Focus on ART

Primate models for assisted reproductive technologies

Laura Hewitson

Departments of Obstetrics/Gynecology and Reproductive Sciences, and Environmental and Occupational Health, Pittsburgh Development Center, 204 Craft Avenue, Pittsburgh, Pennsylvania 15213, USA

Correspondence should be addressed to L Hewitson; Email: Lhewitson@pdc.magee.edu

Abstract

Although the deliberate creation of human embryos for scientific research is complicated by ethical and practical issues, a detailed understanding of the cellular and molecular events occurring during human fertilization is essential, particularly for understanding infertility. It is clear from cytoskeletal imaging studies of mouse fertilization that this information cannot be extrapolated to humans because of unique differences in centrosomal inheritance. However, the cytoskeletal rearrangements during non-human primate fertilization are very similar to humans, providing a compelling animal model in which to examine sperm–egg interactions. In order to address this key step in primate fertilization and to avoid the complexities in working with fertilized human zygotes, studies are now exploring the molecular foundations of various assisted fertilization techniques in a monkey model. While intracytoplasmic sperm injection with ejaculated or testicular sperm is quite successful in primate models, there are some specific differences when compared with standard IVF that warrant further investigation, particularly in regards to nuclear remodeling, genomic imprinting, Y-chromosome deletions and developmental outcomes. Similarly, primate models have been useful for examining spermatid function during fertilization but these have met with limited success. One area of primate reproductive research that has yet to be mastered is reproductive cloning. Genetically identical primates would provide the ultimate approach for accelerating stem cell-based therapies for a number of neurodegenerative diseases such as Alzheimer's and Parkinson's disease, as well as targeted gene therapies for various metabolic disorders.

Reproduction (2004) **128** 293–299

Introduction

Intracytoplasmic sperm injection (ICSI), first reported in 1992 (Palermo *et al.* 1992), has revolutionized methods for overcoming male factor infertility, offering many couples who were previously unable to conceive through *in vitro* fertilization (IVF) a chance of parenthood. The success of ICSI was largely driven by clinical and patient demands. There was no body of historical evidence, including animal studies, to determine the safety of potential clinical extrapolations. ICSI was hastily adopted into clinical practice and soon extended to include the use of sperm with abnormal morphology, such as in Kartagener's syndrome (von Zumbusch *et al.* 1998), as well as epididymal and testicular sperm (Craft *et al.* 1993). Over the last decade, the number of ICSI cycles in the USA has increased dramatically and accounts for more than 50% of all IVF cycles. Some clinics use ICSI exclusively whenever assisted reproduction is warranted (Van Steirteghem *et al.* 1993).

Notwithstanding the success of ICSI, questions remain about the dangers of passing on traits responsible for male infertility (Kent-First *et al.* 1996), sex and autosomal chromosome aberrations (In't Veld *et al.* 1995, Van Opstal *et al.* 1997, Bonduelle *et al.* 1999) and mental, physical and reproductive normalcy (Bonduelle *et al.* 1998a, Bowen *et al.* 1998). Perhaps the Y-chromosome deletions are among those best explored (Vogt *et al.* 1992, Reijo *et al.* 1996, Mulhall *et al.* 1997, Pryor *et al.* 1997, Kent-First *et al.* 1999) and are of particular concern, since a son conceived by ICSI may also suffer from male infertility (Kent-First *et al.* 1996, Kamischke *et al.* 1999). It has also been suggested that ICSI might interfere with the establishment of the maternal imprint in the oocyte or embryo increasing the risk of imprinting defects. Cox *et al.* (2002) report on two children who were conceived by ICSI who developed Angelman syndrome, a neurogenetic disorder, caused by loss of function of the maternal allele of the *UBE3A* gene. Beckwith–Wiedemann syndrome (BWS), another imprinting disorder, has been associated with

both IVF and ICSI procedures, suggesting that loss of maternal allelic methylation in BWS may be associated with *in vitro* embryo culture (Maher *et al.* 2003). Future studies are required to assess the possible association between assisted reproductive technologies (ART) and imprinting disorders. Several studies examining the health of ICSI offspring have now been completed and the data are reassuring (Bonduelle *et al.* 1998b, 1999, Devroey & Van Steirteghem 2004) although there is no experience yet with potential generational effects of ICSI in humans. Nudell *et al.* (2000) reported that, in some infertile men demonstrating meiotic arrest, there was a higher rate of mutations in the genes necessary for DNA repair in their testicular DNA samples than in testicular DNA isolated from fertile men with normal spermatogenesis. The same DNA repair problem was also found in malignant tumor cells of some cancer patients, suggesting that ICSI offspring produced from the sperm from these patients may also be infertile and, more importantly, at a higher risk for certain cancers (Nudell *et al.* 2000).

Animal models for ART

'Technical challenges' were most likely responsible for the lack of a mouse ICSI model prior to its clinical application. Survival of mouse oocytes after ICSI is low (Ron-El *et al.* 1995) without the aid of a piezo-driven microinjection pipette (Kimura & Yanagimachi 1995) and although hamsters are a good model for understanding pronuclear formation after ICSI, their use is hampered because of the difficulty in culturing manipulated hamster oocytes to the desired stage for embryo transfer. Furthermore, fertilization in rodent species is dependent on maternal centrosomes (Schatten *et al.* 1985), whereas in humans the centrosome is paternally derived (Simerly *et al.* 1995). This is especially relevant with regard to heterologous ICSI to assay sperm function (Asada *et al.* 1995), since human sperm injected into hamster oocytes will not nucleate microtubules (Hewitson *et al.* 1997), as they do after injection into human oocytes (Simerly *et al.* 1995).

Rabbits have been successfully used to produce ICSI offspring (Iritani *et al.* 1988) but have not been routinely used as a model for studying human ICSI. Bovine ICSI has proved to be technically challenging (Hewitson *et al.* 1998) with low success rates and, prior to the first clinical reports in 1992, had resulted in the birth of a single calf (Goto *et al.* 1990). Furthermore, bovine oocytes are unable to complete meiotic maturation after ICSI without an additional chemical stimulus such as incubation in calcium ionophore (Keefer *et al.* 1990). Conversely, primate oocytes share several morphological similarities with human oocytes, including size, cytoskeletal architecture and cytoplasmic clarity. Oocyte manipulations for non-human primate ART procedures are almost identical to those used clinically (Hewitson *et al.* 1996, 1998) and offspring can be derived at similar success rates (Hewitson

et al. 1999, 2000). While primate species are certainly expensive to maintain, their benefits as preclinical models for ART procedures are tremendous.

Cytoskeletal architecture during primate IVF

Understanding the cellular and molecular events during human fertilization is essential for the field of developmental biology, as well as for clinical applications, including infertility treatments, contraception and the avoidance of developmental abnormalities. Using conventional and confocal epifluorescence microscopy of fixed oocytes and embryos, it has been well established that fertilization of rhesus oocytes requires a paternally derived centrosome (Sutovsky *et al.* 1996, Wu *et al.* 1996). The sperm centrosome is introduced into the oocyte during sperm incorporation where it is transformed into a zygotic centrosome, capable of nucleating microtubules (Simerly *et al.* 1995, Sathananthan *et al.* 1996). The microtubule-based 'sperm aster' is a radially arrayed three-dimensional structure that is found adjacent to and affixed to the sperm nucleus. The sperm aster has three essential roles: first, its growth subjacent to the egg cortex pushes the sperm nucleus towards the egg center, next, when the distal ends of the dynamic microtubules contact the egg nucleus, it undergoes a sudden and swift translocation to the center of the sperm aster where it meets the sperm nucleus and, finally, the continuing elongation of the sperm astral microtubules moves the now adjacent pronuclei towards the egg center. Following the migration and union of the male and female pronuclei, the centrosome also defines the site of first bipolar mitotic spindle assembly within the activated cytoplasm and participates in spindle organization by serving as a dominant microtubule organizing center at the spindle poles.

The cytoskeletal arrangements observed during meiotic maturation and fertilization of rhesus oocytes closely mirror events observed in humans (Simerly *et al.* 1995, Wu *et al.* 1996) but not in mice, which rely on maternal centrosomal sources. In addition, the role of the sperm centrosome during fertilization should not be minimized, as certain forms of fertilization failures appear to be due to defects in the organization or functioning of the sperm aster (Simerly *et al.* 1995, Rawe *et al.* 2000). There is great utility in performing studies on centrosome function in a primate model as it overcomes the complicated ethical, political, financial and practical issues regarding the deliberate creation of human zygotes and embryos for scientific research.

Cytoskeletal architecture during primate ICSI

With the advent of clinical ICSI, primate oocytes were invaluable for better understanding cytoskeletal dynamics during ICSI fertilization. Using sperm from fertile rhesus macaques, centrosomal function, particularly sperm aster

Figure 1 Microtubule and chromatin dynamics in rhesus ICSI zygotes. The only microtubules present in the unfertilized rhesus oocyte are those found in the metaphase-arrested, meiotic spindle (A). After the injection of a sperm, the oocyte activates and the sperm and egg pronucleus begin to form (B). As the sperm decondenses an aster of microtubules emanates from the base of the sperm head. These microtubules elongate to fill the entire cytoplasm during pronuclear formation and apposition (C). By prophase, most of the cytoplasmic microtubules disassemble leaving a small tuft of microtubules associated with the introduced centrosome (arrow, D). The centrosome duplicates and separates to form a bipolar array (E). The chromosomes align across the metaphase spindle (F) in preparation for first mitosis. Transmission electron microscopy reveals that asynchronous decondensation of sperm chromatin is an unusual feature of ICSI. The apical chromatin is unable to decondense synchronously with the basal region of chromatin, perhaps due to the physical restrictions imposed by the still intact acrosome (G). However, as the sperm continues decondensation, a normal male pronucleus will eventually form (H). F, female pronucleus; M, male pronucleus; C, chromatin; A, aster; AC, acrosome. Magnification: (A and B) and (D–F) bar = 20 μm; (C) bar = 25 μm; (G and H) bar = 3 μm. Reprinted with permission from Hewitson *et al.* (1996).

formation, appeared normal (Hewitson *et al.* 1996, 1998; Fig. 1). Sperm microinjected into rhesus oocytes nucleated microtubules and completed repositioning of the maternal and paternal pronuclei in a manner similar to that of rhesus oocytes fertilized by IVF (Wu *et al.* 1996). Since this study used *in vitro* matured oocytes obtained from older, unstimulated females, a number of fertilization failures were also noted (Hewitson *et al.* 1996). These included: (i) the inability to resume meiosis, shown by metaphase II arrest and premature chromosome condensation and (ii) centrosomal defects characterized by microtubule nucleation arrest, premature detachment of the sperm axoneme and aster from the paternal pronucleus and sperm aster microtubule growth defects. Similar cytoskeletal anomalies are seen in human fertilization failures in infertility clinics (Simerly *et al.* 1995) and further emphasize the importance of normal sperm centrosomal function regardless of fertilization method.

While the cytoskeletal events that occur during ICSI fertilization are similar to those reported for IVF, there are some distinct differences. Since ICSI by-passes many of

the upstream events that prime the sperm for fertilization, such as capacitation and the acrosome reaction, it is not surprising that an injected sperm undergoes unusual nuclear remodeling (Hewitson *et al.* 1996, 1999, Ramalho-Santos *et al.* 2000; Fig. 2). The acrosome may remain intact post-ICSI; consequently, the digestive enzymes usually released to the exterior are instead internalized. While most healthy cells have specific ways to handle hydrolytic enzymes, the introduction of the concentrated acrosomal enzymes raises concerns regarding the manner in which they are immediately neutralized upon their introduction into the egg, as well as their ultimate fates.

The perinuclear theca, a structure located immediately subjacent to the acrosome, is normally lost during sperm incorporation (Sutovsky *et al.* 1997). However, after rhesus ICSI it persists throughout pronuclear formation, constricting the apical region of the sperm head, resulting in an asynchrony in DNA decondensation. Additionally, the retention of vesicle-associated membrane protein (VAMP) on the injected sperm further separates the condensed and

Figure 2 Unusual nuclear remodeling after rhesus ICSI. Membrane VAMP (A) is detected as a constricting ring around the paternal pronucleus separating the condensed and decondensing regions (inset, arrow). The perinuclear theca (B and arrow in inset) persists for up to 12 h post-ICSI, constricting the apical DNA (arrow). NuMA (C) is excluded from the condensed, apical region of the paternal pronucleus (main arrow), which is showing asynchronous chromatin decondensation (inset, arrow). M, male pronucleus; F, female pronucleus. Bar in (C) represents 20 μm (A) and 10 μm (B and C). Reprinted with permission from Hewitson *et al.* (1999).

decondensing regions of DNA by forming a two-piece collar around the sperm head (Ramalho-Santos *et al.* 2000). There are also noteworthy architectural differences in the manner in which certain nuclear proteins are handled. For example, nuclear mitotic apparatus protein (NuMA), which is important during interphase and in maintaining spindle architecture, typically enters the sperm nucleus as it decondenses during fertilization. After ICSI, however, NuMA is initially excluded from the regions of paternal chromatin which remain condensed, perhaps because of the physical constraints imposed by the still-intact acrosome and perinuclear theca (Hewitson *et al.* 1999).

Furthermore, DNA synthesis, as detected by bromodeoxyuridine incorporation, and pronuclear migration can be delayed by several hours after ICSI in both pronuclei when the paternal pronucleus is still undergoing decondensation in the apical region, identifying a unique G_1/S cell cycle checkpoint (Hewitson *et al.* 1999). Conversely, after IVF, pronuclear migration has been completed within 12 h post-insemination and DNA synthesis is detected in both pronuclei. The significance of this asynchronous decondensation is not completely understood but it may lead to a diminished ability of the oocyte to express, or be exposed to, important paternal genes or gene products, thus leading to anomalies such as imprinting defects.

The positioning of the sperm after ICSI, in contrast to its normal entrance at the egg surface, may also expose the sperm to a different cytoplasmic environment. The use of time-lapse video microscopy to study pronuclear migration during fertilization has revealed that, after primate ICSI, the sperm which is typically deposited in the center of the oocyte migrates to a more cortical cytoplasmic position before attracting the female pronucleus, whereas sperm entering the oocyte following IVF remain in a cortical position during sperm aster assembly (C Simerly, personal communication). Dynamic imaging of rhodamine-labeled spindle microtubules of human oocytes revealed that the first polar body can be as much as 95° displaced from the spindle compared with 20° for control oocytes (Hewitson *et al.* 1999). Since the first polar body is not anchored firmly, mechanical manipulations such as those required for cumulus cell removal may result in its lateral displacement within the perivitelline space (Hardarson *et al.* 2000). With this in mind, the position of the polar body prior to ICSI should not be considered a reliable indicator of the position of the meiotic spindle. However, recent advances in polarization microscopy now permit the direct visualization of the meiotic spindle in living eggs prior to ICSI (Silva *et al.* 1999, Cohen *et al.* 2004). While alterations in pronuclear remodeling after ICSI do not prevent pronuclear formation, the onset of DNA synthesis and pronuclear migration may be delayed (Sutovsky *et al.* 1997, Hewitson *et al.* 1999) with, as yet, unknown consequences.

Primate oocytes have also been useful for examining centrosomal function of testicular sperm during fertilization (Hewitson *et al.* 2000). While sperm aster formation

appeared normal, fertilization rates were slightly lower than for ICSI with ejaculated sperm (Hewitson *et al.* 1998, 2000). Similarly, elongated spermatids have been used for fertilization of rhesus oocytes, although failure in oocyte activation was commonly observed. This may be associated with the production of abnormal calcium oscillations after spermatid injection, although this problem generally improves with the maturation of the spermatids (Tesarik *et al.* 2000, Yazawa *et al.* 2000). When oocyte activation was successfully accomplished, sperm aster formation and pronuclear apposition in rhesus oocytes were observed, suggesting that elongated spermatids contain a functional centrosome. A single rhesus macaque has been derived from the injection of elongated spermatids (Hewitson *et al.* 2000).

Round spermatids, which are considered experimental by most, are used in ART for injection in cases of spermatogenic arrest by some clinics. Several normal clinical pregnancies following round spermatid injection have been reported (Antinori *et al.* 1997, Kahraman *et al.* 1998, Barak *et al.* 1998), although disappointingly poor fertilization and subsequent embryonic development are common outcomes (Levrin *et al.* 2000, Vicdan *et al.* 2001). Fertilization of cynomolgus macaque oocytes with round spermatids has been reported; however, the only pregnancy established aborted at day 103 (Ogonuki *et al.* 2003). Round spermatids differ biologically from mature sperm in several important ways, such as immaturity of certain cytoplasmic and nuclear proteins (Ziyyat & Lefevre 2001), which probably contributes to the poor pregnancy rates (Ghazzawi *et al.* 1999). With regard to genomic imprinting, the exact timing of events in human spermatids is unclear. However, the expression of several paternally and maternally imprinted genes in mouse embryos fertilized with round spermatids has been reported (Shamanski *et al.* 1999) although these authors also suggested that some minor elements of genomic imprinting might be incomplete even after spermiogenesis. Because the safety and effectiveness of round spermatid injections for human ART are still far from certain, non-human primate models are valuable experimental systems for assessing the application of this technique.

Nuclear transfer in non-human primates: challenges and possibilities

Experimental cloning research in non-human primates is well justified for many reasons, e.g. to accelerate HIV/AIDS vaccine trials, to explore the potential of human embryonic stem (ES) cells in a species more closely related than mice and to investigate the molecular basis of human disease. Cloning by somatic cell nuclear transfer (SCNT) has now been confirmed in domestic species, mice, rabbits and cats, and was successful once with embryonic nuclei in non-human primates (Meng *et al.* 1997), but not yet somatic nuclei (Simerly *et al.*

2003). SCNT in rhesus macaques results in unanticipated defects in centrosomes (Simerly *et al.* 2003) and mitochondria (St. John & Schatten 2004). While rhesus cloned embryos are able to enter first mitosis, chromosomes misalign on disarranged spindles and, despite seemingly normal appearance of eight-cell embryos, no pregnancies resulted after transfer (Simerly *et al.* 2003). Imaging of cloned 'embryos' revealed that non-human primate SCNT fails due to imbalances between the chromosome sets, spindle pole numbers, microtubule-based molecular motors and the acquired somatic cell centrosome (Simerly *et al.* 2003).

The utility of non-human primate ES cells

Human ES cell lines are predicted to be valuable for the provision of therapeutic cells whose growth and differentiation can be controlled for the treatment of disease. However, for ES cell-based therapy to become a clinical reality, translational research involving non-human primates is essential. Several investigators have established ES cell lines from a variety of non-human primate species (Thomson *et al.* 1995, 1996, Suemori *et al.* 2001). These lines have been successfully maintained in an undifferentiated state with a normal karyotype for many months of culture. Pluripotent ES cell lines derived from non-human primates are a useful resource for preclinical human ES cell research, including allogeneic transplantation studies for researching primate models of human disease.

A recent report by scientists in South Korea describes the isolation of an ES cell line derived from a cloned human embryo (Hwang *et al.* 2004). In doing so, these researchers have overcome some of the obstacles that to date have hampered primate cloning (Simerly *et al.* 2003) and accelerated research for potential ES cell-based therapies. While this work provides the first evidence for generating human ES cells from a somatic cell isolated from a living person, it is also likely to re-ignite the smoldering debate over how such research should be regulated.

Conclusions

While ICSI has been an extremely important development for couples suffering from male infertility, many critical questions still need to be addressed. These include concerns about the physiological, behavioral and psychological integrity of the ICSI children, whether they will experience unexpected degenerative diseases in later life as well as the reproductive potential of the ICSI offspring, particularly sons. The use of immature testicular spermatogenic cells raises another set of problems, including the accurate definitions and criteria for cells selected and whether alteration of the normal pattern of genomic imprinting might result in an increasing incidence of diseases such as Angelman syndrome. Non-human primate models are valuable experimental systems for assessing

novel ART techniques for human infertility, as well as assisting captive breeding programs to aid in the survival of endangered species. Although somatic cell nuclear transfer in rhesus macaques has yet to be reported, technological advances in cloning procedures are likely to accelerate this goal. Finally, primate ES cells are instrumental for understanding the regulative mechanisms of stem cell differentiation *in vivo* and *in vitro*, and hold tremendous potential for understanding the therapeutic value of differentiated human ES cell lines.

Acknowledgements

I am grateful to the Pittsburgh Development Center faculty and staff whose research is described herein including: Drs Saverio Capuano, Kowit-yu Chong, Chris Navara, Gerald Ruppenthal, Paul Sammak, Gerald Schatten and Calvin Simerly. I am also grateful to our collaborators, Drs Justin St. John, João Ramalho-Santos and Yukihiro Terada. The sponsorship of the NIH in the support of the fundamental research cited in the article is gratefully acknowledged.

References

- Antinori S, Versaci C, Dani G, Antinori M, Pozza D & Selman HA 1997 Fertilization with human testicular spermatids: four successful pregnancies. *Human Reproduction* **12** 286–291.
- Asada Y, Baka SG, Hodgen GD & Lanzendorf SE 1995 Evaluation of the meiotic spindle apparatus in oocytes undergoing intracytoplasmic sperm injection. *Fertility and Sterility* **64** 376–381.
- Barak Y, Kogosowski A, Goldman S, Soffer Y, Gonen Y & Tesarik J 1998 Pregnancy and birth after transfer of embryos that developed from single-nucleated zygotes obtained by injection of round spermatids into oocytes. *Fertility and Sterility* **70** 67–70.
- Bonduelle M, Joris H, Hofmans K, Liebaers I & Van Steirteghem A 1998a Mental development of 201 ICSI children at 2 years of age. *Lancet* **351** 1553.
- Bonduelle M, Aytöz A & Van Assche E 1998b Incidence of chromosomal aberrations in children born after assisted reproduction through intracytoplasmic sperm injection. *Human Reproduction* **13** 781–782.
- Bonduelle M, Camus M, De Vos A, Staessen C, Tournaye H, Van Assche E, Verheyen G, Devroey P, Liebaers I & Van Steirteghem A 1999 Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children. *Human Reproduction* **14** (Suppl 1) 243–264.
- Bowen JR, Gibson FL, Leslie GI & Saunders DM 1998 Medical and developmental outcome at 1 year for children conceived by intracytoplasmic sperm injection. *Lancet* **351** 1529–1534.
- Cohen Y, Malcov M, Schwartz T, Mey-Raz N, Carmon A, Cohen T, Lessing JB, Amit A & Azem F 2004 Spindle imaging: a new marker for optimal timing of ICSI? *Human Reproduction* **19** 649–654.
- Cox GF, Burger J, Lip V, Mau UA, Sperling K, Wu BL & Horsthemke B 2002 Intracytoplasmic sperm injection may increase the risk of imprinting defects. *American Journal of Human Genetics* **71** 162–164.
- Craft I, Bennet V & Nicholson N 1993 Fertilizing ability of testicular spermatozoa. *Lancet* **342** 864.
- Devroey P & Van Steirteghem A 2004 A review of ten years experience of ICSI. *Human Reproduction Update* **10** 19–28.
- Ghazzawi IM, Alhasani S, Taher M & Sousa S 1999 Reproductive capacity of round spermatids compared with mature spermatozoa in a population of azoospermic men. *Human Reproduction* **14** 736–740.
- Goto K, Kinoshita A, Takuma TY & Ogawa K 1990 Fertilization by sperm injection in cattle. *Theriogenology* **33** 238.
- Hardarson T, Lundin K & Hamberger L 2000 The position of the metaphase II spindle cannot be predicted by the location of the first polar body in the human oocyte. *Human Reproduction* **15** 1372–1376.
- Hewitson LC, Simerly C, Tengowski MW, Sutovsky P, Navara CS, Haavisto AJ & Schatten G 1996 Chromatin and DNA configurations after intracytoplasmic sperm injection in the rhesus monkey: failures and successes. *Biology of Reproduction* **55** 271–280.
- Hewitson L, Haavisto A, Simerly C, Jones J & Schatten G 1997 Microtubule organization and chromatin configurations in hamster oocytes during fertilization and parthenogenetic activation, and after insemination with human sperm. *Biology of Reproduction* **57** 967–975.
- Hewitson L, Takahashi D, Dominko T, Simerly C & Schatten G 1998 Fertilization and embryo development to blastocysts after intracytoplasmic sperm injection in the rhesus monkey. *Human Reproduction* **13** 3449–3455.
- Hewitson L, Dominko T, Takahashi D, Martinovich C, Ramalho-Santos J, Sutovsky P, Fanton J, Jacob D, Monteith D, Neuringer M, Battaglia D, Simerly C & Schatten G 1999 Unique checkpoints during the first cell cycle of fertilization after intracytoplasmic sperm injection in rhesus monkeys. *Nature Medicine* **5** 431–433.
- Hewitson L, Martinovich C, Simerly C, Takahashi D & Schatten G 2000 Is round spermatid injection (ROSI) a therapy for male infertility? ROSI in the rhesus monkey is unsuccessful. *Fertility and Sterility* **74** Suppl 3S O–181.
- Hwang WS, Ryu YJ, Park JH, Park ES, Lee EG, Koo JM, Jeon HY, Lee BC, Kang SK, Kim SJ, Ahn C, Hwang JH, Park KY, Cibelli JB & Moon SY 2004 Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. *Science* **303** 1669–1674.
- In't Veld P, Brandenburg H, Verhoeff A, Dhont M & Los F 1995 Sex chromosomal abnormalities and intracytoplasmic sperm injection. *Lancet* **346**.
- Iritani A, Utsumi K, Miyake M, Hosoi Y & Saeki K 1988 *In vitro* fertilization by a routine method and by micromanipulation. *Annals of the New York Academy of Sciences* **541** 583–590.
- Kahraman S, Polat G, Samli M, Sozen E, Ozgun OD, Dirican K & Ozbicer T 1998 Multiple pregnancies obtained by testicular spermatid injection in combination with intracytoplasmic sperm injection. *Human Reproduction* **13** 104–110.
- Kamischke A, Gromoll J, Simoni M, Behre HM & Nieschlag E 1999 Transmission of a Y chromosomal deletion involving the deleted in azoospermia (DAZ) and chromodomain (CDY1) genes from father to son through intracytoplasmic sperm injection: case report. *Human Reproduction* **14** 2320–2322.
- Keefer CL, Younis AI & Brackett BG 1990 Cleavage development of bovine oocytes fertilized by sperm injection. *Molecular Reproduction and Development* **25** 281–285.
- Kent-First MG, Kol S, Muallem A, Ofir R, Manor D, Blazer S, First N & Itskovitz-Eldor J 1996 The incidence and possible relevance of Y-linked microdeletions in babies born after intracytoplasmic sperm injection and their infertile fathers. *Molecular Human Reproduction* **2** 943–950.
- Kent-First M, Muallem A, Shultz J, Pryor J, Roberts K, Noltén W, Meisner L, Chandley A, Gouchy G, Jorgensen L, Havighurst T & Grosch J 1999 Defining regions of the Y-chromosome responsible for male infertility and identification of a fourth AZF region (AZF_d) by Y-chromosome microdeletion detection. *Molecular Reproduction and Development* **53** 27–41.
- Kimura Y & Yanagimachi R 1995 Intracytoplasmic sperm injection in the mouse. *Biology of Reproduction* **52** 709–720.
- Levrán D, Nahum H, Farhi J & Weissman A 2000 Poor outcome with round spermatid injection in azoospermic patients with maturation arrest. *Fertility and Sterility* **74** 443–449.
- Maher ER, Brueton LA, Bowdin SC, Luharia A, Cooper W, Cole TR, Macdonald F, Sampson JR, Barratt CL, Reik W & Hawkins MM

- 2003 Beckwith–Wiedemann syndrome and assisted reproduction technology (ART). *Journal of Medical Genetics* **40** 62–64.
- Meng L, Ely JJ, Stouffer RL & Wolf DP 1997 Rhesus monkeys produced by nuclear transfer. *Biology of Reproduction* **57** 454–459.
- Mulhall JP, Reijo R, Alagappan R, Brown L, Page D, Carson R & Oates RD 1997 Azoospermic men with deletions of the DAZ cluster gene are capable of completing spermatogenesis, fertilization, normal embryonic development and pregnancy with retrieved testicular spermatozoa and ICSI. *Human Reproduction* **2** 503–508.
- Nudell D, Castillo M, Turek PJ & Pera RR 2000 Increased frequency of mutations in DNA from infertile men with meiotic arrest. *Human Reproduction* **15** 1289–1294.
- Ogonuki N, Tsuchiya H, Hirose Y, Okada H, Ogura A & Sankai T 2003 Pregnancy by the tubal transfer of embryos developed after injection of round spermatids into oocyte cytoplasm of the cynomolgus monkey (*Macaca fascicularis*). *Human Reproduction* **18** 1273–1280.
- Palermo G, Joris H, Devroey P & Van Steirteghem A 1992 Pregnancies after intracytoplasmic sperm injection of single spermatozoon into an oocyte. *Lancet* **340** 17–18.
- Pryor JL, Kent-First M, Muallem A, Van Bergen AH, Nolten WE, Meisner L & Roberts KP 1997 Microdeletions in the Y-chromosome of infertile men. *New England Journal of Medicine* **336** 534–539.
- Ramalho-Santos J, Sutovsky P, Simerly C, Oko R, Wessel GM, Hewitson L & Schatten G 2000 ICSI choreography: fate of sperm structures after monospermic ICSI and first cell cycle implications. *Human Reproduction* **15** 2610–2620.
- Rawe VY, Olmedo SB, Nodar FN, Doncel GD, Acosta AA & Vitullo AD 2000 Cytoskeletal organization defects and abortive activation in human oocytes after IVF and ICSI failure. *Molecular Human Reproduction* **6** 510–516.
- Reijo R, Alagappan RK, Patrizio P & Page DC 1996 Severe oligozoospermia resulting from deletions of azoospermia factor gene on Y chromosome. *Lancet* **347** 1290–1293.
- Ron-El R, Liu J, Nagy Z, Joris H, Van den Abbeel E & Van Steirteghem A 1995 Intracytoplasmic sperm injection in the mouse. *Human Reproduction* **10** 2831–2834.
- St. John JC & Schatten G 2004 Paternal mitochondrial DNA transmission during non-human primate nuclear transfer. *Genetics* (In Press).
- Sathananthan AH, Ratnam SS, Ng SC, Tarin JJ, Gianaroli L & Trounson A 1996 The sperm centriole: its inheritance, replication and perpetuation in early human embryos. *Human Reproduction* **11** 345–356.
- Schatten G, Simerly C & Schatten H 1985 Microtubule configurations during fertilization, mitosis, and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. *PNAS* **82** 4152–4156.
- Shamanski FL, Kimura Y, Lavoie MC, Pedersen RA & Yanagimachi R 1999 Status of genomic imprinting in mouse spermatids. *Human Reproduction* **14** 1050–1056.
- Silva CP, Kommineni K, Oldenbourg R & Keefe DL 1999 The first polar body does not predict accurately the location of the metaphase II meiotic spindle in mammalian oocytes. *Fertility and Sterility* **71** 719–721.
- Simerly C, Wu G, Zoran S, Ord T, Rawlins R, Jones J, Navara C, Gerrity M, Rinehart J, Binor Z & Schatten G 1995 The paternal inheritance of the centrosome, the cell's microtubule-organizing center, in humans and the implications for infertility. *Nature Medicine* **1** 47–53.
- Simerly C, Dominko T, Navara C, Payne C, Capuano S, Gosman G, Chong KY, Takahashi D, Chace C, Compton D, Hewitson L & Schatten G 2003 Molecular correlates of primate nuclear transfer failures. *Science* **300** 297.
- Suemori H, Tada T, Torii R, Hosoi Y, Kobayashi K, Imahie H, Kondo Y, Iritani A & Nakatsuji N 2001 Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. *Developmental Dynamics* **222** 273–279.
- Sutovsky P, Hewitson L, Simerly C, Tengowski MW, Navara CS, Haavisto A & Schatten G 1996 Intracytoplasmic sperm injection for rhesus monkey fertilization results in unusual chromatin, cytoskeletal and membrane events, but eventually leads to pronuclear development and sperm aster assembly. *Human Reproduction* **11** 1703–1712.
- Sutovsky P, Oko R, Hewitson L & Schatten G 1997 The removal of the sperm perinuclear theca and its association with the bovine oocyte surface during fertilization. *Developmental Biology* **188** 75–84.
- Tesarik J, Mendoza C & Greco E 2000 The activity (calcium oscillator?) responsible for human oocyte activation after injection with round spermatids is associated with spermatid nuclei. *Fertility and Sterility* **74** 1245–1247.
- Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA & Hearn JP 1995 Isolation of a primate embryonic stem cell line. *PNAS* **92** 7844–7848.
- Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP & Hearn JP 1996 Pluripotent cell lines derived from common marmoset (*Callithrix jacchus*) blastocysts. *Biology of Reproduction* **55** 254–259.
- Van Opstal D, Los FJ, Ramlakhan S, Van Hemel JO, Van Den Ouweland AM, Brandenburg H, Pieters MH, Verhoeff A, Vermeer MC, Dhont M & In't Veld PA 1997 Determination of the parent of origin in nine cases of prenatally detected chromosome aberrations found after intracytoplasmic sperm injection. *Human Reproduction* **12** 682–686.
- Van Steirteghem AC, Nagy Z, Joris H, Liu J, Staessen C, Smits J, Wisanto A & Devroey P 1993 High fertilization and implantation rates after intracytoplasmic sperm injection. *Human Reproduction* **8** 1061–1066.
- Vicdan K, Isik AZ & Delilbasi L 2001 Development of blastocyst-stage embryos after round spermatid injection in patients with complete spermiogenesis failure. *Journal of Assisted Reproduction and Genetics* **18** 78–86.
- Vogt P, Chandley AC & Hargreave TB 1992 Microdeletions in interval 6 of the Y chromosome of males with idiopathic sterility point to disruption of AZF, a human spermatogenesis gene. *Human Genetics* **89** 491–496.
- Wu G, Simerly C, Zoran S, Funte LR & Schatten G 1996 Microtubule and chromatin configurations during fertilization and early development in rhesus monkeys, and regulation by intracellular calcium ions. *Biology of Reproduction* **55** 269–271.
- Yazawa H, Yanagida K, Katayose H, Hayashi S & Sato A 2000 Comparison of oocyte activation and Ca^{2+} oscillation-inducing abilities of round/elongated spermatids of mouse, hamster, rat, rabbit and human assessed by mouse oocyte activation assay. *Human Reproduction* **15** 2582–2590.
- Ziyyat A & Lefevre A 2001 Differential gene expression in pre-implantation embryos from mouse oocytes injected with round spermatids or spermatozoa. *Human Reproduction* **16** 1449–1456.
- von Zumbusch A, Fiedler K, Mayerhofer A, Jessberger B, Ring J & Vogt HJ 1998 Birth of healthy children after intracytoplasmic sperm injection in two couples with male Kartagener's syndrome. *Fertility and Sterility* **70** 643–646.