Emerging Concepts

Although a wealth of information is available, the definitive molecular mechanisms underlying uterine receptivity, uterine nonreceptivity, embryo-uterine signaling, and decidualization remain to be resolved. The advent of genomics creates opportunities to revisit implantation research on a global scale. Microarray (*22*) screens and laser capture microdissection may identify cell-specific genes relevant to implantation. Single cell isolation and proteomics may identify critical molecules for implantation. The creation of uterine-specific conditional knockouts may revolutionize implantation research, because deletion of many of the implantation-associated genes produces embryonic lethality, precluding studies on implantation. Another novel approach to isolate the contribution of a single factor is to selectively deliver the product directly into the uterus via blastocyst-sized gelatin beads (Fig. 2), mimicking local changes elicited by a living blastocyst and allowing in vivo functional analysis (*23*). New insights into the mechanisms of implantation will enhance the efficiency of reproductive technologies relevant to fertility regulation.

References and Notes

1. Reviewed in D. D. Carson *et al.*, *Dev. Biol.* **223**, 217 (2000).
2. Reviewed in H. Lim *et al.*, *Vitam. Horm.* **64**, 43 (2002).
3. Reviewed in E. R. Norwitz, D. J. Schust, S. J. Fisher, *N. Engl. J. Med.* **345**, 1400 (2001).
4. L. C. Giudice, *Hum. Reprod.* **14**, 3 (1999).
5. A. Psychoyos, *Vitam. Horm.* **31**, 201 (1973).
6. A. J. Wilcox, D. D. Baird, C. R. Weinberg, *N. Engl. J. Med.* **340**, 1796 (1999).
7. J. F. Couse, K. S. Korach, *Endocr. Rev.* **20**, 358 (1999).
8. B. Mulac-Jericevic *et al.*, *Science* **289**, 751 (2000).
9. M. B. Renfree, G. Shaw, *Annu. Rev. Physiol.* **62**, 353 (2000).
10. S. J. Kimber, C. Spanswick, *Semin. Cell Dev. Biol.* **11**, 77 (2000).
11. L. A. Salamonsen, E. Dimitriadis, L. Robb, *Semin. Reprod. Med.* **18**, 299 (2000).
12. C. L. Stewart *et al.*, *Nature* **359**, 76 (1992).
13. M. Ernst *et al.*, *J. Exp. Med.* **194**, 189 (2001).
14. J. V. Bonventre *et al.*, *Nature* **390**, 622 (1997).
15. H. Lim *et al.*, *Cell* **91**, 197 (1997).
16. H. Lim *et al.*, *Genes Dev.* **13**, 1561 (1999).
17. R. Mechoulam, E. Fride, V. Di Marzo, *Eur. J. Pharmacol.* **359**, 1 (1998).
18. B. C. Paria *et al.*, *J. Biol. Chem.* **276**, 20523(2001).
19. M. Maccarrone *et al.*, *Mol. Hum. Reprod.* **8**, 188 (2002).
20. W. Ma *et al.*, *Mol. Endocrinol.* **15**, 1983 (2001).
21. T. H. Vu, Z. Werb, *Genes Dev.* **14**, 2123 (2000).
22. J. Reese *et al.*, *J. Biol. Chem.* **276**, 44137 (2001).
23. B. C. Paria *et al.*, *Proc. Natl. Acad Sci. U.S.A.* **98**, 1047 (2001).
24. Reviewed in L. Ma *et al.*, *Dev. Biol.* **197**, 141 (1998).
25. W. Wang, T. Van DeWater, T. Lufkin, *Development* **125**, 621 (1998).

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REVIEW

The Science of ART

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The methods of gamete manipulation used in assisted reproductive technology (ART) are rapidly proliferating and in some instances outpacing the underlying science. In this review, we discuss two major advances in the ART laboratory—intracytoplasmic sperm injection and extended embryo culture before embryo transfer. We outline the rationale for these approaches, discuss results of experiments obtained from animal model systems and human preimplantation embryos that provide the scientific basis for these procedures, and point out potential concerns that have arisen from these studies.

About 35 to 70 million couples worldwide are infertile and have turned to ART to overcome their infertility. Central to the practice of ART are procedures for egg and sperm collection, fertilization in vitro, and embryo transfer. ART's perceived safety and success have led to an increasing demand for its use (Fig. 1). ART procedures performed in the United States in 1999 accounted for ~1 out of every 150 children born (*1*), and ~1,000,000 children worldwide have been conceived by ART procedures since 1978.

In ~40% of infertile couples, the etiology of infertility is ascribed in part to the male. "Male factor" infertility is often due to a decreased sperm count and/or sperm motility, or abnormal sperm morphology, and is sometimes associated with known genetic defects. Intracytoplasmic sperm injection (ICSI) was developed to circumvent the inability of these sperm to fertilize an egg (*2*) and revolutionized the treatment of male factor infertility. In ICSI, laboratory personnel directly inject a selected sperm into the egg's cytoplasm (Fig. 2, A and B). Although ICSI requires micromanipulation, it is a relatively simple, straightforward, and robust procedure that is rapidly gaining widespread acceptance and is now used to treat infertility in cases not ascribed to male factor infertility. For example, in some U.S. metropolitan areas, ICSI is performed in 60 to 80% of ART procedures (*1*).

The major concern regarding ICSI is that it bypasses almost all the natural selection mechanisms that sperm encounter during the course of a natural conception. There is also the added risk of mechanical injury to the spindle that could potentially lead to aneuploidy. Polarized microscopy to noninvasively locate the position of the birefringent spindle would, in principle, solve this problem (*3*). Other concerns focus on numerous differences between normal fertilization and ICSI. In primate ICSI, sperm head decondensation is asynchronous such that the apical portion remains condensed when control inseminated eggs have formed a male pronucleus (Fig. 2, C and D). Moreover, DNA replication of the paternal genome after ICSI is delayed, because it only initiates after complete chromosome decondensation (*4*). These differences, and the preferential localization of the sex chromosomes to the anterior sperm head (*5*), may underlie the reported increase in sex chromosome abnormalities associated with ICSI (*6*). This increase also

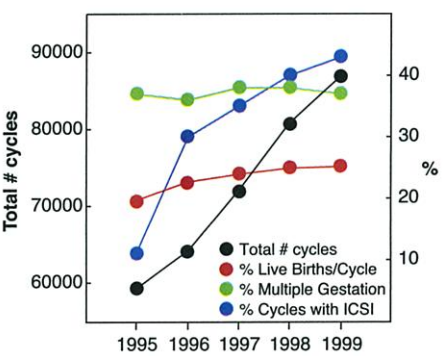

Fig. 1. Increasing use of ART in the United States. "Total # cycles" includes fresh nondonor, frozen nondonor, and donor cycles. An ART cycle typically initiates with ovarian hyperstimulation and concludes with embryo transfer. The "% multiple gestation" (of live births), "% cycles with ICSI," and "% live births/cycle" were calculated from fresh, nondonor cycles only. Data are from (*1*).

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could reflect increased karyotypic abnormalities in sperm from these infertile men (*6*). Other differences include introducing into the egg's cytoplasm sperm components (e.g., acrosome) and media components (e.g., polyvinylpyrrolidone) that normally do not enter the egg. Moreover, the kinetics of sperm factor release that initiates the calcium oscillations responsible for egg activation may differ between ICSI and fertilization after sperm-egg plasma membrane interactions (*7*).

The relative ease of microinjecting human eggs has spurred interest in developing procedures to overcome the detrimental effects of aging on egg-embryo quality that may be related to compromised mitochondrial function. Using the mouse as a model system, microinjection of healthy egg cytoplasm into oxidatively stressed eggs, whose developmental competence is markedly reduced due to compromised mitochondrial function, partially restores their ability to develop (*8*). In an example of an ART procedure outpacing the underlying science, such cytoplasmic transfers have resulted in the birth of a child with mitochondrial heteroplasmy (*9*) that has unknown consequences. Although this ART technique is now prohibited in the United States, further research with model systems will evaluate the suitability of this procedure as a clinical therapy by establishing its underlying scientific basis.

A major dilemma in ART is the incidence of multiple gestation. Multiple embryos are generated during each ART treatment cycle, and varying numbers of the "best" embryos from this pool are chosen for transfer to ensure a reasonable probability of pregnancy yet minimize multiple gestation (i.e., >one fetus). Nevertheless, multiple gestation pregnancies account for ~50% of the children born from ART procedures. These pregnancies, as compared to singletons, result in a much higher incidence of pregnancy complications, low birth weight, and learning disabilities (*10*). In addition, there are economic costs associated with managing these high-risk pregnancies and the intensive care of the premature babies. As cogently argued by Gerris and Van Royen, the obvious solution is single embryo transfer (SET), in particular for patients at high risk for multiple gestation (*11*). Accepting SET as the solution highlights the problem of identifying the "best" embryo.

Several morphological criteria are used to identify the "best" embryos. For example, the timing of pronuclear apposition and alignment of the nucleoluslike bodies and the embryo cleavage rate are correlated with the incidence of clinical pregnancy (*11*). Although these parameters may provide a prospective method to evaluate "embryo quality," the molecular and cellular bases for these observations are unknown.

A more recent approach is to transfer blastocysts rather than earlier cleavage–stage embryos. The assumption is that further development to the blastocyst stage permits selection of the one or two embryos with the highest developmental potential for transfer. Moreover, transfer of blastocysts into the uterus provides the appropriate temporal synchronization of the embryo and uterus. Development to the blastocyst stage, however, requires culture for 5 to 6 days (instead of 2 to 3 days). Because culture in vitro can affect gene expression and embryo metabolism in model systems, concerns have arisen about the effects of extended culture on these human embryos that may in part be responsible for the increased risk of monozygotic twinning after blastocyst transfer (*12*).

In the mouse, the global pattern of gene expression is perturbed by modest changes in media composition [e.g., the presence of an osmolyte (*13*)]. Embryo culture conditions also can affect the expression of imprinted genes (i.e., genes that are differentially expressed from the maternal and paternal genomes). A global loss of imprinting in human eggs can result in molar pregnancy [i.e., placental tissue but no fetus (*14*)]. Loss of imprinting is also associated with Prader-Willi and Angelman syndromes. Several imprinted genes are implicated in growth regulation (e.g., *Igf2* and *Igf2r*). Inclusion of serum in mouse embryo culture media can result in both altered expression of several imprinted genes and reduced developmental potential after embryo transfer (*15*). Culture of mouse embryos to the blastocyst stage under suboptimal conditions results in biallelic expression of the *H19* gene, which is normally expressed only from the maternal allele (*16*). In bovine and ovine embryos, culture to the blastocyst stage before embryo transfer can result in a higher incidence of fetal and perinatal loss (*17*). These abnormalities have been attributed to embryo culture conditions and in sheep may be linked to reduced expression of the imprinted *Igf2r* gene (*18*). Whether culture conditions used in human ART programs affect the expression of imprinted genes, and if so, whether there are long-term consequences for the children, is unknown.

Embryo culture also results in shifts in energy metabolism. In vivo, the early cleavage–stage, relatively metabolically quiescent embryo uses pyruvate/lactate, but not glucose, as a sole energy source. In contrast, blastocysts are highly metabolically active and use glucose as a sole energy source (*19*). Embryo culture results in a stress response that is manifested by changes in gene expression (e.g., expression of heat-shock genes), apoptosis, and metabolism. For example, mouse blastocysts that develop in vivo convert 40 to 50% of the glucose to lactate, whereas blastocysts that develop in vitro from the morula stage convert ~100% of the glucose to lactate. Early cleavage–stage embryos also exhibit an increase in glycolysis in response to culture that is associated with reduced implantation and development after embryo transfer. Of particular interest is that inclusion of amino acids in the medium prevents this metabolic change and restores the embryo's ability to implant and develop after transfer (*20*).

These findings prompted development of

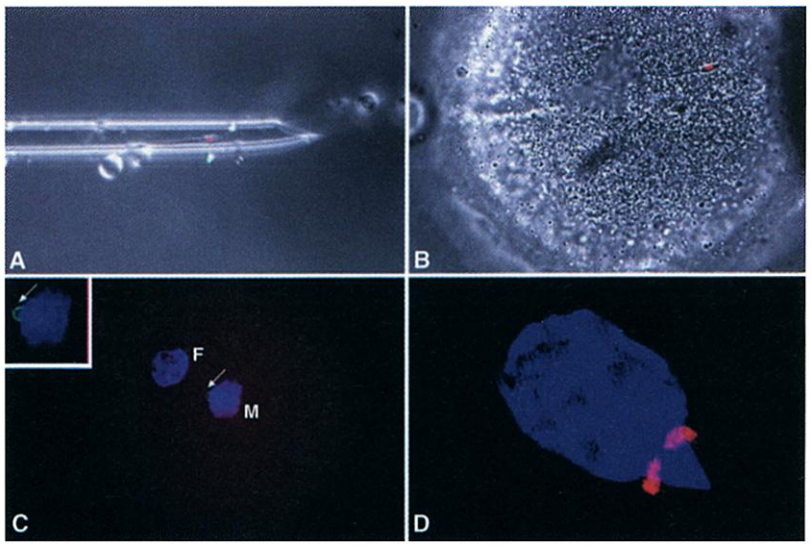

Fig. 2. (**A**) Rhesus monkey sperm (labeled with rhodamine) drawn up into an ICSI pipette. (**B**) Rhesus monkey egg subjected to ICSI. Note the entire sperm is deposited into the egg. (**C**) Twelve hours after ICSI, the perinuclear theca (green, arrow) is still observed and constricts the apical region of the DNA (blue). F, female pronucleus; M, male pronucleus. Inset: higher magnification of the male pronucleus. (**D**) Staining for the acrosomal vesicle-associated membrane protein (VAMP) (red) reveals that decondensation of the apical portion of the sperm head is inhibited by the VAMP collar. (C) is reprinted with permission from (*25*).

completely defined, albeit sometimes proprietary, culture media for human embryos that mimic the changing environment the embryo experiences during its passage from the oviduct (high pyruvate, low glucose) to the uterus (high glucose, low pyruvate). In addition, oxidative stress is being minimized by including EGTA and culturing in 5% oxygen. Such changes in culture conditions that are based on model systems may account for dramatic increases in the ART success rate in many centers (*1*). Although other centers have similar success rates using alternative media supplements such as maternal serum or co-culture (i.e., culturing the embryos in the presence of somatic cells), these conditions are difficult to replicate. There is clearly a need to develop standard embryo-culture protocols that use defined nonproprietary media. Studies of embryo metabolism also provide the scientific impetus to assess the predictive value of noninvasive measurements of glycolysis as a marker for the developmental potential of single embryos. Noninvasive measurements of uptake and/or secretion of specific amino acids in early cleavage–stage embryos are also being evaluated as predictors of development to the blastocyst stage (*21*). In the future, these noninvasive methods may be used to minimize the length of culture while enabling transfer of a single cleavage-stage embryo with the highest developmental potential.

The question as to whether ART increases the incidence of congenital malformations or has subtle, long-term, and adverse effects on behavior and cognition remains contentious. This issue was highlighted in three recent studies that compared children born after ART to those naturally conceived. One study reported an increase in the incidence of neurological abnormalities (*22*), whereas a second study found an increased incidence of major congenital abnormalities (*23*); these differences could not be solely accounted for by multiple gestation, prematurity, or low birth weight. A third study reported an increase in the incidence of low birth weight in singleton pregnancies (*24*). Although these findings could be specific to these patient populations and need to be confirmed, they nevertheless serve as an impetus to refine ART procedures to minimize multiple gestation and any other potential detrimental effects of ART. This could be done by developing noninvasive methods, coupled with morphological embryo assessment, to select single embryos for transfer, and by experimentally refining ART procedures to minimize the differences between manipulated, cultured embryos and embryos that develop in vivo.

References and Notes

1. See the National Center for Chronic Disease Prevention and Health Promotion's report on assisted reproductive technology, available online at www.cdc.gov/nccdphp/drh/art.htm.
2. G. Palermo, H. Joris, P. Devroey, A. C. Van Steirteghem, *Lancet* **340**, 17 (1992).
3. W. H. Wang, L. Meng, R. J. Hackett, R. Odenbourg, D. L. Keefe, *Fertil. Steril.* **75**, 348 (2001).
4. J. Ramalho-Santos *et al.*, *Hum. Reprod.* **15**, 2610 (2000).
5. C. M. Luetjens, C. Payne, G. Schatten, *Lancet* **353**, 1240 (1999).
6. M. Bonduelle *et al.*, *Hum. Reprod.* **17**, 671 (2002).
7. P. Sutovsky, R. Oko, L. Hewitson, G. Schatten, *Dev. Biol.* **188**, 75 (1997).
8. L. Liu, D. L. Keefe, *Biol. Reprod.* **62**, 1828 (2000).
9. J. A. Barritt, C. A. Brenner, H. E. Malter, J. Cohen, *Hum. Reprod.* **16**, 513 (2001).
10. M. Hack, A. A. Fanaroff, *Early Hum. Dev.* **53**, 193 (1999).
11. J. Gerris, E. Van Royen, *Hum. Reprod.* **15**, 1884 (2000).
12. Y. J. Menezo, D. Sakkas, *Hum. Reprod.* **17**, 247 (2002).
13. Y. Ho, A. S. Doherty, R. M. Schultz, *Mol. Reprod. Dev.* **38**, 131 (1994).
14. H. Judson, B. E. Hayward, E. Sheridan, D. T. Bonthron, *Nature* **416**, 539 (2002).
15. S. Khosla, W. Dean, D. Brown, W. Reik, R. Feil, *Biol. Reprod.* **64**, 918 (2001).
16. A. S. Doherty, M. R. Mann, K. D. Tremblay, M. S. Bartolomei, R. M. Schultz, *Biol. Reprod.* **62**, 1526 (2000).
17. E. Behboodi *et al.*, *Theriogenology* **44**, 227 (1995).
18. L. E. Young *et al.*, *Nature Genet.* **27**, 153 (2001).
19. D. K. Gardner, *Theriogenology* **49**, 83 (1998).
20. M. Lane, D. K. Gardner, *Hum. Reprod.* **13**, 991 (1998).
21. F. D. Houghton *et al.*, *Hum. Reprod.* **17**, 999 (2002).
22. B. Strömberg *et al.*, *Lancet* **359**, 461 (2002).
23. M. Hansen, J. J. Kurinczuk, C. Bower, S. Webb, *N. Engl. J. Med.* **346**, 725 (2002).
24. L. A. Schieve *et al.*, *N. Engl. J. Med.* **346**, 731 (2002).
25. L. Hewitson *et al.*, *Nature Med.* **5**, 431 (1999).

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