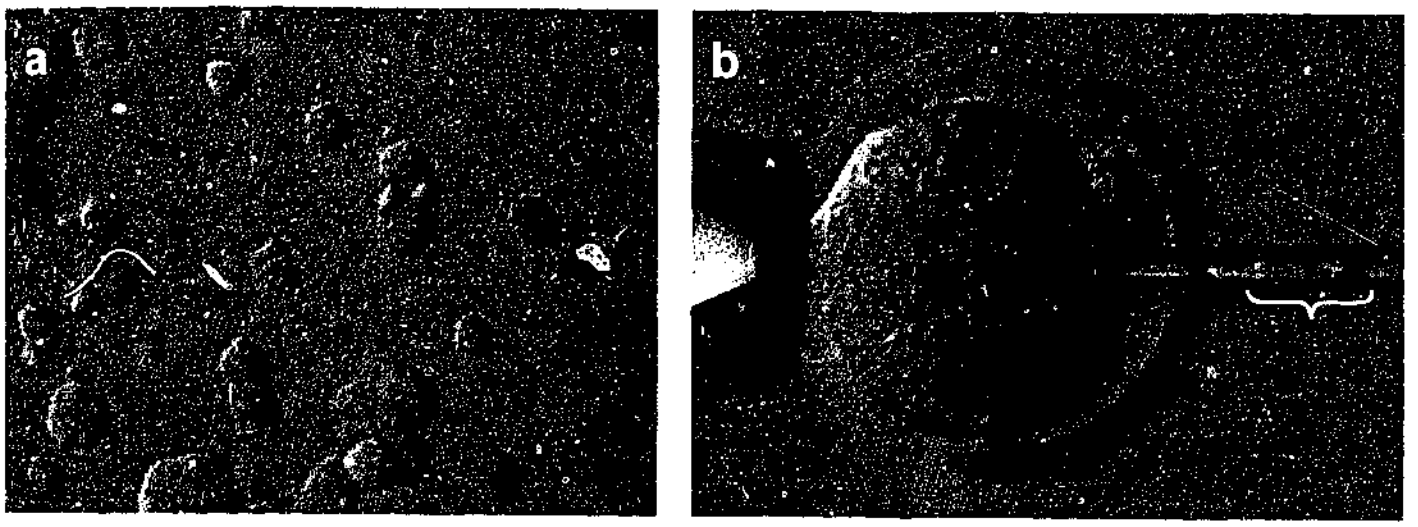


Figure 6.11 (a) Male fibroblasts in culture. (b) Injection of a male fibroblast (white bracket) into an intact oocyte (400X).

6.7 Fertilization and embryonic cleavage assessment

Haploidization and fertilization were confirmed by observation of two distinct PN and a PB (containing chromatin) when female somatic cells were used. The observation of two distinct PNs and simultaneous extrusion of two PBs were indicators of successful fertilization when male somatic cells were injected. Embryo development was assessed at 24 hour intervals by observing and recording the number and characteristics of blastomeres as well as formation of a blastocoele. Random blastomeres isolated from cleaving embryos were processed for cytogenetic analysis.

6.8 Development of artificial oocytes

From an initial population of 247 MII mouse oocytes, 237 (96.0%) were successfully enucleated, and the 210 (88.6%) that survived somatic cell injection were then subjected to ICSI. Following this, 149 of 192 (77.6%) surviving oocytes displayed 2PN and a PB. Although experimentally fertilized oocytes underwent the first cleavage division at the same time as the normal *in vivo* matured MII serving as control, later cleavage was often impaired, and only 6.0% eventually formed a blastocyst (compared to 78.9% of the controls) (Table 6-II). Among 14 of the experimental embryos analyzed cytogenetically, 12 (85.7%) presented with numerical chromosomal abnormalities while the other two were normal.

Table 6-II. *In vitro* development of oocytes generated from somatic nuclei

No. of (%)	Control oocytes	'Manufactured' oocytes
Zygotes	38	149
2-cell	38 (100)	137 (91.9)
4-cell	38 (100)[a]	109 (73.1)[b]
Morula	36 (94.7)[c]	84 (56.4)[d]
Blastocyst	30 (78.9)[e]	9 (6.0)[f]

[a vs b, c vs d, e vs f] χ^2, 2 x 2, 1 *df*, Effect of the haploid genomic origin (natural versus induced) on embryonic cleavage, $P < 0.001$

6.9 Utilization of a mouse fibroblast as a male gamete

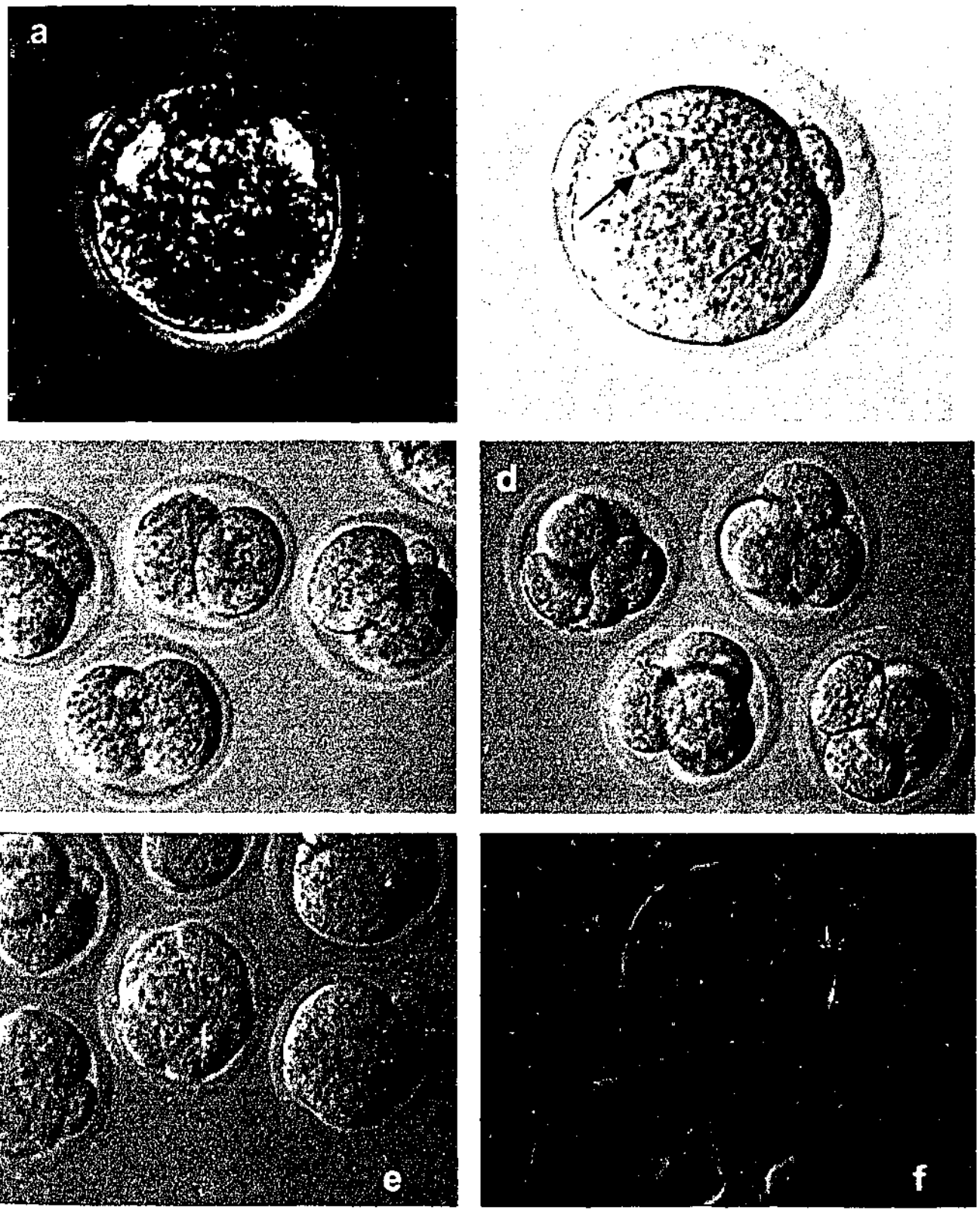


Figure 6.12 (a) A double spindle at metaphase after injection of mouse male fibroblast visualized by polarized light microscopy – prior to oocyte activation; (b) fertilized oocyte after male fibroblast injection with 2PN (black arrows). Preimplantation embryo development: (c) 2-cells, (d) 4-cells, (e) compacted morulae, and (f) expanded blastocysts.

A total of 260 reconstituted MII oocytes was divided into two groups according to the gender origin of the somatic cell. The fertilization rate and the incidence of 2-cell embryos were significantly higher after cumulus cell injection than after fibroblast injection ($P < 0.05$), though the rates of blastocyst formation were comparable (Table 6-III) (Figure 6.12a-f). Preliminary cytogenetic assessment revealed a 15.8% (3/19) normal numerical chromosomal distribution (Figure 6.13).

Figure 6.13 Mouse diploid karyotype (Giemsa) obtained from a 2-cell embryo generated by somatic cell injection.

Table 6-III. Embryo development of manufactured oocytes according to the gender of the injected cell

	Somatic cell origin	
Oocytes/embryos (%)	Cumulus cell	Male fibroblast
Inseminated/stimulated	105	155
Fertilized	71 (67.6)[a]	74 (47.8)[b]
2-cell stage	68 (64.8)[c]	70 (45.2)[d]
Blastocyst	8 (7.6)	13 (8.4)

[a vs b, c vs d] χ^2, 2 x 2, 1 *df*, Effect of the haploid genomic origin (male versus female) on fertilization and embryonic cleavage, $P < 0.01$

6.10 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. As described in Chapter 3, transplantation of an isolated GV into an enucleated 'young' ooplast is practicable, and such reconstituted oocytes can undergo nuclear maturation, fertilization, early cleavage as well as pre- and post-implantation development. However, oocyte availability remains as a limiting factor, since older patients generally produce only a small number.

In order to overcome this limitation, I 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 chromosome spreads (Kubelka and Moor, 1997; Takeuchi et al., 2000a). Karyotyping shows that the transplanted somatic nucleus can undergo spontaneous segregation with extrusion of a PB. Oocytes that failed to extrude PB displayed two sets of chromosomes with two spindle centers (Kubelka and Moor, 1997).

The activation of a mature ooplast can induce segregation of the diploid chromosomes of a transplanted fibroblast nucleus within it, allowing apparently normal fertilization and even blastocyst formation. The production of full-term offspring in experimental animals represents the next step towards the accomplishment of generating artificial male gametes (Takeuchi et al., 2002a).

The construction of entire oocytes from donor ooplasts would represent a definitive advantage where only a few 'aged' oocytes are available, since zygotes generated from haploidized somatic cells can undergo development to the blastocyst stage (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, it differs in requiring a biparental 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. The finding 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 and develop an apparently normal MII spindle at an acceptable rate. However, more detailed analysis of the haploidized nuclei is needed, the behavior of the somatic cell centrosome must be clarified, and whether the imprinting pattern of such reconstructions is comparable to that in normal gametes needs to be established. Although the safety of nuclear transplantation procedures can only be confirmed by obtaining healthy offspring, the results obtained so far certainly justify further research.

In normal meiosis I of mammals, replicated homologous chromosomes physically attach by chiasmata at sites of chromatin exchange and segregate from each other to the two opposite spindle poles (Hodges et al., 2002; Eichenlaub-Ritter, 2003). In meiosis II, the sister chromatids are affixed at kinetochores to the two opposite spindle poles, ensuring a high fidelity of

chromosome segregation at the second meiosis (Sandalinas et al., 2002). In contrast, the injected G_0/G_1 diploid somatic cell consisted of two sets of physically unattached chromosomes. Therefore, pairing, recombination, or the cohesion of centromeres essential for correct reduction segregation between homologous chromosomes is missing (Paliulis and Nicklas, 2000). This could explain that in experiments with MII oocytes receiving either murine or Chinese hamster somatic cell nuclei, extrusion of the PB failed to have a haploid number of chromosomes in the oocyte (Tateno et al., 2003a).

The imprinting of male gametes is accomplished in advance of the second meiotic division (Shamanski et al., 1999). As discussed in Chapter 8, correct imprinting during gametogenesis and epigenetic reprogramming during the preimplantation period are essential for normal developmental potential. They involve DNA methylation, modification of histones, and a chromatin remodeling that regulates gene expression in early embryos (DeRycke et al., 2002). Importantly, the epigenetic status of the donor nucleus is different from that of the gametes. Therefore, the other major determinant of success would hinge on the epigenetic modification of somatic DNA in the cytoplasm of oocytes (Wakayama et al. 1998; Chen et al., 2004). In the somatic cell, only one allele is imprinted, either maternally or paternally. In the case of its haploidization, there is a fifty percent chance that it contains the wrong imprint, leading to either a biallelic expression or to a repression of imprinted regions, similar to that which gives rise to congenital abnormalities and disease in cases of uniparental disomy, e.g. Prader-Willi or Angelman syndrome (Eichenlaub-Ritter, 2003). Even when a normal haploid set of chromosomes is artificially created, it is extremely unlikely that such a chromosome complement would contain

exclusively maternally or paternally imprinted genes (Tateno et al., 2003a), making this unpredictable. It cannot be excluded that the oocyte might be able to erase and re-establish imprint (Tateno et al., 2003b), though, it is unlikely that the MII oocyte can do so. It remains to be determined whether GV stage oocytes can fully erase and restore genomic imprinting information. A recent report suggested that normal chromosome segregation may occur in GV stage oocytes (Palermo et al., 2002a). However, other authors claim that it is unlikely that fully grown GV oocytes would retain the capacity to erase and restore genomic imprints, therefore, haploidization would not occur with the GV (Fulka et al., 2002a; Heindryckx et al., 2004).

Chapter 7

Construction and Fertilization of Reconstituted Human Oocytes

Declaration for Thesis Chapter 7

In the case of Chapter 7, contributions to the work involved the following:

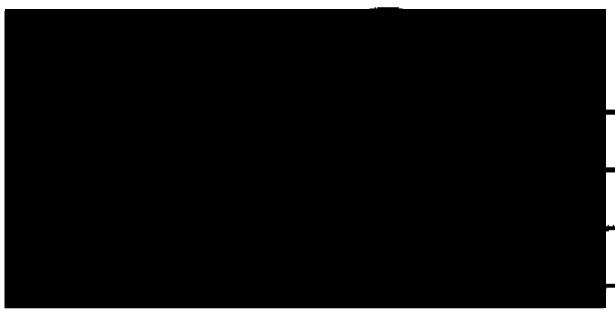
	Name	% contribution	Nature of contribution
1	**Gianpiero D. Palermo**	55	Conception and design, collection, analysis, and writing of manuscript
2	**Takumi Takeuchi**	20	Execution of work, collection and data analysis
3	**Gail Stetten**	15	Execution of work
4	**Marcos Reis (deceased)**	10	Execution of work

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Weill Medical College of Cornell University, The Center for Reproductive Medicine, New York, NY, USA



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Signature 2 _____ **Date** 11-19-2004

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Signature 4 _____ **Date** —

7.1 Summary

The ability to reduce the chromosomes of a somatic cell in half by transferring it into an enucleated oocyte, provides the premise for the construction of artificial gametes. As noted in Chapter 6, it may be possible to harness this ability of the mammalian ooplast to "haploidize" producing gametes with either female or male somatic nuclei. This study examines the possibility of constructing viable human gametes, and their potential for participation in normal fertilization.

Spare GV-stage oocytes were donated by consenting patients undergoing ICSI. After micromanipulation, about 41% of injected oocytes generated 'haploid' pronuclei. In human oocytes, however, this was never accompanied by extrusion of a PB, but resulted in the formation of two "haploid" pronuclei. Consequently, after ICSI, selective removal of a female pronucleus marker was required to restore a balanced haploid content in the cell. Human zygotes derived from somatic cell injection proved to be haploid in approximately 38% of the cases. However, on karyotypic analysis, blastomeres isolated from cleaving embryos evidenced a chaotic distribution of chromosomes.

Oocytes were able to induce haploidization of transplanted somatic cell nuclei independently of the gender of the donor cell. Fertilization of artificial oocytes was followed by embryonic cleavage even if blastocyst development rates and their chromosomal content were compromised.

7.2 Introduction

One problematic cause of infertility is the higher incidence of oocyte aneuploidy in older women (Dailey et al., 1996). Independent of the initial indication or the ART used to treat it, the pregnancy rate follows a downward slope starting at 35 years (Tietze, 1957). As discussed in previous chapters, 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 aging and the non-disjunction of bivalent chromosomes during meiosis (Dailey et al., 1996), it is likely that such aging compromises the meiotic apparatus (Battaglia et al., 1996; Volarcik et al., 1998), perhaps via a 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 mtDNA 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 MII oocytes from younger women (van Uem et al., 1987; Chen, 1988; Porcu et al., 1997) or of the entire ovarian cortex taken at a younger age (Gosden et al., 1994; Newton et al., 1996; Oktay and Gosden, 1996; Oktay et al., 1998; 2004; Shaw et al., 2000; Kim et al., 2002; Gook et al., 2003). Another quite novel approach has been the use of nuclear transplantation techniques (Zhang et al., 1999; Takeuchi et al., 1999a; 2001a).

Although nuclear transfer has been successful in producing some animal offspring, its overall efficiency remains extremely low (Takeuchi et al., 1999a; 2004b; Li et al., 2001). The main bottle-neck for 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 aging 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., 1999c; 2000a; 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., 1999c; 2000a; Tsai et al., 2000) and would provide a definitive treatment for age-related infertility.

I have previously shown (Chapter 6) that the mouse ooplast can 'haploidize' a somatic nucleus. 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. This involved injection of mature human ooplasts with somatic cell nuclei and their subsequent exposure to physical and chemical stimuli to induce

haploidization. The efficiency of each step as well as the oocyte activation procedures was evaluated according to pronucleus formation and cytogenetic assessment. Haploidization of a somatic cell nucleus by a human ooplast consistently resulted in the formation of two distinct pronuclei, but no polar body 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 labeling. Thereafter, development and genomic status of the resultant embryos were assessed.

7.3 Oocytes and somatic cell isolation in humans

Spare human GV oocytes were obtained from consenting patients undergoing ICSI. The procedures as well as the handling of patient material were performed in accordance with an approved research protocol (see Section 2.5.11). Oocytes were retrieved following treatment with a GnRHa and gonadotropins (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 HTF supplemented with 0.4% HSA for 24 hours to allow maturation to MII.

7.4 Nuclear transplantation and activation of human oocytes

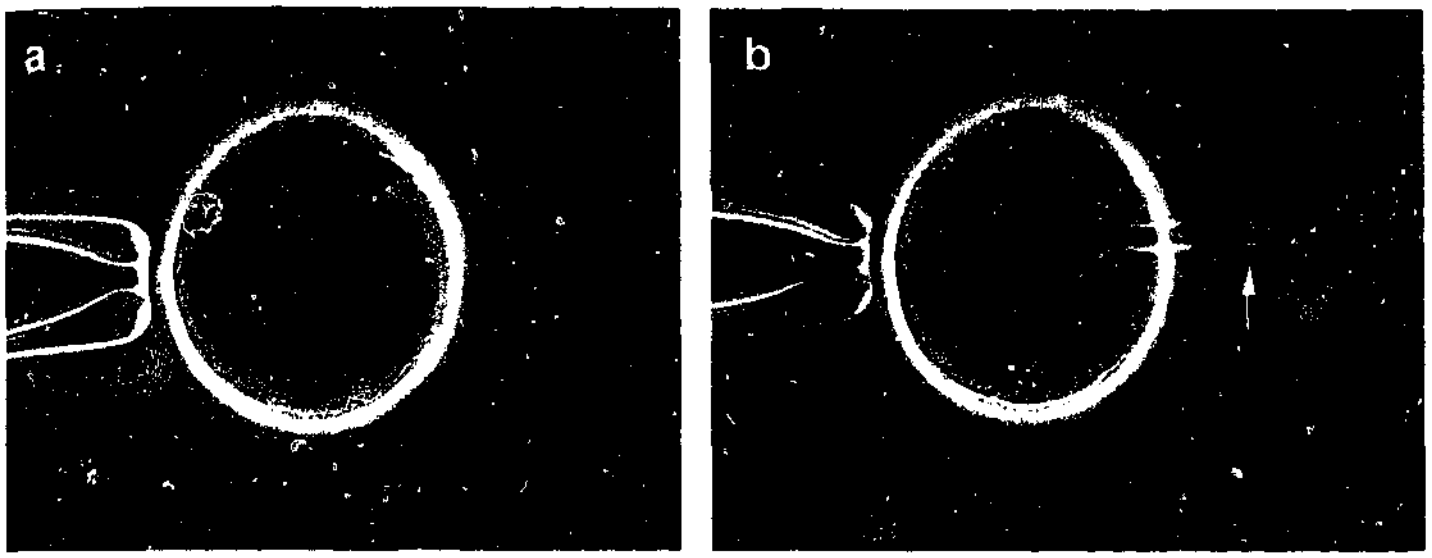

Figure 7.1 (a) Human MII meiotic spindle. (b) Removal of a spindle (arrow). Visualized by polarizing microscope.

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 CCB the metaphase chromosomes together with first 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) or a polarizing light microscope was used to monitor this step (Figure 7.1a,b), so 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 7.2). The reconstituted oocytes were then cultured in HTF medium for at least one hour. Oocyte activation for initiation of meiosis-like division was performed by a brief exposure to calcium ionophore A23187 (5 μM, 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 30 min intervals from 1 – 5 hours after reconstitution and returned to culture.

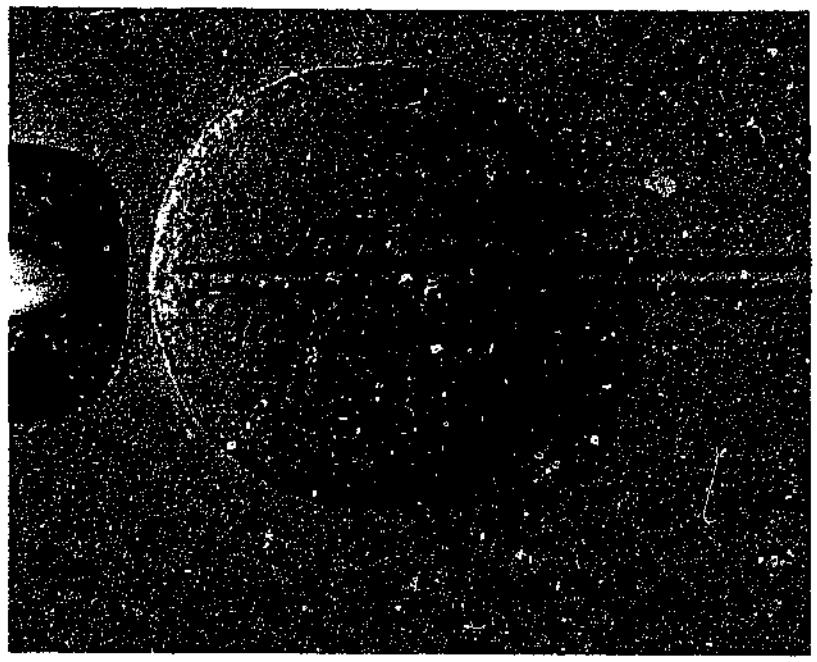

Figure 7.2 Injection of a somatic cell (arrow) into a human oocyte.

7.5 Assessment of pronucleus formation and cytogenetic analysis of zygotes

Activated oocytes were cultured for 16 – 20 hours in HTF medium and scored for morphological indicators such as extrusion of a PB or the appearance of a pronucleus. Because DNA replication occurs a few hours after pronucleus formation (Capmany et al., 1996) and followed by syngamy, the assessment of the original chromosomal complement of each individual PN required the prompt isolation of a PN leaving, therefore, a monopronucleated "haploid" oocyte (Figure 7.3). For cytogenetic analysis, some

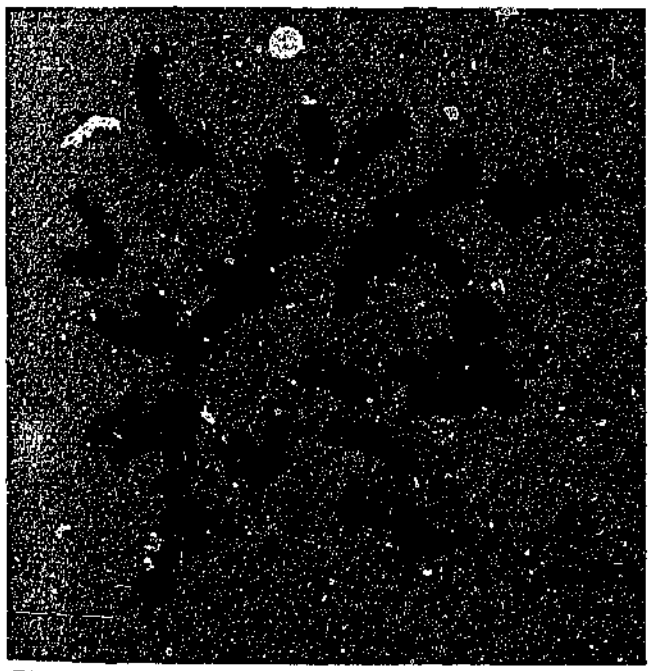

Figure 7.3 Twenty-three human metaphase chromosomes after fixation of a manipulated oocyte generated by injecting a human cumulus cell in a human ooplast (Giemsa) (600X).

activated oocytes were processed for two round FISH analysis with specific probes for chromosome 13, 14, 15, 16, 18, 21, 22, and X (Figure 7.4a,b) or incubated further for up to 24 hours to enter metaphase and then stained for individual chromosomes.

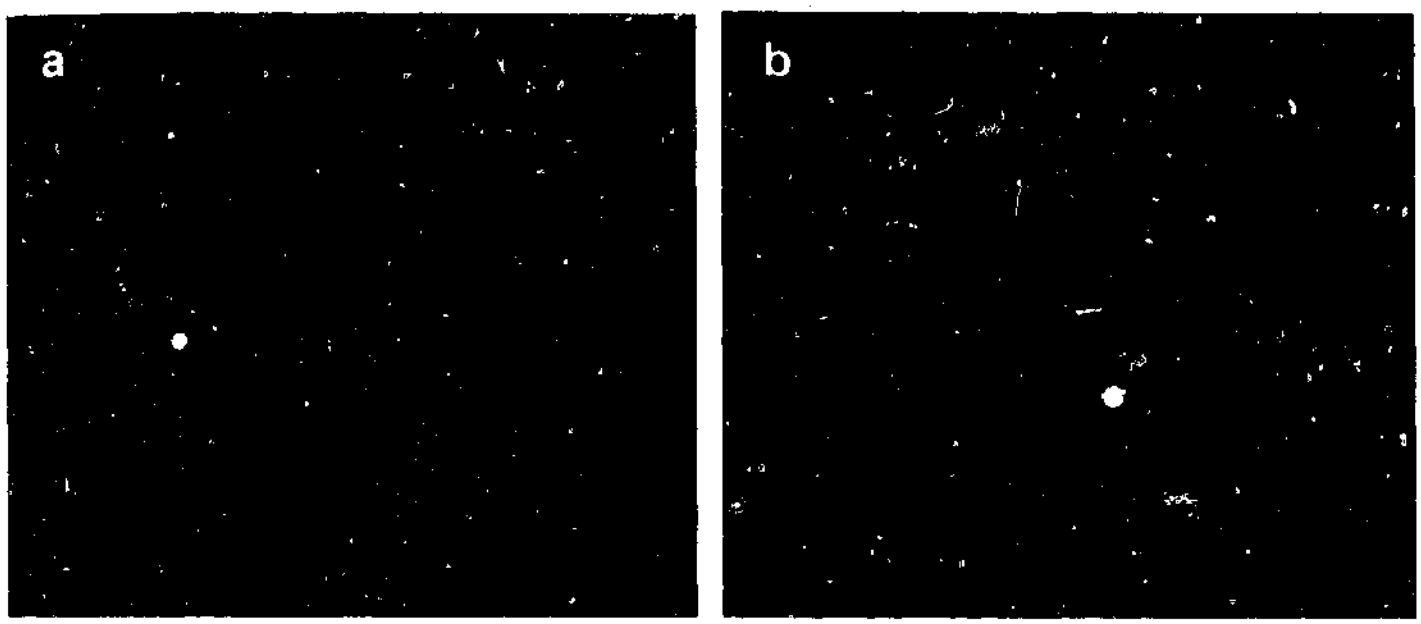

Figure 7.4 Cytogenetic analysis of a human haploidized pronucleus after somatic cell injection. **(a)** First round – red identifies chromosome 21 (1 signal), chromosome 18 appears in aqua (1 signal), green fluorochrome identifies chromosome 13 (1 signal), and yellow corresponds to chromosome X (1 signal). **(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).

7.6 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 18 – 20 hours 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 7-I).

Table 7-I. 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 vs b] χ^2, 2 x 2, 1 *df*, Effect of incubation time on survival, $P < 0.01$

[c vs d] χ^2, 2 x 2, 1 *df*, Effect of incubation time on activation, $P < 0.01$

[e vs f] χ^2, 2 x 2, 1 *df*, Effect of incubation time on 2PN, $P < 0.01$

As shown above, a pre-incubation period of more than 2 hours 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 2PNs had undergone haploidization.

7.7 The efficiency of nuclear transplantation and pronucleus formation in human oocytes

Of a total of 295 GV oocytes retrieved, 192 had reached MII after 24 hours incubation. Enucleation was successful in 159/192 and 119 ooplasts survived cumulus cell injection. However, while none of the activated survivors extruded a PB, 33 displayed 1PN, and 32 had 2PNs (Table 7-II). 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 analyzed. The monopronuclear oocytes were diploid as expected, and the remaining non-activated oocytes presented with diploid nuclei arrested at interphase or metaphase.

Table 7-II. The 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)

7.8 Sperm labeling, insemination, and fertilization assessment

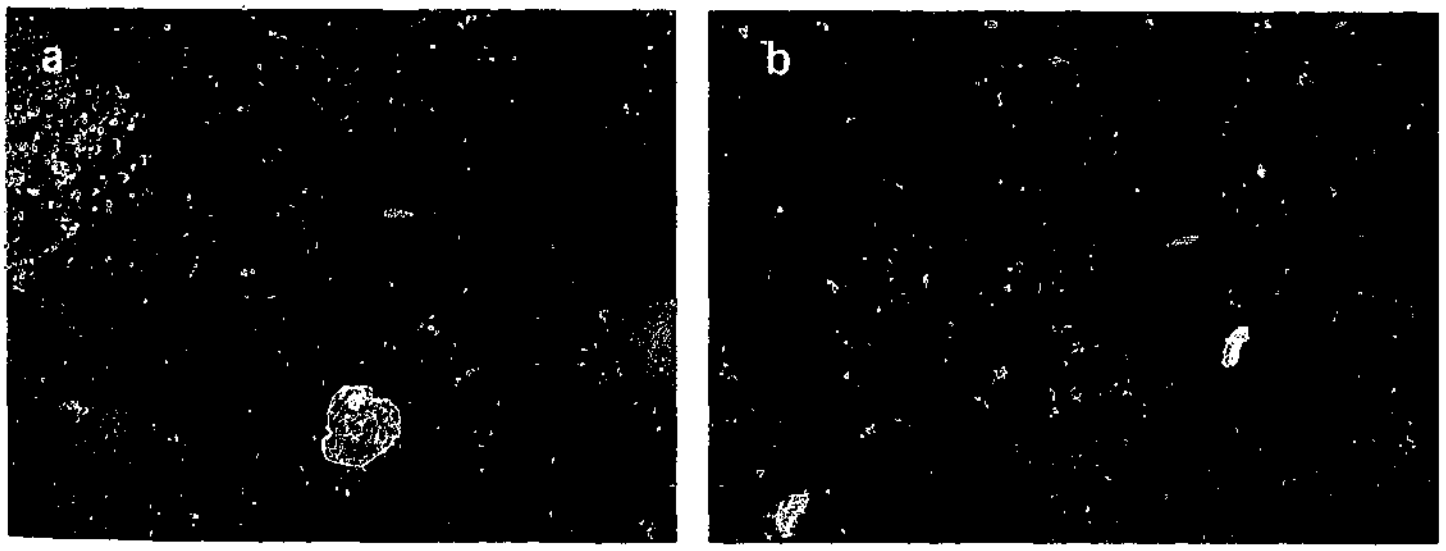

Figure 7.5 **(a)** Fluorescent labeling of the sperm mitochondrial sheath in free spermatozoa and **(b)** a spermatozoon in an injection pipette.

In some oocytes, a spermatozoon injected two hours after somatic cell transfer was tracked by midpiece mitochondrial labeling using MitoTracker Red® to identify the male PN (Figure 7.5a,b). Fertilization was assessed at 16 – 20 hours after ICSI. Haploidization was considered successful according to the development of three PNs — two from the haploidized somatic nucleus and one from the male gamete (Figure 7.6a,b).

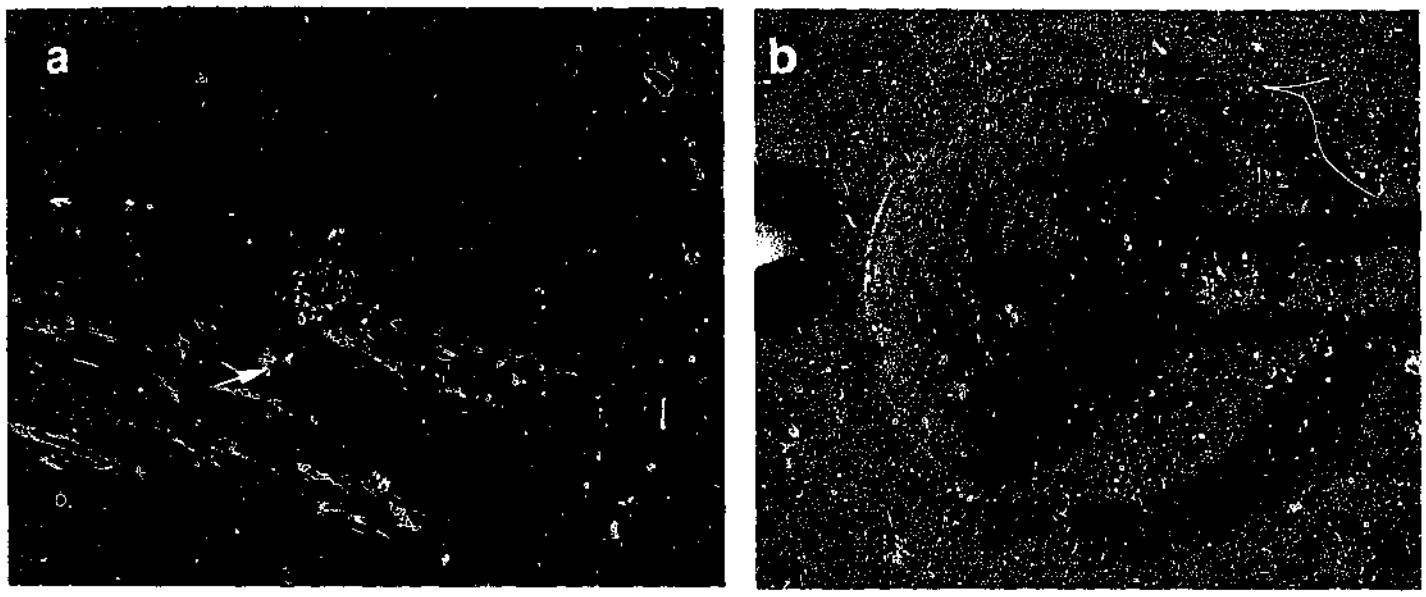

Figure 7.6 (a) The male pronucleus shows sperm mitochondrial labeling (white arrow) under fluorescence microscopy. (b) Removal of the additional female pronucleus (black arrow).

7.9 Restoration of diploidy and cytogenetics of human embryos generated from somatic cell haploidization

As in previous experiments with human oocytes matured *in vitro*, haploidization of somatic cell nuclei resulted in the formation of 2PNs, but without extrusion of a PB. The morphological characteristics of the PNs, 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%) such oocytes were activated — six had one PN, three had 2PNs, and four formed 3PNs. After identification of the male PN based on MitoTracker® Red labeling, a female PN was aspirated from each of the three zygotes, which later developed as far as the 6-8 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 7.7).

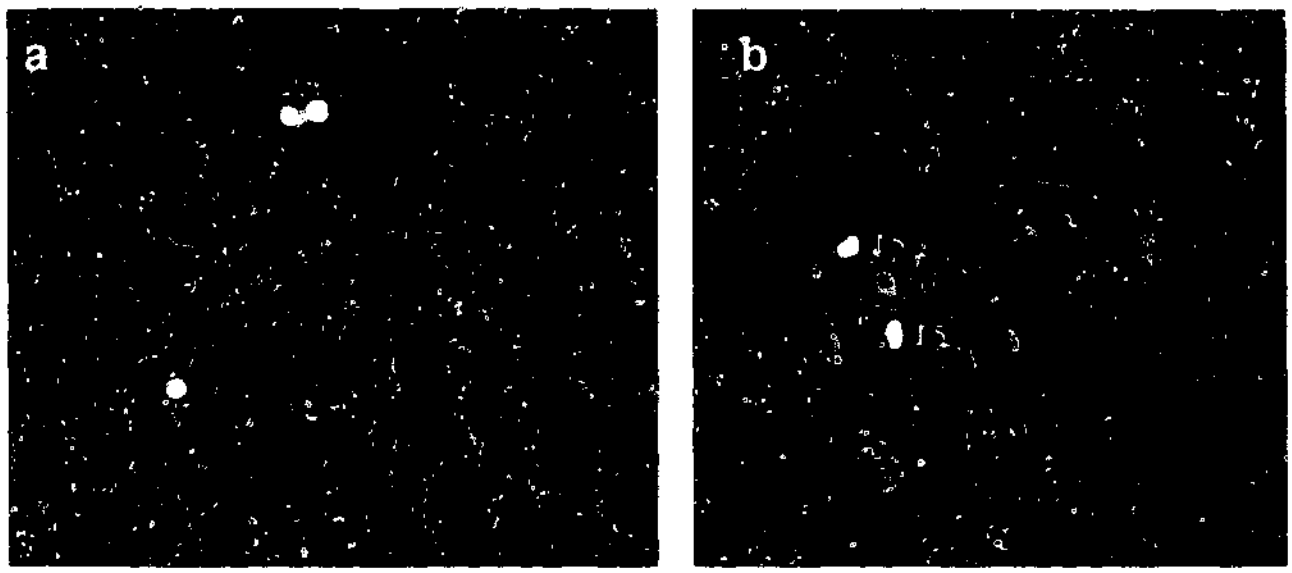

Figure 7.7 Cytogenetic analysis of an abnormal embryo obtained from the fertilization of a constructed human oocyte. Image generated an image analyzing software for FISH of a day 3 embryo. **(a)** First round FISH – yellow signal corresponds to chromosome Y (2 signals) and chromosome X appears blue (1 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).

7.10 Discussion

The morphological characteristics of the PNs, PBs, and blastomeres after 'manufacturing' mouse 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 complete preimplantation development. The impaired blastocyst formation seen in many such cases may be attributed to an unbalanced chromosomal segregation (Takeuchi et al., 2002b).

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). 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.

Regarding human oocytes, the 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 hours) for the somatic genome with the host ooplast is considered critical for reprogramming of transferred diploid

nuclei. Therefore, I used at least a 2 hour incubation for later experiments. Mature metaphase II 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 haploidization of human cumulus cells injected into mature human ooplasts (Takeuchi et al., 2001b; Tesarik et al., 2001).

Somatic cell nuclei can be successfully haploidized, forming distinct pronuclei in enucleated mature human oocytes (Takeuchi et al., 2001b; Kaneko et al., 2001), but somatic cell haploidization of *in vitro* matured human oocytes resulted in the formation of two PNs, without PB extrusion (Takeuchi et al., 2002b). This may be explained by an inability of the cytoplasm in this case to develop a proper meiotic spindle or by the inability of the chromosome's kinetochore to bind appropriately to it, and so a poor chromosomal segregation in the manufactured oocytes. Thus, the ability of GV and MII mouse oocytes to haploidize and extrude a polar body contrasts with that of human oocytes, which did not support haploidization while at the GV stage, nor generate a PB. This situation is similar to that in which pronucleate oocytes are generated after ICSI, i.e., decondensation of the chromatin to become a pronucleus instead of a polar body (Palermo et al., 1996b; 1996c).

Labeling 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 or even the presence of two competing centrosomes derived from the somatic cell and the spermatozoon, respectively (Takeuchi et al., 2002b).

Correct chromosome segregation is crucial in artificial haploidization. Meiotic chromosomes in an oocyte display a behavior different from that of the mitotic chromosomes in the somatic cell (Fulka et al., 2002a). 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 (Paliulis and Nicklas, 2000; Fulka et al., 2002a; 2002b). 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). The precise way in these single chromatids in G_0/G_1 cells separate correctly remains to be determined. Nevertheless, some artificially created oocytes are apparently chromosomally normal, as proven by FISH with a limited number of chromosomes-specific probes (Palermo et al., 2002a; 2002b). This unexpected behavior 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).

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). Moreover, the imprinted status of the somatic cell nucleus may be subjected to trans-modification of germ cell cytoplasm, as demonstrated in germ-somatic hybrids (Surani, 1999), or may be affected by epigenetic events in the early embryo (Reik et al., 1993). 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. The extent to which the genome's epigender is reflected in a gene expression pattern or a developmental phenotype depends on the cellular environment (Spielman et al., 2001), but because imprinting appears to be established very early during oogenesis (Obata and Kono, 2002), it is unlikely that fully grown GV stage oocytes would retain the capacity to erase and restore genomic imprints. 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.

Chapter 8

Genomic Imprinting

Declaration for Thesis Chapter 8

In the case of Chapter 7, contributions to the work involved the following:

	Name	% contribution	Nature of contribution
1	**Gianpiero D. Palermo**	70	Conception and design, collection, analysis, and writing of manuscript
2	**Yukiko Katagiri**	5	Execution of work
3	**Takumi Takeuchi**	5	Execution of work
4	**Scott Coonrod**	5	Execution of work
5	**Queenie V. Neri**	5	Execution of work
6	**Jenny Xiang**	5	Execution of work
7	**Salim Wehbe**	5	Execution of work

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Weill Medical College of Cornell University, The Center for Reproductive Medicine, New York, NY, USA

Signature 1 **Date** 10-29-2004
Signature 2 **Date** 11-19-2004
Signature 3 **Date** 11-19-2004
Signature 4 **Date** 11-19-2004
Signature 5 **Date** 11-11-2004
Signature 6 **Date** 11-22-2004
Signature 7 **Date** 11-24-2004

8.1 Summary

Manipulation of the types discussed heretofore may bring problems related to epigenetic factors that can bear on development. Offspring born from nuclear transfer-derived and *in vitro*-produced animal embryos may display multiple abnormalities, a high birth weight and an extended gestation length being predominant features of a phenomenon often called 'large offspring syndrome'. This untoward complications appear to result from epigenetic modifications, and primarily aberrant DNA methylation patterns. In mammals, the methylation state of DNA determines the pattern of transcription during development and cell differentiation, and it has been established that DNA methylation and histone modifications at specific imprinted gene loci play a key role in regulating gene expression.

Recent reports suggest an increased incidence of altered gene expression also in ART children, possibly as a result of gamete manipulation and/or exposure of embryos to extended *in vitro* culture that may produce epigenetic changes in the embryo and consequent developmental abnormalities. For example, an imprinting disorder involving genes for insulin-like growth factor II (*Igf2*) and *H19*, the Beckwith-Wiedemann Syndrome (BWS) (also known as the large infant syndrome), has been reported at a six-fold incidence in such children.

In an attempt to establish whether disorders observed following ART procedures are comparable to those in cloned offspring, we have studied mouse embryos generated *in vivo*, by parthenogenesis, by ICSI, and by cloning. To establish whether the micromanipulation culture

conditions and chemical exposure have any effect, I have performed histone modification analysis on conceptuses and have quantified fluorescence by specific monoclonal antibodies against histone tails. To establish a quantitative method of monitoring gene expression, we assessed *Igf2*, *H19*, and *Igf2r* in mature oocytes in cloned blastocysts, and in fetal tissues generated by nuclear transplantation.

Three types of embryos were compared. MII oocytes retrieved from oviducts of B6D2F1 strain 10 week old female mice and were fertilized by ICSI. Other oocytes were activated by exposure to Sr^{++}, and a third group enucleated, injected with cumulus cell nuclei then activated. Oocytes and zygotes were evaluated by immunofluorescence, utilizing antibodies against static and dynamic epigenetic marks of tails on histone H4, and studied by deconvolution microscopy. Embryos created *in vivo* served as controls. Total RNA was isolated from oocytes, embryos, and placentae of fetal tissues generated by 'rescuing' mitochondrially damaged oocytes by germinal vesicle transfer (GVTr, see Chapter 5). The genes assayed by reverse transcription and quantitative PCR were *Igf2*, *H19*, and *Igf2r*, with *Gapdh* expression serving as a reference.

Parthenogenetic activation was induced in a total of 37 oocytes, of which 32 displayed 2PN, and 27 developed into expanded blastocysts. Of 68 oocytes subjected to ICSI, 82.8% reached the blastocyst stage. Of 77 oocytes cloned by injecting G_0 cumulus cell nuclei into a previously enucleated MII ooplast, 50 constructs finally displayed 2PNs. Of those, 43 underwent a first cleavage and 15 became blastocysts. Histone modification analysis of the somatic cells prior to injection demonstrated an imprinting pattern that was reset after their transfer into fresh

ooplasm, as evidenced by deacetylation and demethylation of histone tails. The respective histone tail patterns were similar for all specimen types. After tissue dissection, the cell concentration was assessed, the total amount of RNA was estimated, and its quality determined. For the genes assessed, a high level of expression was identified in the GVTr-derived fetal *adnexa*.

Although cloned embryos reached the blastocyst stage and histone acetylation patterns were similar to those of *in vivo*-derived embryos, they developed poorly compared to those generated through ICSI or parthenogenesis. Histone modification analysis revealed that the ooplast can reprogram a somatic nucleus, therefore allowing it to behave as a gamete at least at the zygote level. Amplification analysis of the transcripts detected a loss in the GVTr generated fetuses of the imprinting pattern for key genes involved in placentation and early growth.

8.2 Introduction

Two decades have passed since the experiments that established the principle of nuclear non-equivalency (McGrath and Solter, 1983; Surani et al., 1984) indicating that uniparental diploid mouse embryos were non-viable. In fact, gynogenetic embryos failed to develop functional fetal *adnexa* while androgenetic conceptuses were retarded and failed to support embryonic development (McGrath and Solter, 1984; Surani et al., 1984). The explanation for this non-viability lay in the differential epigenetic modifications of maternal and paternal gametes (termed

imprinting), leading to incomplete development of either the embryo or the placenta (Reik and Walter, 2001). In other words, neither spermatozoa nor oocytes possess a full complement of the genes required for normal development, some of which are suppressed in the male and others in the female gamete. Such imprinting patterns and X chromosome inactivation provide the best examples of epigenetic regulation of gene expression in mammals.

The abnormalities often observed in cloned animals are considered to reflect the inappropriate expression of specific genes. Since imprinted genes are involved in regulating not only gamete maturation and implantation, but also embryonic development, imprinting errors can result in phenotypic abnormalities ranging from trophoblastic to single organ dysfunction (Jaenisch and Wilmut, 2001; Humphreys et al., 2002; Wilmut et al., 2002). The large majority of these abnormalities are early miscarriages occurring mainly as a result of abnormalities of the trophoblast (Hill et al., 2000; Inoue et al., 2002). While in such cases of full term development, many offspring die within the first 24 hours after birth due to respiratory distress and cardiovascular abnormalities (Wilmut et al., 2002). The offspring that survived were susceptible to infection or displayed some dysfunction of the central nervous system, or of other organs (Wilmut et al., 2002).

Germline epigenetic modifications in mammalian oocytes result in the repression of certain maternally inherited genes, defined as maternal imprinting (Reik and Walter, 2001). The paternal genome must pair for these maternally imprinted genes, therefore resulting in reciprocal imprints (McGrath and Solter, 1984; Surani et al., 1984). As a consequence of this process, both parental

genomes are essential for normal development, with experiments in the mouse suggesting that the paternal component is more important for development of the trophectoderm, and the maternal genome for the embryo proper (McGrath and Solter, 1983; Surani et al., 1984).

The sex-related imprinting patterns are modified at two points of development. The first takes place during germ cell maturation where a loss of DNA methylation is linked to an erasure before subsequent resetting of the imprint that characterizes the developing gametes (Monk et al., 1987; Hajkova et al., 2002; Lee et al., 2002). The second occurs throughout the preimplantation period, during which imprinting is maintained despite a general loss of DNA methylation. Thus a series of epigenetic modifications are reflected in the oocyte both prior to, and immediately after, fertilization (Surani, 1998; Wright, 1999).

Nuclear transplantation has shown that maternal imprinting occurs during preovulatory growth of oocytes (Kato et al., 1999; Kono et al., 1996; Obata et al., 1998). At this time, the modification of the paternal X chromosome ensures that it will later be inactivated in trophectoderm and primary endoderm cells (Tada et al., 2000). Prior to fertilization, the DNA of both oocyte and sperm is highly methylated. In the zygotes, however, whereas the full pronucleus is the site of further *de novo* methylation (Monk, 1987; Mayer et al., 2000; Oswald et al., 2000), a remarkable loss of methylation occurs in the early decondensation stage of the male pronucleus (Mayer et al., 2000; Oswald et al., 2000; Dean et al., 2001; Santos et al., 2002; Lane et al., 2003).

Transplantation of a somatic nucleus into the oocyte has the potential to restore its totipotency by reversion to an embryonic pattern of gene expression (Solter, 2000). While imprinted genes may be largely unaffected during such reprogramming, there are nevertheless instances where imprints may be erased, leading to anomalies of fetal and placental growth (Solter, 2000). Although it appears that telomere length is restored in the somatic nucleus in most of such cases, much of the loss of DNA methylation cannot be replaced. Although, such mutation may be tolerated in a differentiated cell, it can be lethal at earlier stages, explaining the low rates of development following transplantation of somatic nuclei. This contrasts with the higher rate of development following transplantation of nuclei from pluripotent embryonic stem (ES) cells (Rideout et al., 2000), suggesting that the frequency of normal development could be improved by techniques capable of selecting and modifying the epigenetic status of somatic nuclei. Some of the anomalies encountered in cloned embryos point to a disruption of imprinted gene expression at this level (Tamashiro et al., 2000).

As noted above, imprinted genes regulate crucial aspects associated with reproduction, embryogenesis, fetal growth, placentation, energy homeostasis, lactation, and behavior (Bartolomei and Tilghman, 1997; Tilghman, 1999; Lefebvre et al., 1998; Li et al., 1999). So far, 73 imprinted genes have been identified in mice, and 49 in humans. To take two specific examples, the gene for insulin-like growth factor 2, *Igf2*, is active only in the paternal genome (De Chiara et al., 1991), the converse being true for *H19* (Ferguson-Smith and Surani, 2001; Reik and Walter, 2001; Tilghman, 1999). The *Igf2* codes for a mitogenic peptide with important roles in the control

of embryonic and placental growth (Efstratiadis, 1998), and is also implicated in the pathogenesis of some human cancers as well as the overgrowth associated with BWS (Maher and Reik, 2000; Feinberg et al., 2002; Tycko and Morison, 2002). *Igf2* (or Somatomedin A) constitutes with *H19* (or Adult Skeletal Muscle Gene; ASM1), a set of physically and functionally linked imprinted genes located in an evolutionarily conserved chromosome region (human 11p15.5 and mouse distal 7) (Brannan and Bartolomei, 1999; Reik et al., 2001).

In the normal placenta, *H19* is abundantly expressed in the villous stroma (Ariel et al., 1994) as well as in the villous cytotrophoblast and the intermediate trophoblastic cells of the cell columns and implantation site, but not in syncytiotrophoblast which is the end stage of differentiation of the trophoblastic cell lineage (Mutter et al., 1993; Ariel et al., 1994). Jinno et al. (1995) found that *H19* is monoallelically (maternally) expressed in the human placenta after 10 weeks of gestation, whereas it is biallelically expressed at earlier stages. Regardless of *H19*'s biallelic or monoallelic expression, *Igf2* is monoallelically (paternally) expressed in the placenta. The *H19* gene is expressed also in a number of other tissues during a restricted period of fetal development, and in embryonic carcinoma cells after induction of differentiation. This complex organization of imprinted genes means that any disruption of such clusters through chromosomal translocation or epigenetic mutations can result in their anomalous expression within the cluster in some cases, and this may account for some of the disorders associated with growth, neurogenetic disorders, and diabetes (Tilghman, 1999; Ferguson-Smith and Surani, 2001; Reik and Walter, 2001). Quantitative PCR may represent a valuable tool through which to assess expression of key genes

including *H19* and *Igf2* in conceptuses established by gamete manipulation, or exposed to extended *in vitro* culture (Ogawa et al., 2003 ; Katagiri et al., 2004).

Caution should be taken in assessing risks related to imprinting. In all cases, syndromes related to this have arisen through an aberrant methylation or demethylation of maternal imprints. BWS is associated with identical twinning, which itself carries a risk of anomalies or death for developing fetuses (Clayton-Smith et al., 1992). Imprinting mutations leading to Angelman Syndrome (AS) occur at a frequency of 5 – 8% in ART patients, an approximately 10-fold increase over that in the general population (Cox et al., 2002; Ørstavik et al., 2003). Maternal imprints at the 15q11-13 sites displayed inappropriate hypomethylation without complete deletion; moreover they were not gametic and arose during oocyte maturation or fertilization, perhaps due to IVF, ICSI or *in vitro* culture (Cox et al., 2002). Most of the evidence points to factors related to *in vitro* culture as the primary causes of imprinting defects. The large offspring syndrome observed in cattle and sheep is considered to be related to the serum components of the culture medium, which may hypomethylate the element controlling *Igf2r* imprinting in preimplantation embryos (Hoshi, 2003). Paternally expressed *Igf2* in mammalian placentae, along with other imprinted genes, regulates placental supply and fetal nutrition (Constancia et al., 2002). Imprints arise variously; some are related to the sex of the gamete, others are imposed on oocytes and early embryos, and some arise during differentiation at particular developmental stages (Reik and Walter, 2001). Interference with them can distort growth patterns and lead to birth defects

(Niemitz and Feinberg, 2004). Exposing animal and human embryos to media containing serum could impair the development of normal imprinting patterns.

At the very heart of the issues related to epigenetic reprogramming, and some raised by the experiments described here, is the restoration of developmental plasticity in cells that have already proceeded along a path of differentiation, and so to a loss of totipotency. The sequential restrictions involved in the latter are associated with changes that influence the transformation of euchromatin to facultative heterochromatin, in which transitional DNA methylation is thought to play a critical role (Bird, 2002; Jenuwein and Allis, 2001). For chromatin components, the information that dictates the shift to active or inactive transcriptional configurations is embodied in the modifications of the amino terminal tails of core histones of the nucleosome. These covalent modifications, which include acetylation, phosphorylation, methylation, poly-adenosine diphosphate (ADP)-ribosylation, and ubiquitination, have been suggested to represent an epigenetic lexicon or 'histone code' (Jenuwein and Allis, 2001; Turner, 2000; Strahl and Allis, 2000). Acetylation of the histones occurs at the ε-amino group of specific lysine within the N-terminus. Specific histone acetyltransferases transfer an acetyl group from acetyl-CoA (co-enzyme A) as a donor molecule to the histones. As the turnover of acetylated lysine is relatively high, this modification is perfectly suited for a rapid regulation of chromatin structure (Imhof and Becker, 2001). In 1964, Allfrey and coworkers at Rockefeller University observed a preferential association of acetylated histone isoforms with actively transcribed gene loci. Another post-translational modification, which occurs on the tail domain(s), is the phosphorylation of serine residues (Imhof and Becker, 2001). Phosphorylation correlates with mitosis and provides an

excellent marker for dividing cells in immunohistochemistry studies. Less is known about the impact of histone methylation on the structure and function of chromatin. Nevertheless, as it is also a modification of the tail domain, it may have a structural impact similar to the one caused by the better-characterized acetylation (van Holde, 1988). The major differences between methylation and acetylation are the turnover rate and the fraction of nucleosomes modified *in vivo*. Whereas histones are rapidly acetylated and deacetylated again with only a small percentage being acetylated at more than one site, the methylation of histones seems to be a rather stable modification (Imhof and Becker, 2001). Poly-ADP-ribosylation is a rare nuclear modification that is catalyzed by the nuclear poly-ADP-ribose polymerase. This enzyme catalyzes the synthesis of large polymers of ADP-ribose consisting of up to 250 residues on various nuclear substrates such as histones, high mobility group (HMG) proteins, topoisomerases, and itself (Boulikas, 1992) using nicotinamide adenine dinucleotide (NAD^+) as a substrate. The ADP-ribose polymer is thought to resemble a DNA molecule and to lead to a displacement of the nucleosome allowing the repair machinery to gain access to the damaged sites (Althaus, 1992). Ubiquitin is a small, 76 amino acid long polypeptide, which is attached as a polyubiquitin polymer to the ε-amino group of lysine residues in polypeptides targeted for proteasomal degradation. However, ubiquitination of histones has a different effect. Again, the effect of this modification on the structure and function of chromatin is not known (Imhof and Becker, 2001). It has been observed that ubiquitinated H2A and H2B disappear during metaphase and reappear during anaphase suggesting an involvement of this modification in chromatin condensation (Mueller et al., 1985).

Recently it has been demonstrated that the status of certain histone modifications (such as acetylation) at key imprinting gene loci is important for silencing of imprinting diseases such as Angelman, Prader-Willi, and Beckwith-Wiedemann syndromes (Higashimoto et al., 2003). In that regard, specific amino acid residues within the N-terminal histone tail region are targets for a number of post-translational modifications (Strahl and Allis, 2000). Histone acetylation and deacetylation by histone acetyltransferases (HAT's) and histone deacetylases (HDACs) respectively, provide for a rapid and reversible mechanism for regulation of gene expression (Kuo and Allis, 1998). Methylation of histone arginine residues represents a more recently characterized post-translational histone modification which, as with acetylation, is associated with activation of gene expression (Wang et al., 2001). In contrast to the dynamic nature of histone acetylation, histone methylation has previously been thought to be relatively "permanent", similar to that of DNA methylation (Bannister et al., 2002).

Given this current background, I focused on epigenetic markers for a selected small group of genes in mouse embryos generated *in vivo* by parthenogenesis, by ICSI, and by cloning to establish rigorously whether disorders observed in the ART system and in cloned offspring are comparable. A sophisticated microgenetic procedure of histone modification analysis was utilized to establish whether the micromanipulation culture conditions, and chemical exposure have any effect as well.

The genes in question being explored were *Igf2*, *H19*, and *Igf2r*, these being assessed in different mouse tissues as well as in oocytes matured *in vivo*. Placental tissue generated by germinal

vesicle transfer was also assessed and compared with fetuses conceived *in vivo*. Similarly, blastocysts obtained by cloning were assessed by the same methodology.

8.3 Specimen isolation and processing for histone modification analysis

MII oocytes were retrieved from oviducts of B6D2F1 mice after ovarian stimulation, and freed from cumulus cells by a brief exposure to hyaluronidase. Oocytes were fertilized by ICSI using spermatozoa obtained from the cauda epididymides of B6D2F1 strain. Other MII oocytes were parthenogenetically activated by exposure to Sr^{++}. Finally, a third group was enucleated, injected with cumulus cell nuclei, and then activated, with cleavage being followed up to the blastocyst stage as described in Chapter 7. Oocytes were processed for immunofluorescence analysis with several different anti-histone antibodies for stable epigenetic marks, namely H4/H2A phosphoserine 1 [Ph(Ser1)H4/H2A] (H4/H2A-S1), and for dynamic marks, histone H4 acetyl lysine 5 [Ac(Lys5)H4] (H4-K5), histone H4 methyl arginine 3 [Me(Arg3)H4] (H4-R3), and observed under a deconvolution microscope (Table 8-I). For anti-histone modification analysis, as positive control, somatic cells were derived from an established cell line (Kim et al., 2003). As a negative control, permeabilized mouse spermatozoa were used (Figure 8.1). To confirm adequate membrane permeabilization monoclonal anti-protamine antibody was utilized (Sarmento et al., 2004). Embryos obtained *in vivo* served as controls. Spare human oocytes donated from

consenting patients undergoing ICSI treatment were studied at either the germinal vesicle stage or the MII stage after *in vitro* maturation.

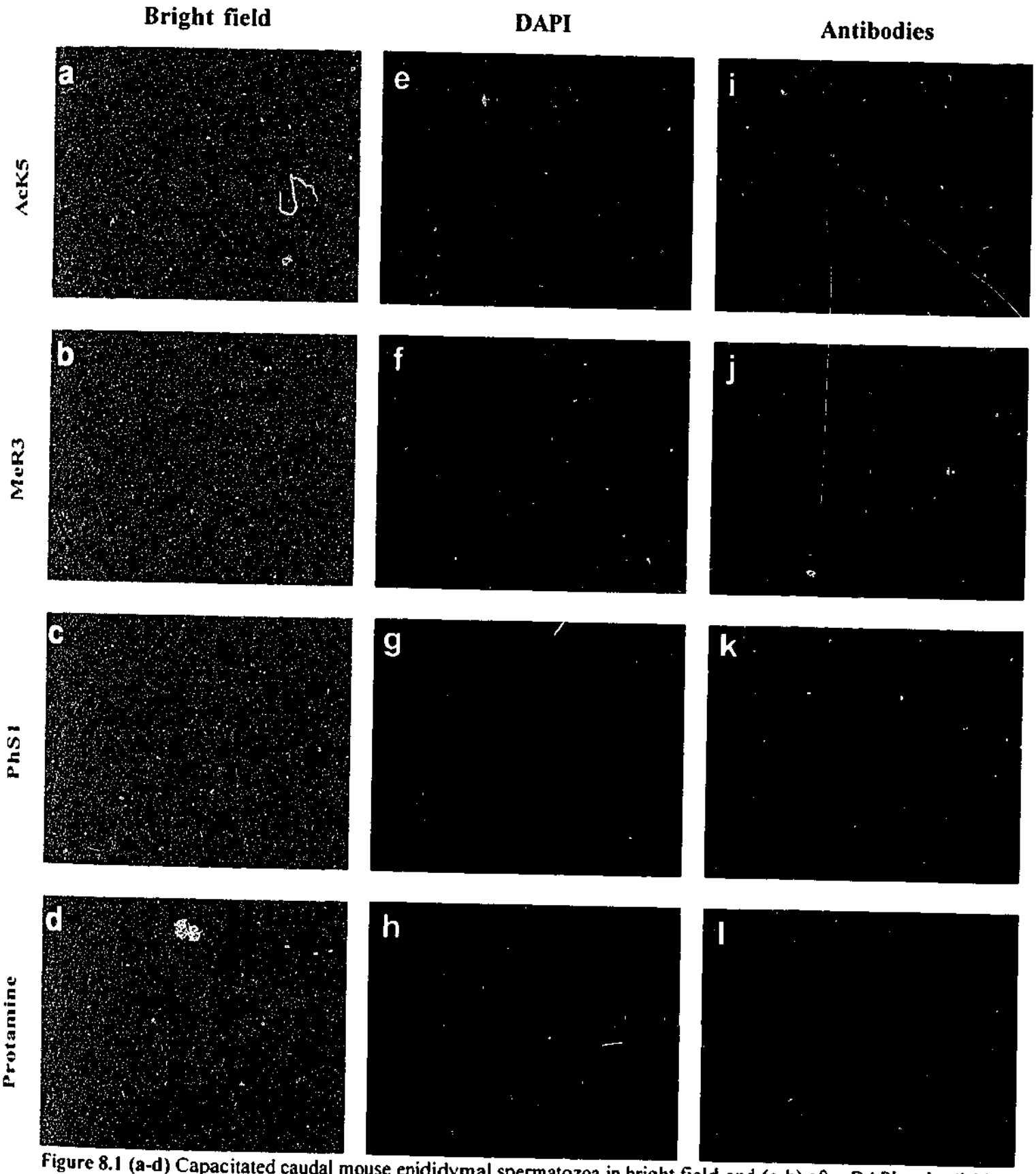

Figure 8.1 (a-d) Capacitated caudal mouse epididymal spermatozoa in bright field and **(e-h)** after DAPI stain. **(i-k)** Antihistone modification polyclonal antibodies did not react with mouse sperm proteins. (l) The protamine control was included to ensure that the permeabilization protocol was sufficient to allow for antibody access to chromatin.

Table 8-I. Effect of specific histone modifications on gene activity

Histone modification	Gene activity	Reversible	Antibodies	Abbreviation
Methylation	**expressed**	no	Arg-3 H4	H4-R3
Acetylation	**expressed**	yes	Lis-5 H4	H4-K5
Phosphorylation	**expressed**	yes	Ser-1 H4/H2A	H4/H2A-S1

Oocytes and embryos were fixed for 20 min in a solution of 4% paraformaldehyde in PBS and then washed three times with 1% BSA-supplemented PBS. Fixed oocytes and embryos were permeabilized in 0.5% Triton-X100 in 1% BSA-supplemented PBS for 20 min, washed, and incubated overnight at 4°C in the appropriate antibody diluted in 1% BSA-supplemented PBS. The anti-histone (rabbit) antibodies were used in the following dilutions: H4/H2A-S1 1:2000, H4-K5 1:500, H4-R3 1:200. The embryos and oocytes were then extensively rinsed and incubated in Texas red-conjugated donkey anti-rabbit IgG (2.5 μg/ml) for 1 hour at room temperature. Embryos and oocytes were again rinsed, placed in a 'slow-fade' equilibration medium for approximately 1 min, mounted on slides in glycerol 'slow-fade' mounting solution, and studied at 1000X using an Axiovert 200 3D Deconvolution fluorescent microscope (Axiovision; Carl Zeiss, Göttingen, Germany).

The intensity of fluorescence was quantified as previously described (Aoki et al., 1997). In brief, the pixel value of fluorescence was measured within a constant area from five different regions of

chromosomes and five different regions of cytoplasm, and the average cytoplasmic value was subtracted from the average chromosomal value.

8.4 Processing of specimens for RNA extraction

Ten week old B6D2F1 strain female and male mice were sacrificed by CO_2 overdose and tissues were dissected in PBS (Invitrogen #14040-141; Gibco, Carlsbad CA USA) with sterile microforceps and needles. PBS was reconstituted with 0.1% diethylpyrocarbonate (DEPC) (Sigma #D5758) treated water. To remove any nucleic acid contaminant, DEPC treatment included an overnight incubation at room temperature and then autoclaving for 20 min prior to use. Cell concentrations were assessed with CELL VU® disposable counting chambers (Millennium Sciences Corp, New York, NY, USA). Cell integrity and characteristics were assessed by Testsimplets® (Boehringer Mannheim, Mannheim, Germany). MII oocytes were retrieved 15 hours after an injection of hCG given 48 hours after PMSG. The cumulus oophorus was removed partly by brief exposure to hyaluronidase (300 IU/ml, Type IV-S; Sigma Chemical Co.) and finally by aspirating it through a calibrated glass pipette. Disposable, sterile, DNase/RNase-free pipette tips (Molecular BioProducts, San Diego, CA, USA) and culture safe disposable plasticware (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) were used during the entire procedure. Two fetuses and their *adnexa* generated by nuclear transfer rescue of photoirradiated oocytes (see Chapter 5) were also processed for RNA isolation. The fetuses

were delivered at day 19 – 20 by cesarean section. Three similarly aged fetuses obtained by *in vivo* conception served as controls.

8.5 RNA isolation, quantification, and qualification

Total RNA was isolated from tissue by Absolutely RNA® Microprep Kit (Stratagene, La Jolla, CA, USA) and from pooled MII oocytes by Absolutely RNA® Nanoprep Kit (Stratagene), including a DNase treatment step. Elution buffer was warmed to 60°C to increase the RNA yield. RNA was stored at -80°C until use, with a small portion ranging between 1 – 5 µl being used for the quantification and qualification analysis. The total RNA was quantified by NanoDrop® Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Total RNA was qualified using Agilent Bioanalyzer 2100 with either a Nano LabChip or Pico LabChip (Agilent Technologies, Palo Alto, CA, USA) according to RNA content.

8.6 Primer design

Primers were custom-designed by OligoPerfect™ Designer software (Invitrogen) with a final primer concentration of 200 nM, a TM of 60°C, and a salt concentration of 73.8 mM. The design was based on mRNA sequences obtained from the GeneBank: *Gapdh* [Accession No. NM008084] served as an endogenous control and the assayed genes were *Igf2* [Accession No.

NM010514], *H19* [Accession No. AK003142], and *Igf2r* [Accession No. NM010515] (Table 8-II) and analysis was carried out by using an ABI Prism 7900HT (Applied Biosystems, Foster City, CA).

Table 8-II. Primer sequences utilized for qRT-PCR

Genes		Sequence	Amplicon length (bp)
Gadph	F	5′-GAT GAC ATC AAG AAG GTG GT-3′	177
	R	5′-ATA CCA GGA AAT GAG CTT GA-3′	
Igf2	F	5′-GGC AAG TTC TTC CAA TAT GA-3′	124
	R	5′-CCT CTC TGA ACT CTT TGA GC-3′	
H19	F	5′-CTG CAA TCA GAA CCA CTA CA-3′	227
	R	5′-GAA AAA GAC AGG AGG GAG AT-3′	
Igf2r	F	5′-GCT GCA TAA GAA GGA GAG AA-3′	300
	R	5′-GAG TGA ACT TTC ACC TCT GG-3′	

F = forward; R = reverse

8.7 Reverse transcription & real-time PCR

Reverse transcription (RT) and quantitative PCR (qPCR) were performed utilizing SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen) capable of cDNA synthesis from 10 pg to 1 µg total RNA. Real-time PCR was performed using the ABI PRISM 7900 sequence detector (Applied Biosystems) where templates were amplified up to 45 cycles

with denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. Uracil-DNA glycosylase (UDG) treatment was performed at 50°C for 2 min and immediately followed by PCR amplification. The qPCR results were plotted by the Sequence Detection System Analysis Software v. 2.0 (Applied Biosystems). Gene expression was reported as a percentage calculated on the cycle threshold against *Gapdh* considered to represent 100% expression. Samples were always analyzed in replicates.

8.8 Analysis of histone tail modification

Parthenogenesis was induced in a total of 37 oocytes, of which 32 displayed 2PN and 27 reached the fully expanded blastocyst stage. Of 68 oocytes fertilized by ICSI, 94.1% survived and were fertilized, and of these 82.8% reached the blastocyst stage. Of the 77 oocytes cloned by injecting somatic nuclei into MII ooplasts, 89.6% survived the procedure with 50 constructs finally displaying 2PNs. Of those, 43 underwent first cleavage and 15 became fully expanded blastocysts — a significantly lower rate than that in the parthenotes and ICSI treated oocytes ($P < 0.0001$) (Table 8-III).

Table 8-III. Development of *in vivo*, ICSI, cloned, and parthenogenetic embryos

No of (%)	*In vivo*	ICSI	Clone	Parthenote
Intact		68	77	37
Activated/fertilized	55	64 (94.1)	50 (64.9)	32 (86.5)
2-cell	54 (98.2)	64 (100)	43 (86.0)	31 (96.9)
Blastocyst	52 (94.5)	53 (82.8)	15 (30.0)	27 (84.4)

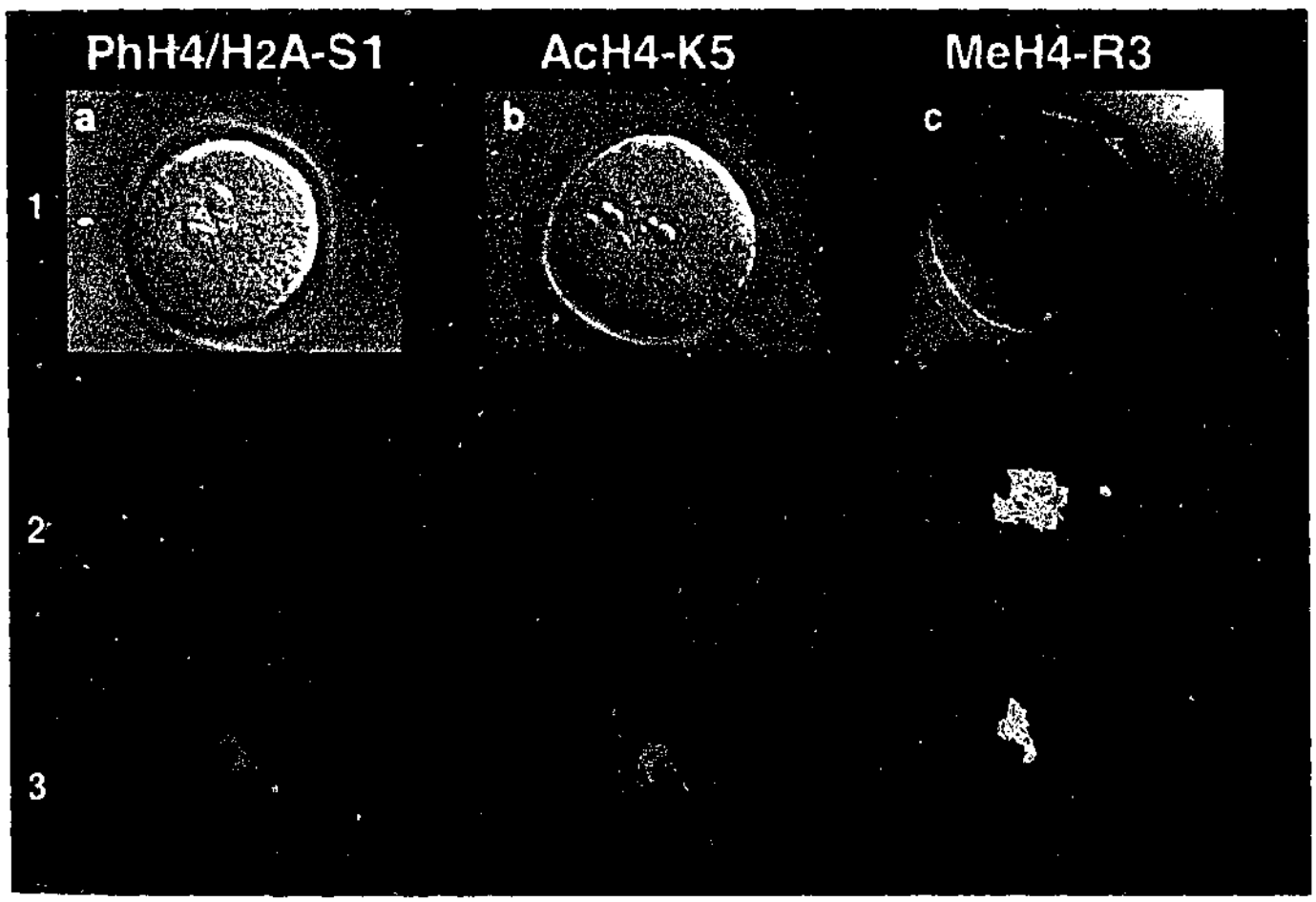

Figure 8.2 Immunofluorescent histone modification markers — **(a)** phosphorylation [PhH4/H2A-S1], **(b)** acetylation [AcH4-K5], **(c)** methylation [MeH4-R3]) — **(a,b)** in mouse zygotes, and **(c)** an enucleated mouse MII oocytes immediately after injection with a cumulus cell nucleus. **(1, a-c)** Bright field microscopy; **(2, a-c)** DAPI; and **(3, a-c)** Texas Red.

Histone modification analysis of the somatic cells prior to injection demonstrated an imprinting pattern that was reset after the transfer into fresh ooplasm, as evidenced by deacetylation and demethylation of histone tails (Figure 8.2). Zygotes created *in vivo*, parthenogenetically activated, ICSI, and cloned, were similar for all three histone marks (Table 8-IV).

Table 8-IV. Assessment of histone modification in somatic cells, mature oocytes, *in vivo* generated, and cloned conceptuses

	Antibodies		
Specimen	PhH4/H2A-S1	AcH4-K5	MeH4-R3
SC (interphase)	+	+	+
SC (metaphase)	+	+	+
GV oocytes	+	+	+
MII oocytes	+	-	-
NT construct (within 30 min)	+	+	+
NT construct (at 3 hours)	+	-	-
In vivo 2PN	+	+	-
Parthenotes 2PN	+	+	-
ICSI 2PN	+	+	-
Cloned 2PN	+	+	-

In order to evaluate the effect of nuclear cloning on the global abundance of specific histone modifications during the formation of metaphase chromatin, oocytes were fixed and stained

either: 1) immediately after somatic cell transfer, 2) after 3 hours incubation, and in another group, 3) 9 hours later or 6 hours after the activating stimulus. As with germinal vesicle stage oocytes, staining was positive for the H4/H2A-S1, H4-K5, and H4-R3 positions in the microinjected somatic cell nuclei. Following 3 hours incubation, there was no apparent reduction in the phosphorylation level of H4/H2A-S1 in the metaphase chromatin of cloned embryos as well as the MII eggs, while no acetylation was apparent for the H4-K5, nor methylation for H4-R3. Thus, the histone modification appeared similar in the cloned- and *in vivo*-derived embryos. As judged by the modifications of the histone tails, it appears that somatic nuclei underwent reprogramming and re-imprinted correctly in cloned conceptuses. Levels of PhH4/H2A-S1 were also maintained in human oocytes during *in vitro* maturation from the GV to the MII stage.

8.9 Gene array

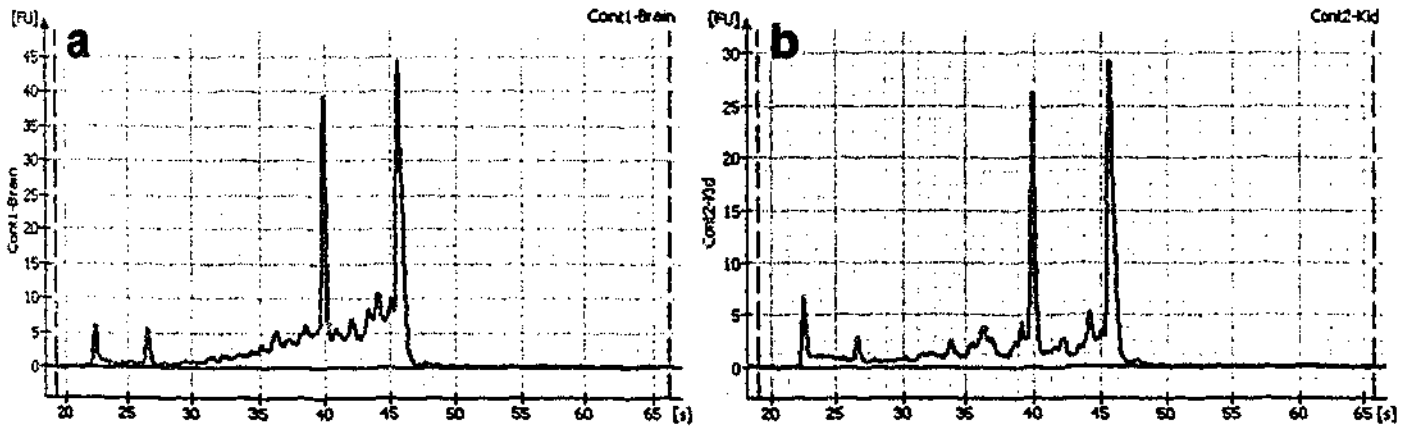

Figure 8.3 RNA qualification with the correct ratio of the two ribosomal subunits, 18S and 28S, in mouse (a) brain and (b) kidney.

After its homogenization, the cell concentration ranged between 5.7 x 10^6/ml to 0.8 x 10^6/ml depending on the tissue examined. The amount of RNA estimated per cell ranged between 25.3 pg (testis) and 31.2 pg (ovary). The concentration for a single oocyte or blastocyst was not assessable. Qualitative analyses of rRNA showed two clear peaks at 18S and 28S (Figure 8.3a,b).

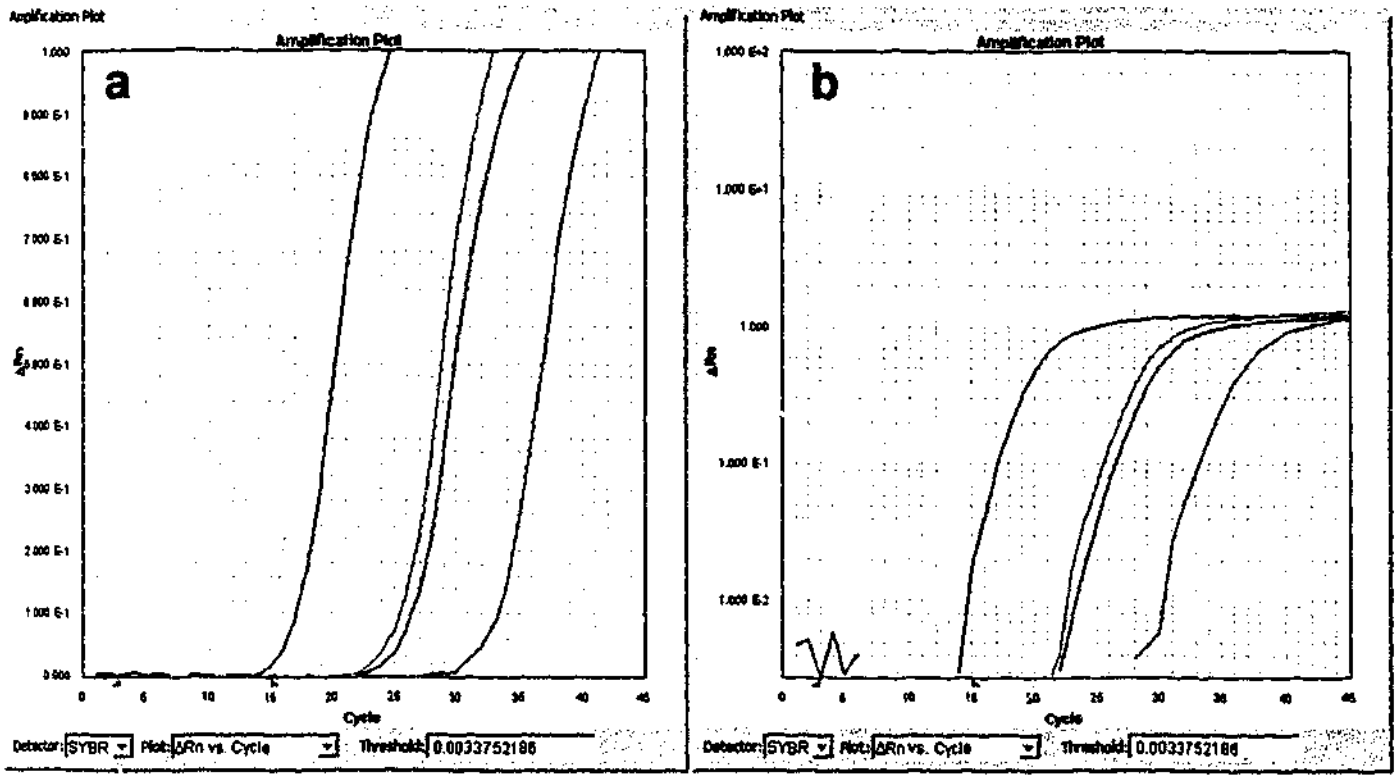

Figure 8.4 Gene expression plotted as cycle threshold in relation to amplicon content. **(a)** Linear and **(b)** logarithmic plotting of control gene (*Gapdh*) and study genes (*Igf2, H19, Ifg2r*).

Once qRT-PCR was run, linear and logarithmic plotting (Figure 8.4a,b) revealed that expression of *Gapdh*, the internal control, was consistent in all samples with an average cycle threshold ranging from 15.3 to 22.9, and was considered to be completely expressed. Expression of *Igf2* in ovary was 65.2%, in testis 64.3%, and in the oocytes 83.2%, but this was not expressed in the liver. For *H19*, the same pattern of expression was observed for each of these tissues (66.0%, 62.4%, and 66.3%, for liver, ovary, and testis, respectively) and with no expression in oocytes. *Igf2r* expression was 67.6% in liver, 60.5% in ovary, 61.6% in testis, and at 78.3% (P = 0.03)

was highly expressed in MII oocytes. Although there was a trend to higher expression for all genes analyzed, there was a significant difference of expression in the *Igf2* (P = 0.01) and *H19* (P = 0.0006) in GV transferred oocyte placentae (Figure 8-5). As predicted by the histone modification analysis, the expression pattern of cloned blastocysts was comparable to that in the controls.

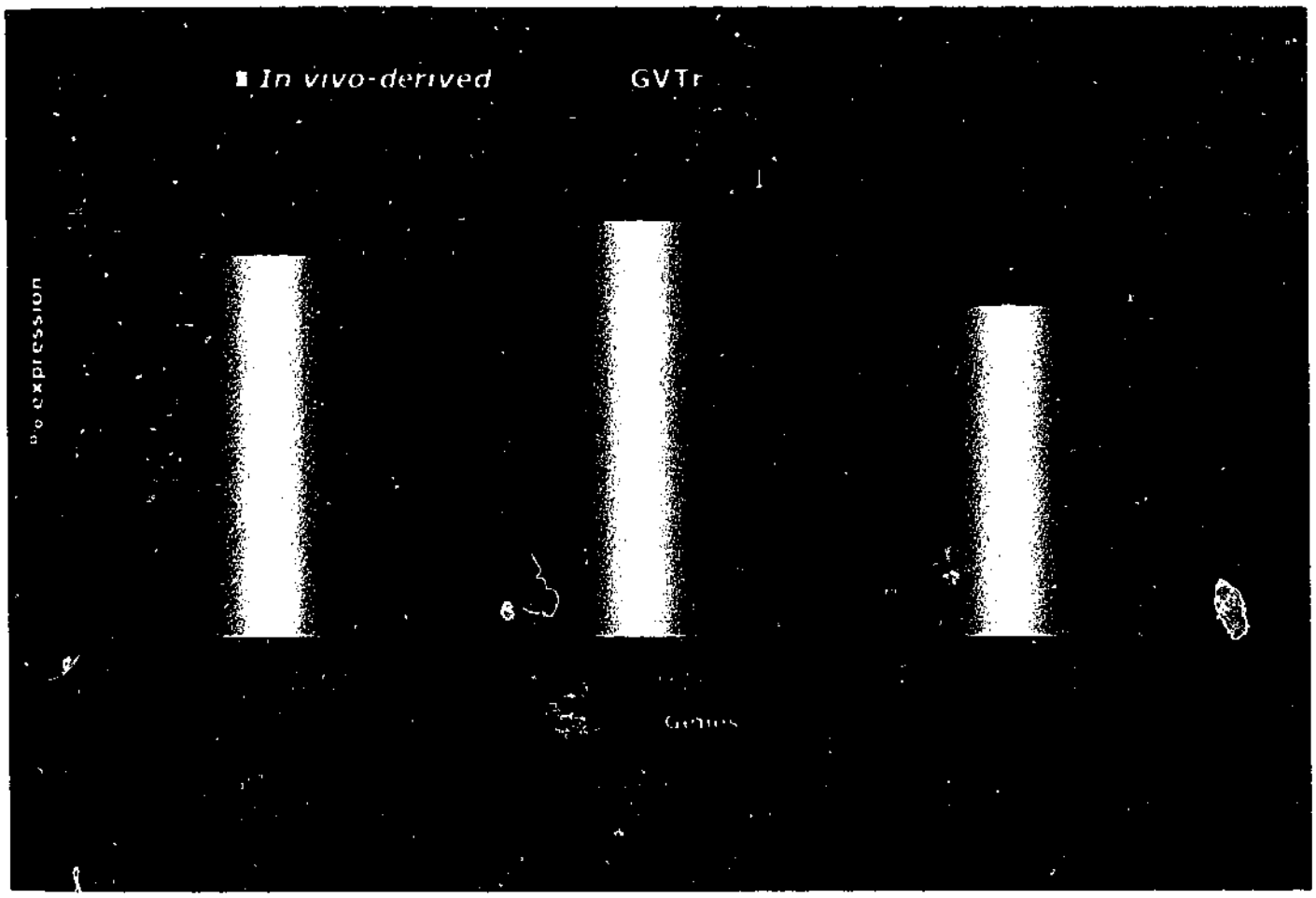

Figure 8.5 Gene expression of mouse GVTr and *in vivo*-derived placentae.

Three fetuses generated by spontaneous conception were delivered at day 19 by cesarean section with fetal weights of 1.4, 1.3, and 1.8 g. The respective placental tissues weighed 0.13, 0.12, and 0.15 g. Fetuses generated from photoirradiated oocytes rescued by nuclear transfer were delivered

at day 20 by cesarean section. Their weights were 2.0 g and 1.4 g for fetus 1 and 2, respectively. The placental tissues weighed at 0.51 g for fetus 1 and 0.20 g for fetus 2.

8.10 Discussion

The present study of a linked group of genes was stimulated by an increasing concern that some of the procedures involved now (or in the future) in ART may predispose to defects in the imprinting pattern of the conceptus. Although 30% of cloned embryos reached the blastocyst stage, their development *in vitro* was significantly impaired in comparison with embryos derived from ICSI or parthenogenetic activation. Nevertheless, their histone acetylation patterns were similar, demonstrating that the ooplast can reprogram a somatic nucleus. Moreover, in the zygote at least, the behavior of somatic nuclei was similar to that of the sperm nucleus. These findings suggest that during the early stages of development, *in vitro* conditions may influence histone tail modifications and consequently changes in gene expression patterns. Phosphorylation of histone H4/H2A, acetylation of lysine 5 on histone H4, and methylation of arginine 3 on histone H 4 are all present in the germinal vesicle, while only H4/H2A-S1 is maintained in MII egg chromatin (Sarmento et al., 2004). Thus, not only acetylation but also methylation may be reversible in the chromatin exposed to ooplasmic factors during entry into mitosis.

The present study indicates also that during meiosis, human oocytes maintain stable phosphorylation patterns, as do mouse oocytes (Takeuchi et al., 2004c). Transplanted somatic

nuclei underwent general histone modifications similar to those observed in maturing oocytes. This finding suggests that somatic cell nuclei can initiate the epigenetic reprogramming required for embryonic development already 3 hours after exposure to the ooplasm, and indicates that ooplasmic factors are capable of resetting imprinting patterns and so of modulating the expression of genes within differentiated somatic nuclei.

It was possible to isolate good quality RNA from different cells and tissues. However, although the expected RNA concentration in each oocyte and blastocyst is approximately 30-50 pg, we were unable to quantify it. Expression of *H19*, and *Igf2r* did not differ among the various tissues. *Igf2* gene expression was similar in the testis and ovary, but it was undetected in liver. As expected, there was no expression of *H19* in oocytes (Villar et al., 1995; Obata et al., 1998). However, there was a remarkable expression of *Igf2r* in oocytes, though only at the MII stage. In spite of the undetectable amounts of RNA in some specimens, our amplifications confirmed that quantitative PCR represents a valuable tool through which to assess expression of key genes.

Nuclear transplantation, as described in Chapters 3 and 4, is a technique which has the promise to improve the outcome of certain problem cases in assisted reproductive technology, and to generate pluripotent stem cells (Takeuchi and Palermo, 2004). The basic technique has proven useful in other ways that include its application to immature eggs as a new approach to the prevention of the aneuploidy common in older women, and in preimplantation genetic diagnosis (Tsai et al., 2000; Palermo et al., 2002a). Thus, while attempts at reproductive cloning in man seem unwarranted and even dangerous at present, this field will require rational rather than

emotional reactions as a basis for legislation if the therapeutic promise of stem cell research and the experimental potential of NT techniques are to be fully realized.

Epigenetic modifications of imprinted genes have been casually linked to the procedures involved in assisted reproductive techniques and gamete micromanipulation. Because present knowledge of related epigenetic changes in human nuclear and mitochondrial genes remains limited, and the associated risks cannot be quantified, caution is warranted. Careful documentation of abnormalities in offspring will be of great importance for our understanding of this relationship and so to improve these procedures. Disclosure of imprinting mutations potentially affecting children conceived from IVF and nuclear transplantation must be included during patient education at centers contemplating these treatments.

In an effort to unravel effects of somatic nuclear transfer on transcriptional activities in cloned embryos, the relative abundance of eight developmentally important gene transcripts measured in reconstructed blastocysts derived from various modifications of the nuclear transfer protocol, has been compared to those of *in vitro*- and *in vivo*-derived embryos (Wrenzycki et al., 2001a). The use of either G_0 or G_1 donor cells significantly modified the relative amounts of certain transcripts. Modifications of the nuclear transfer protocol are associated with distinct alterations in the expression patterns of the resulting embryos. In one study, an aberrant expression pattern in NT-derived embryos was found with respect to stress susceptibility, trophoblastic function and DNA methylation during preimplantation development (Wrenzycki et al., 2001b).

Epigenetic changes such as loss of imprinting and/or alterations of the DNA methylation status clearly bear on the ability of nuclei to initiate and sustain normal development. It has been postulated that epigenetic changes in preimplantation embryos affect gene activity during fetal development and thus the resulting phenotype of the neonate. In cloned bovine embryos a disturbed pattern of epigenetic reprogramming has been demonstrated, with highly aberrant methylation patterns evident in various genomic regions (Dean et al., 2001). Frequently, cloned blastocysts resembled donor cells in their overall genomic methylation status (Kang et al., 2001a; 2001b) whereas trophectodermal cells were undermethylated compared to the ICM. The abnormally high level of DNA methylation in the trophectodermal lineage, which constitutes an integral part of the placenta, could be responsible for the observed placental abnormalities in cloned pregnancies. The mRNA transcription of genes that are exclusively expressed in the trophectoderm was heavily affected in the early embryo already by the cloning procedure (Wrenzycki et al., 2001a). Thus it appears that the type of donor cell and its treatment affect the expression pattern of the reconstituted embryos, and that aberrations of the subtle regulation of the methylation pattern may underlie the large offspring syndrome.

It has been postulated that *in vitro* culture also may tend to induce epigenetic modifications in the genome of the preimplantation embryo. Extended culture of murine embryos in a more basic medium (Whitten's medium) led to bi-allelic expression of the *H19* gene, whereas in an optimized medium (KSOM), the regular pattern, a silent paternal allele, was maintained (Doherty et al., 2000). Furthermore, analysis of multiple growth-related and imprinted genes in murine embryos

cultured in a chemically defined medium (M16) with or without fetal calf serum revealed that culture in the presence of serum affects the regulation of imprinted genes (Khosla et al., 2001). These epigenetic alterations were not corrected during post-implantation development and were associated with aberrant fetal expression of imprinted genes and phenotypic abnormalities (Dean et al., 1998). Other studies have shown that culture conditions affect the well-orchestrated expression pattern of developmentally important genes in bovine embryos (Wrenzycki et al., 1996; 1999; 2001a; 2001b; 2002). Thus, bovine embryos produced *in vitro* differ from their *in vivo*-derived counterparts with regard to mRNA expression.

In the present study, the qualitative RT-PCR assay was tuned to a quantitative level, allowing determination of the relative abundance of specific genes in single embryos. Employing this sensitive approach, Wrenzycki and others (1999; 2001b) investigated the relative abundance of a set of developmentally important 'marker genes' in embryos cultured in media supplemented with either serum or PVA (polyvinyl alcohol, e.g. chemically defined medium). The results of Wrenzycki and colleagues revealed that composition of the medium and its oxygen tension had profound effects on the level of specific transcripts, and while the protein source much less so. There have been investigations also of the effects of two diverging culture systems on the expression of genes from the insulin-like growth factor family (*Igf1* and *Igf2*, *Igf-1r* and *Igf-2r*), known to play an important role in the regulation of embryonic and fetal development (Yaseen et al., 2001). Recently, it was possible for the first time to link mRNA expression patterns with *in vivo* development of embryos derived *in vitro* (Lazzari et al., 2002). As implied in discussing large offspring syndrome, birth weights of calves derived from *in vitro* produced (IVP) embryos

were significantly higher than normal. In general, the results support the hypothesis that early deviations in gene expression patterns are causally involved in this syndrome (Lazzari et al., 2002).

Recent studies have revealed also that abnormalities in gene expression of preimplantation embryos are not related to the oocyte maturation phase (Knijn et al., 2002; Dieleman et al., 2002), suggesting that this is not the major step at which these genes are modulated in the post-fertilization period *in vitro*. Moreover, it was found that gene expression, at least in the bovine blastocyst, is different in relation to the two lineages, the inner cell mass (ICM) and trophectoderm (TE; Wrenzycki and Niemann, 2003). As demonstrated in cloned mice, a spatial perturbation of gene expression patterns could be another source of aberrant development (Boiani et al., 2002).

The data presented here, support the hypothesis that the GVT environment and the nuclear transfer procedure itself can have profound effects on mRNA expression patterns in embryos created this way. Possible explanations for the abnormal phenotypes frequently observed in offspring born after transfer of IVF and NT-derived embryos include reprogramming errors, epigenetic dysregulation during *in vitro* culture prior to their replacement and, in the case of the NT-derived embryos, undefined factors associated with the nuclear transfer procedure *per se*. The common occurrence of aberrations in IVP or NT-derived embryos suggest that current *in vitro* production systems and/or the cloning technology may lead to persistent alterations of gene expression patterns during development, perhaps as a result of changes in the methylation

patterns (Niemann and Wrenzycki, 2000), and accordingly, deregulation of gene expression appears to perturb placental and fetal growth (Bartolomei and Tilghman, 1997; Reik and Walter, 2001). Interestingly, changes in a single imprinted gene does not seem sufficient to induce the significant overgrowth (Humphreys et al., 2001). The widespread dysregulation of imprinted and non-imprinted genes in NT- and IVP-derived embryos that survived to term suggest that mammalian development can tolerate a substantial degree of epigenetic abnormality. In fact, as suggested in analysis of the histone patterns, epigenetic deviations and associated phenotypes may occur in all mammals including man (Jaenisch and Wilmut, 2001). Therefore, the molecular mechanisms underlying epigenetic reprogramming during early embryonic development require further investigation. Transcript production is only the first expression of genome activity, and only the final protein defines the phenotype. Since the new protocols used in human ARTs involve extended culture to the blastocyst stage and allow transfer of only one or two embryos, this may also introduce a new set of problems in infertility treatment (De Rycke et al., 2002; Powell, 2003) as exemplified, perhaps, by recent reports on imprinting defects of ART children (Cox et al., 2002; DeBaun et al., 2002; Gicquel et al., 2003; Palermo et al., 2004).

Chapter 9

Conclusions

Declaration for Thesis Chapter 9

In the case of Chapter 9, contributions to the work involved the following:

	Name	% contribution	Nature of contribution
1	**Gianpiero D. Palermo**	100	Conception and design, collection, analysis, and writing of manuscript

Declaration by co-authors

The undersigned hereby certify that:

(6) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(7) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(8) there are no other authors of the publication according to these criteria;
(9) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(10) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Weill Medical College of Cornell University, The Center for Reproductive Medicine, New York, NY, USA

Signature 1 [redacted] **Date** 10-29-2004

Conclusions

In the last 25 years, IVF and its related procedures have undergone a remarkable development and are now a recognized branch of medicine. These successes have also stimulated researchers to adopt micromanipulation for clinical purposes, as well as experimental work in the field of human infertility, where it has become an integral part of IVF and now PGD. Today, micromanipulation is an essential tool as well in the treatment of male infertility, with ICSI now having become the method of choice for fertilizing oocytes *in vitro* where any problems seem likely to be present. A remaining challenge, however, rests in the problem of age-related infertility where the failure of the embryo to implant is often due to chromosomal aneuploidy. The use of micromanipulation procedures have allowed the isolation of polar bodies or blastomeres and together with genetic screening, provides the means to select normal oocytes and embryos. However, it does not allow for correction of the problem.

This thesis proposes nuclear transplantation as a more direct option for correction of the oocyte aneuploidy that affect patients in advanced reproductive age. Nuclear transplantation and other micromanipulation procedures were first developed in the field of veterinary medicine, and have been instrumental in reproductive manipulation in farm animals. Nuclear transplantation has proven to be a highly efficient procedure also in mice, in that > 90% of reconstituted oocytes were able to extrude a polar body and displayed a normal chromosomal constitution. With human oocytes, however, lower maturation rates have been the rule, probably due to the suboptimal procedures currently available for their *in vitro* maturation. Nonetheless, nuclear transplantation might ultimately provide an attractive approach to the age-related aneuploidy seen especially in poor responders and in older patients. The limited availability of human oocytes often makes it

difficult to draw firm conclusions. Therefore, we have designed a mouse model to simulate the ooplasmic damage of aged human oocytes that involves the selective disruption of the mitochondria and subsequent rescue of the nucleus of the impaired oocyte by transferring it to a healthy ooplast. This approach has shown in principle that nuclear transplantation can rescue nuclei isolated from a damaged ooplasm, with reasonable efficiency and can generate offspring. However, the limit on the number of human oocytes available still remains as a confounding factor for immediate application of this radical approach to the correction of oocyte aneuploidy in man.

In the second part of my work, an even more radical approach to this question relates to the creation of genetically normal gametes by bringing a somatic nucleus to a haploid state. This procedure in which haploidization is achieved using mouse GV oocytes requires, once attempted in humans, activated MII oocytes reduce to half the chromosomal content of the transplanted somatic cell nuclei. Although normal fertilization and development to the blastocyst stage can be readily accomplished, however, such blastocysts did not implant. The reason for this failure may reside in the present inability of this system to consistently reprogram the somatic nucleus and so to appropriately reset the imprinting process, reproducing a situation similar to that observed in cloned embryos.

While this work suggest that alternative sources of gametes are not merely the stuff of science fiction, but a reality, most of the manipulations involved are unlikely to be applied to man in the very near future. However, while stressing that the genetic normality of the offspring and the

safety of the procedures tested in animal experiments must first be firmly established, the experimental results obtained so far would seem to justify further research. Toward the end, we need to know more about the mechanism and patterns of heteroplasmy, how the blueprint of gene expression is maintained, and which cell cycle stage will favor reprogramming of a nucleus once transplanted into an ooplast. It is hoped that the type of nuclear transplantation procedures conducted here will also allow, and eventually provide further insight into the events of meiosis, syngamy, and the mitotic events of early embryo growth and so into further selective manipulation of development.

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4. Palermo GD, Takeuchi T, Rosenwaks Z. **Oocyte-induced haploidization.** *Reproductive BioMedicine Online,4:237-242, 2002*
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6. Takeuchi T, Rosenwaks Z, Palermo GD. **A successful model to assess embryo development after transplantation of prophase nuclei.** *Human Reproduction, 19:975-981, 2004*
7. Takeuchi T, Palermo GD. **The implications of cloning technique for reproductive medicine.** *Reproductive BioMedicine Online, 8:509-515, 2004*
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Abbreviations

A

AA	amino acid supplement
Ac	acetylation
AC	alternate current
Ac(Lys5)H4	acetylation of lysine5 on histone H4
ADP	adenosine diphosphate
Arg	arginine
ART	assisted reproductive technologies
AS	Angelman Syndrome
ASM1	adult skeletal muscle gene
ASRM	American Society for Reproductive Medicine
ATP	adenosine triphosphate

B

BDH	British Drug Houses Company
BSA	N,O-bis(trimethylsily)acetamide
BWS	Beckwith-Wiedemann Syndrome

C

°C	degree Celsius
CCB	cytochalasin B
cDNA	complimentary deoxyribonucleic acid
CEP	centromere probe
CMXRos	chloromethyl-X-rosamine
CoA	coenzyme A
CRI	Cambridge Research & Instrumentation
CSF	cytostatic factor
CZB	Chatot Ziomek Bavister
CZB-HEPES	Chatot Ziomek Bavister + 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

D

D3	day three embryo
DAPI	4',6-diamino-2-phenylindole
DC	direct current
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DPEC	diethylepyrocarbonate

E

EDTA	ethylenediaminetetraacetic acid
EG	embryonic germ cells
ES	embryonic stem cells
ESHRE	European Society for Human Reproduction and Embryology

F

FBS	fetal bovine serum
FISH	fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone

G

g	grams
g	gravitational force
G_0	Gap 0
G_1	Gap 1
G_2	Gap 2
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
GM	Gentamicine sulfate
GnRH	gonadotropin releasing hormone
GnRHa	gonadotropin releasing hormone agonist
GV	germinal vesicle
GVBD	germinal vesicle breakdown
GVT	germinal vesicle transfer/transplantation
GVTr	germinal vesicle transplanted and 'rescue'; rescue germinal vesicle transplantation

H

h	hour(s)
H1-4	histone 1-4
H19	adult skeletal muscle gene (ASM1)
HAT	histone acetyltransferases
hCG	human chorionic gonadotropin
HDAC	histone deacetylases
HDMT	histone demethyltransferases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hMG	human menopausal gonadotropin
HMG	high mobility group
HSA	human serum albumin
HTF	human tubal fluid
HTF-HEPES	human tubal fluid + 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HTF-HSA	human tubal fluid + human serum albumin
HTF-PVP	human tubal fluid + polyvinylpyrrolidone

I

i.p.	intraperitoneal
IBMX	3-isobutyl-1-methylxanthine
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
Igf2	Insulin-like growth factor 2 (somatomedin A)
Igf2r	Insulin-like growth factor 2 receptor
IRB	Internal Review Board
IU	International units
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* produced

K

K	lysine
KSOM	potassium simplex optimized medium
KSOM^{AA}	potassium simplex optimized medium with amino acid supplement
kV	kilo volts
kV/cm	kilo volts/centimeter

L

LSI	locus specific probe
LSM	laser scanning microscope
Lys	lysine

M

µl	microliter(s)
µM	micromole(s)
µs	microsecond(s)
M	mole(s)
M199	Medium 199 or TCM199
M199-IBMX	Medium 199 + 3-isobutyl-1-methylxanthine
M2	Medium 2 (HEPES buffered standard embryo culture medium)
M2-CCB	Medium 2 + cytochalasin B
M2-IBMX	Medium 2 + 3-isobutyl-1-methylxanthine
Me	methylation
Me(Arg3)H4	methylation of arginine 3 on histone H4
MeR3	methylation of arginine 3
mg	milligram(s)

MI	metaphase I
MII	metaphase II
ml	milliliter(s)
mm	millimeter(s)
mM	millimole(s)
mOsm	milliosmole(s)
mOsm/l	milliosmole(s) per liter
mPCR	multiplex polymerase chain reaction
MPF	maturation promoting factor
M-phase	mitotic phase
MRI	Medical Research International
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid

N

n	haploid number of chromosomes
4n	tetraploid number of chromosomes
NAD	nicotinamide adenine dinucleotide
nM	nanomole(s)
NT	nuclear transplanted

P

P	significance level
PA	pyruvic acid sodium salt
PB	polar body
PBD	phosphate buffer detergent
PBS	phosphate buffered saline
PCC	premature chromosome condensation
PCN-G	Penicillin G potassium salt
PCR	polymerase chain reaction
PGD	preimplantation genetic diagnosis
Ph	phosphorylation
pH	potential of hydrogen
Ph(Ser1)H4/H2A	phosphorylation of serine 1 on histone H4/H2A
pHi	intracellular potential of hydrogen
PhS1	phosphorylation of serine 1
PMSG	pregnant mare serum gonadotropin
PN	pronucleus/pronuclei
1PN	one pronucleus
2PN	two pronuclei
3PN	three pronuclei; tripronucleate
PVA	polyvinyl alcohol

PVP	polyvinylpyrrolidone
PZD	partial zona dissection

Q

qPCR	quantitative PCR
qRT-PCR	quantitative real-time PCR

R

R	arginine
RNA	ribonucleic acid
ROSI	round spermatid injection
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
RT-PCR	real-time polymerase chain reaction

S

S	serine
SAM	senescence accelerated mouse
SART	Society for Assisted Reproductive Technologies
SAS	Statistical Analysis System
SC	somatic cell
SCH	somatic cell haploidization
SD	standard deviation
Ser	serine
S-phase	synthesis phase
$SrCl_2$	strontium chloride
S-S	disulfide bond
SSC	standard saline citrate
SSS	synthetic serum substitute
ST	Streptomycin sulfate
SUZI	subzonal injection

T

TCM199	Tissue Culture Medium 199
TE	trophectoderm
TEM	transmission electron microscopy
T M	melting point
tRNA	transfer ribonucleic acid

U

UDG	uracil-deoxyribonucleic acid glycosylase
USP	United States Pharmacopeia
USSC	United States Surgical Suture

V

v	volume
v/v	volume to volume
V	volts
V/cm	volts per centimeter

W

W	watts
WHO	World Health Organization

Z

ZD	zona drilling