(*16*). These findings show that fertilin β and cyritestin are not individually or together required for gamete membrane fusion (*14–16*). In addition, eggs carrying a deletion of the gene for the α6 integrin subunit can bind to and fuse normally with sperm (*32*). Thus, none of the specific proteins acting in the current ADAM-integrin model for adhesion/fusion are required for sperm-egg fusion, and other molecules must exist on the surface of gametes that can act in sperm-egg fusion. These could be other members of the ADAM and integrin families or entirely different proteins.

Research on other egg surface proteins has pointed in two new directions. Egg surface proteins with a GPI anchor have been implicated because PI-PLC treatment releases these proteins from the surface and blocks gamete fusion. Two egg GPI-anchored proteins have been detected, with relative molecular masses of ~70 and ~35 to 45 kD, but have not yet been identified (*33*). More compelling evidence establishes an essential role for egg surface CD9. Female mice carrying a gene knockout for CD9 are infertile; they produce eggs that mature normally, but are defective in sperm-egg fusion (*34–36*). CD9, a member of the tetraspanin family, spans the plasma membrane four times, having two extracellular loops (one small, one large) and short cytoplasmic NH_2-terminal and COOH-terminal tails. One defined role of tetraspanins is to organize functional, multimolecular complexes on the surface of the cell expressing the tetraspanin. In other cases, tetraspanins may (also) bind a soluble ligand or a ligand on an adhering cell (*37*, *38*). Recent evidence suggests that CD9 on eggs may act in cis by interacting with other egg surface molecules (*39*). In addition, CD9-knockout oocytes injected with wild-type CD9 mRNA show a high level of rescue of their fusion ability. However, if the injected CD9 mRNA carries a subtle mutation in the CD9 large extracellular loop (residues 173 to 175, Ser-Phe-Gln→Ala-Ala-Ala), no fusion ability is restored to injected CD9 knockout oocytes. These data suggest that Ser-Phe-Gln is an active site in CD9 that associates with and regulates the egg fusion machinery (*39*).

Sperm-egg fusion stimulates the first signaling pathway(s) in development. The initial events in this pathway, preceding an essential rise in intracellular Ca^{2+} concentration, remain unknown (*40*).

Conclusions. Mammalian fertilization has been inherently difficult to study because of the temperamental nature of in vitro fertilization assays and the small amount of eggs obtainable. Nonetheless, current and emerging strategies—e.g., gene knockout (Table 1), signal peptide traps (*41*), and structural analysis of sperm protein–egg protein complexes—will provide deeper understanding of this fundamental biological process. This increased understanding is needed to generate clinical advances for treatment of infertility and novel contraceptive strategies.

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REVIEW

Deciphering the Cross-Talk of Implantation: Advances and Challenges

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Implantation involves a series of steps leading to an effective reciprocal signaling between the blastocyst and the uterus. Except for a restricted period when ovarian hormones induce a uterine receptive phase, the uterus is an unfavorable environment for blastocyst implantation. Because species-specific variations in implantation strategies exist, these differences preclude the formulation of a unifying theme for the molecular basis of this event. However, an increased understanding of mammalian implantation has been gained through the use of the mouse model. This review summarizes recognized signaling cascades and new research in mammalian implantation, based primarily on available genetic and molecular evidence from implantation studies in the mouse. Although the identification of new molecules associated with implantation in various species provides valuable insight, important questions remain regarding the common molecular mechanisms that govern this process. Understanding the mechanisms of implantation promises to help alleviate infertility, enhance fetal health, and improve contraceptive design.

The success of any species depends on its reproductive efficiency. For sexual reproduction, an egg and sperm must overcome many obstacles to fuse and co-mingle their genetic material at fertilization. The zygote develops into a blastocyst with two cell lineages (the inner cell mass and the trophectoderm), migrates within the reproductive tract, and ultimately implants into a transiently permissive host tissue, the uterus. However, the molecular basis of the road map connecting the blastocyst with the endometrium across species is diverse (*1*) and not fully understood. Recent advances have identified numerous molecules involved in implantation (*1–4*), yet new discoveries have not yielded a unifying scheme for the mechanisms of implantation.

Uterine Preparation and Blastocyst Competency for Implantation

Uterine receptivity is defined as a restricted period when a uterus supports blastocyst attachment (*5*). Although progesterone and estrogen play major roles in a species-specific

manner, the molecular basis of this window of receptivity remains unclear. Evidence suggests that the window is programmed when the receptive state of the uterus is synchronized with the activated state of the blastocyst (*2*). Of equal importance is the transition of the receptive uterus to a nonreceptive state when implantation fails to occur. In humans, increased spontaneous abortions after implantation beyond the putative window of receptivity (*6*) reflect the urgency of the need to resolve the molecular basis of uterine receptivity.

Spatiotemporal elaboration of various growth factors, cytokines, lipid mediators, and transcription factors in the uterus by steroid hormones is thought to play an important role in uterine preparation for implantation (Fig. 1) (*1*, *2*). Estrogen and progesterone actions are primarily mediated by their nuclear receptors estrogen receptor (ER) (ERα and ERβ) and progesterone receptor (PR) (PRA and PRB), which show differential uterine expression. $ER\alpha^{-/-}$ mice are infertile, whereas implantation occurs in $ER\beta^{-/-}$ mice despite reduced ovulation (*7*). Mice lacking both PR isoforms are infertile, whereas selective deletion of PRA allows ovulation but fails to rescue implantation (*8*). Moreover, the responses of ER- or PR-deficient uteri to estrogen or progesterone suggest alternative pathways for steroid actions (*2*, *7*).

A natural delay in the onset of implantation occurs in response to seasonal cues or lactation in certain species (*1*, *2*, *9*). Whether this condition occurs in humans is unknown. Delayed implantation can be experimentally induced by steroid hormonal manipulation. During delayed implantation in mice, the blastocyst undergoes dormancy and the uterus becomes nonresponsive to the presence of a blastocyst. However, the implantation process can be initiated by a single injection of estrogen. Via ER, estrogen transforms the progesterone-primed mouse uterus into a receptive state, and its uterine metabolite, catecholestrogen, activates the blastocyst to an implantation-competent state (Fig. 1) (*2*). Because steroid hormones are central to all aspects of mammalian reproduction, the discovery of new receptor isoforms and classical receptor-independent actions has galvanized interest in the mechanisms of steroid action.

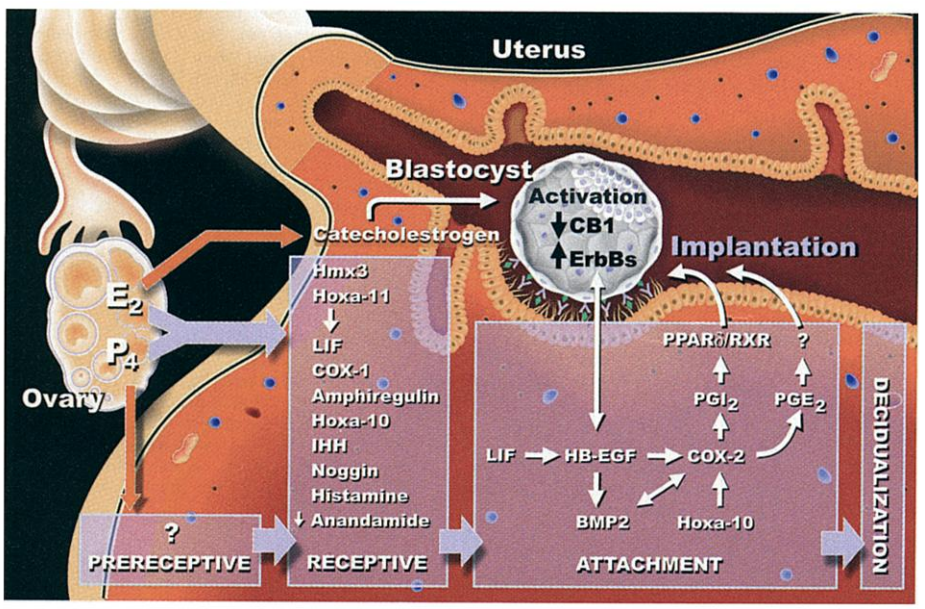

Fig. 1. Molecular signaling during implantation. The prereceptive mouse uterus is unresponsive to blastocysts. Ovarian estrogen (E_2) and progesterone (P_4) transform the prereceptive uterus to a receptive state via a number of uterine factors, whereas uterine-derived catecholestrogen activates the blastocyst to an implantation-competent state. During the attachment phase, sequential signaling events within the uterus lead to blastocyst implantation. Stromal cell decidualization follows the attachment phase. Ligand-receptor interactions and adhesive events (integrins and other cell-surface molecules) are indicated at the blastocyst attachment site.

Molecular and Cellular Prelude to Implantation

Preparation for implantation by ovarian steroids coincides with uterine cellular, molecular, and functional changes. The uterine lumen is lined by a polarized epithelium overlying the stroma and myometrium (Fig. 2). Morphologic changes in the luminal epithelium, including apical microvilli retraction and the emergence of large apical protrusions (pinopodes), mark the transition from a prereceptive to a receptive state. The luminal epithelium is decorated with glycoproteins, which presumably function as implantation barriers. Their unmasking at the implantation site correlates with increased blastocyst adhesiveness to the uterus. Integrins, carbohydrate moieties and their receptors, the trophinin-tastin-bystin complex, and other cell-surface molecules participate in an adhesion cascade to anchor blastocysts at implantation sites (Fig. 1) (*1*–*3*, *10*).

Molecular Messengers in Embryo-Uterine Interaction

The recent revolution in genetics and molecular biology has identified many molecules that are crucial for implantation. Arguably, the most dramatic discoveries have come from genetic manipulation studies in mice.

Numerous growth factors and cytokines and their receptors are implicated in implantation (*2*, *11*). Among the cytokine family, leukemia inhibitory factor (LIF) and interleukin-11 (IL-11) are most pertinent to implantation. Gene targeting, reciprocal embryo transfer, and expression studies show the essential role of maternal LIF in uterine preparation and blastocyst attachment in mice (Fig. 1) (*2*, *12*). LIF is also implicated in human implantation (*2*). Inactivation of gp130, a signaling partner for the LIF receptor, also results in implantation failure (*13*), but the uterine site and action of LIF remain elusive. IL-11 is primarily involved in decidualization, because this process fails in mice lacking its receptor subunit, IL-11Rα (*11*).

Among the EGF-like growth factors, heparin-binding EGF-like growth factor (HB-EGF) is the earliest known marker of implantation in mice, because it is expressed in the uterine luminal epithelium around the preattachment blastocyst (*2*). It promotes embryonic growth via EGF receptors (ErbBs) on the blastocyst cell surface (*2*). It is also expressed in the receptive human uterus and stimulates growth of in vitro fertilized embryos (*1*, *2*). Determination of whether HB-EGF is indispensable for implantation awaits gene targeting experiments. Nevertheless, the gene encoding HB-EGF is one of the genes whose uterine expression is induced by active blastocysts. Identifying the embryonic signal(s) initiating such uterine responses remains a seminal question.

One hallmark of implantation is increased vascular permeability at the implantation site (Fig. 2). Vasoactive agents, including histamine, platelet-activating factor, vascular endothelial growth factor, and eicosanoids, have been studied during implantation. Recent discoveries suggest critical roles for

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prostaglandins (PGs) in female reproduction. The liberation of arachidonic acid by phospholipase A_2 (PLA_2), followed by the action of cyclooxygenases (COX-1 or COX-2), generates PGs. PGs derived from cytosolic PLA_2–COX-2 coupling are most relevant (*14*, *15*). COX-2 is restricted to the implantation site in most species studied, including primates, and *COX-2*$^{-/-}$ mice have defective implantation and decidualization, independent of faulty ovulation and fertilization (*2*, *15*). Deletion of heterotrimeric GTP-binding protein–coupled cell-surface PG receptors does not perturb implantation, but there is evidence that uterine prostacyclin acting via a nuclear receptor, peroxisome-proliferator–activated receptor (PPARδ), participates in implantation (*2*, *16*). More important, genetic and molecular studies with LIF, HB-EGF, and Homeobox A–10 (Hoxa-10) suggest that COX-2 functions as a common downstream pathway (Figs. 1 and 2).

An emerging concept in implantation is the role of endocannabinoids, a group of lipid mediators that are ligands for the cannabinoid receptors CB1 and CB2. *N*-arachidonoylethanolamine (anandamide) represents a model endocannabinoid (*17*). The coordinate down-regulation of uterine anandamide and blastocyst CB1 levels with the onset of implantation suggests a role in modulating the implantation window (*2*). Furthermore, although low doses of anandamide are stimulatory, high doses inhibit blastocyst growth, implying regulated endocannabinoid signaling during implantation (*2*, *18*). Indeed, studies with CB-deficient mice show that regulated endocannabinoid signaling is important for synchronizing embryo development with uterine receptivity (*18*). Spontaneous abortions also occur in women with elevated anandamide levels (*19*).

Uterine stromal cells undergo cellular transformation (decidualization) to regulate embryonic growth and invasion. Angiogenesis and tissue remodeling are well-characterized events whose molecular basis in decidualization is still unfolding (*20*). Among many other molecules, matrix metalloproteinases (MMPs) and their inhibitors appear to be more important for these events. Evidence suggests that MMP-9 and its signaling partners are important and that a balance among select MMPs directs the decidual response (*21*). It is also thought that decidua function as a barrier to maternal immunological responses to semi-allogenic embryos. Although cellular and humoral immune mediators are active during the postimplantation period and placentation (*3*), it is still a mystery how the blastocyst escapes maternal immune surveillance at the time of implantation. Reduced expression of numerous immune-related genes at the onset of implantation in mice suggests that immunomodulation occurs at an early stage and is regulated by blastocyst-derived signals (*22*).

Developmental Genes in Implantation

The complex interplay between the embryo and uterus during implantation shares characteristics of reciprocal epithelial-mesenchymal interactions underlying embryogenesis, involving evolutionarily conserved genes from flies to mammals. There is now evidence that many of these genes, including those encoding fibroblast growth factors, bone morphogenetic proteins, Wnt's, Noggin, and Indian hedgehog, participate in the molecular cross-talk of implantation (*23*).

Homeobox-containing transcription factors, another class of developmental genes, are also important for implantation. Spatial colinearity maps suggest that paralogous groups of *Hox* genes in the caudal region are important in the uterus. Indeed, mice deficient in Hoxa-10 or Hoxa-11 fail to support implantation (*2*, *24*). These genes are steroid hormone–responsive and are up-regulated with the onset of uterine receptivity in mice and humans (*2*). Nonclassical *Hox* genes may also be important in implantation, because *Hmx3*$^{-/-}$ mice have implantation failure (*25*).

Limitations in Implantation Research

Genetic, molecular, and pharmacological studies have provided considerable insight into implantation biology. However, approaches to further resolve the mechanisms of implantation are hindered by the lack of well-defined in vitro systems that faithfully replicate embryo-uterine interactions. Another shortcoming is our inability to distinguish the relationship between critical signaling events that span the interdependent phases of implantation (Fig. 1).

One major task in understanding implantation is to establish a hierarchy of molecular relationships. The difficulty lies in discerning whether signaling pathways operate independently, in parallel, or as a network. The best-recognized sequence of signaling events in implantation has been defined in mice with targeted deletions of *LIF*, *COX-2*, and *Hoxa-10*. Evidence suggests that LIF is essential for preparation of the steroid-primed uterus for the receptive state. LIF signaling permits uterine HB-EGF to interact with blastocyst ErbBs to prepare and direct blastocyst attachment. In turn, this interaction leads to the elaboration of uterine COX-2–derived PGs to fully execute the implantation process. Hoxa-10 in collaboration with PGs then directs the process of decidualization (Figs. 1 and 2). Thus, disruption of any of these pathways can result in implantation failure.

Another challenge is to isolate the critical molecules within a gene family with related functions. Redundancy and compensatory gene expression ensure that essential functions are preserved, but they preclude the identification of individual components. Despite simultaneous deletion of EGF, transforming growth factor–α, and amphiregulin, the presence of other members clouds the importance of individual contributions to implantation (*2*). Targeted gene disruption may also reveal compensation by genes that can adopt similar roles. The absence of implantation abnormalities in *COX-1*$^{-/-}$ mice results from COX-2 compensation during uterine preparation for implantation (*2*).

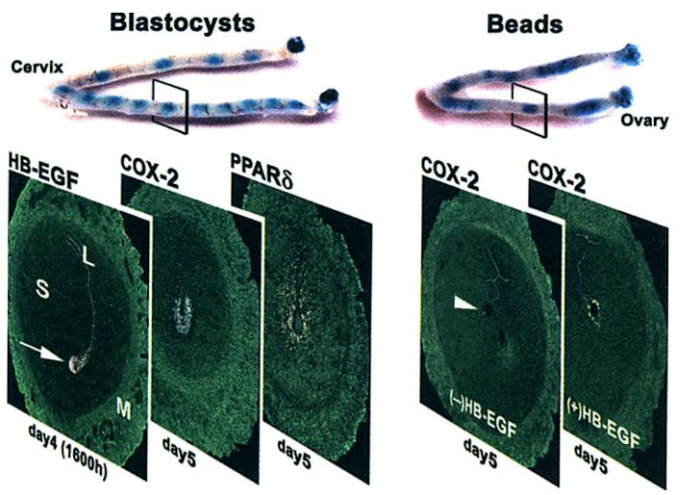

Fig. 2. Hierarchy of gene expression at natural and simulated implantation sites in mice. Increased vascular permeability occurs at implantation sites and was detected by an intravenous injection of blue dye. In situ hybridization shows HB-EGF expression exclusively in the luminal epithelium several hours before blastocyst attachment. COX-2 and PPARδ subsequently appear in the luminal epithelium and stroma surrounding the implanting blastocyst. Blastocyst-sized beads transferred into receptive uteri do not evoke implantation-like responses, whereas beads that had previously absorbed HB-EGF induce responses similar to those produced by living blastocysts, including the induction of COX-2, permitting in vivo analysis of signaling mechanisms. Arrow indicates the blastocyst; arrowhead indicates the bead. L, luminal epithelium; S, stroma; M, myometrium.

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.

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REVIEW

The Science of ART

Richard M. Schultz[1]* and Carmen J. Williams[2]

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

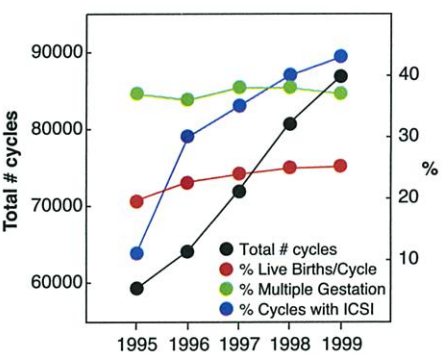

Fig. 1. Increasing use of ART in the United States. "Total # cycles" includes fresh nondonor, frozen nondonor, and donor cycles. An ART cycle typically initiates with ovarian hyperstimulation and concludes with embryo transfer. The "% multiple gestation" (of live births), "% cycles with ICSI," and "%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

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