The Science of ART

In 1978, Robert Edwards and Patrick Steptoe achieved what many likely thought impossible: the birth of a baby conceived outside of a woman’s body, commonly referred to as the first test tube baby. This was not only a breakthrough in biological research and a significant advance in the field’s understanding of human reproduction, but also a cultural and soci-etal leap that released women (and men) from being at the mercy of the reproductive vagaries of their bodies. A solution for many forms of infertility was now available, affording couples previously unable to bear biological children that opportunity. Although controversial at the time, assisted reproductive technology (ART) is now seen as a standard instrument in the toolbox at a doctor’s disposal to assist infertile patients.

However, ART has not been without its challenges, difficulties, and failings. An honest and open discussion of these hurdles as well as the implications of new research and advances is this area was the motivation for a conference that took place in September 2013 in Florence, Italy, and ultimately this publication. Following a year of preparation, in September 2013, we welcomed an international audience to Florence, Italy to hear from the top 10 authors them- selves and challenge their findings. Out of that two day event grew a panel of experts to be presented, and challenged, by peers in the field at this unique two-day meeting.

Topics covered included the high incidence of multiple births follow- ing ART and how this might be addressed through new techniques, improved genetic screening of embryos was also on the agenda, including the challenges regarding testing for diseases with par-tial penetrance and the fear expressed by some of the spectators of so-called designer babies. This publication provides a brief overview of the conference, a first of its kind as far as format and intent is concerned. We also present a review article from each of the authors of the 10 original manuscripts, providing context to their work in the current research landscape. The 10 articles are prefaced by a short perspective from J.L.H. Evers and An-tonio Pellicer, both editors in chief of prestigious reproductive medicine journals and experts in this field, who provide their as-sessment of ART following Edwards and Steptoe’s long-awaited Nobel prize in Medicine in 2010. We close out the booklet with three excellent reviews from leaders in reproductive medicine: Legro and Matzuk who discuss disorders that affect ovarian function and related treatments, Lamb and Lashultz whose re-search has focused on the male role in infertility, and Chu, Vidal-la, Dey, and Simón, who together review the complex topic of the molecular mechanisms involved in embryo implantation in the uterus.

It is our hope that this booklet, made possible by the sponsor-ship and support of the Serono Symposia International Founda-tion, will provide a broad review of the important and impact-ful subject of reproductive medicine, summarizing advances, challenging dogmas, and advancing new paradigms, as seen from the perspective of conference attendees and presenting authors. The intention is not to stop here, but to use this conference as a model to continue the discussion in this field as well as many others that impact our lives and well-being.

Sean Sanders, Ph.D.
Editor
Custom Publishing Office
Science/AAAS

From the Chief Executive of the Serono Symposia International Foundation

At the Serono Symposia International Foundation (SSIF), we believe that it is more important than ever to ensure that the best independent continuing medical education (CME) is avail-able to clinicians, scientists, and researchers. Every day across the world, new research findings and improvements in diagno-sis and treatment mean new opportunities to improve outcomes for patients. This booklet focuses on one very important strand of our work in CME: reproductive medicine. It clearly exemplifies the ap-proach that SSIF takes to all of the areas we cover and the many events we hold each year, sharing the knowledge and wisdom of internationally renowned experts with audiences worldwide through live educational activities and, increasingly, online.

The Top Ten in Reproductive Medicine project has been both exciting and challenging. With so much groundbreaking research worldwide, how could we identify just 10 papers? During the summer of 2012, we brought together an expert panel to select the best papers from a shortlist of 50 produced by a special-ist independent bibliographer. The shortlist featured 25 papers related to basic research and 25 that were clinical and field-based. The papers were chosen by the number of downloads they had received and the number of citations in the past 10 years. Our expert panel of eight leading figures in reproductive medicine then spent many hours debating which would form the final Top Ten. Asking which papers had really challenged tradi-tional views or which had actually changed diagnosis and treatment?

Our aim with the Top Ten project is to high-light research that has taken reproductive medicine forward to the benefit of patients.

Rachel Clark
Chief Executive Officer
Serono Symposia International Foundation
The “Top Ten in Reproductive Medicine” conference highlighted the best papers as an example of how research on human reproduction and infertility should be done.

In September 2013, scientists and clinicians in the field of human reproduction participated in a groundbreaking conference held in Florence, Italy. Over the course of two days, authors of the 10 most cited, viewed, or downloaded research articles in the field presented their papers. Each author was confronted by a “challenger,” after which participants discussed the paper’s findings and significance. The goal: to advance rigorous science in a field dominated by small-scale studies and trial and error. Everyone is aware of the clinical relevance of assisted reproduction technology (ART),” said Carlos Simón, professor of obstetrics and gynecology at the University of Valencia, Spain, and a conference scientific organizer. “We need to increase our scientific relevance.”

In 2009, the World Health Organization officially recognized infertility as a disease. Not all countries, however, have followed suit. Consequently, large-scale funding of fertility treatments has been scarce. Regulation differs among countries, making adoption of standard practices difficult. And on the basic research front, strict embryo protection laws in some countries restrict studies on human embryos. “If you look at all the things we do in the clinic, I would bet half of them have never been proven efficient in multicenter trials,” said Hans Evers, a professor in the department of obstetrics and gynecology at Maastricht University Medical Centre in Maastricht, The Netherlands. Intracytoplasmic Sperm Injection (ICSI) whereby sperm is injected directly into an egg, for example, is now being used to treat unexplained infertility in about half of all treatment cycles, said Evers. “But no one has ever published that ICSI is necessary in unexplained infertility.”

ICSI was an advance initially developed to help men with poor sperm quality become fathers when traditional in vitro fertilization failed. The past 10 years have seen additional advances in technology aimed at helping greater numbers of people become parents, too. Several of the most promising of these were discussed at the conference. Embryo cryopreservation, for example, may make in vitro fertilization (IVF) safer and less stressful by enabling a woman to undergo ovarian stimulation only once to retrieve eggs (See Cobo, page 25). And embryo selection technology that can distinguish healthy embryos from unhealthy ones promises to raise success rates. Further in the future it will be possible to diagnose any number of gene mutations in an embryo and harvest eggs from ovarian stem cells so that older couples can have biological children (see Legro, page 35 and Lamb, page 40). However, the best application of these technologies should be debated before they come into widespread use, said Evers. Moreover, some technologies pose ethical questions that require societal input. For example, while diagnostic tests can detect the presence of a gene, the tests may be meaningless since genes don’t predict with 100% certainty whether a person will actually develop a disease. Also, if women beyond normal reproductive age can indeed one day have biological children, then how old is too old? “You have to think about these things before the possibility arises,” said Evers.

To start addressing these issues, the “Top Ten in Reproductive Medicine” conference highlighted the best papers as an example of how research into human reproduction and infertility should be done. The format, inspired by Simón and developed by the Serono Symposia International Foundation (SSIF), was the first of its kind in the field. By discussing each paper—five clinical and five basic research—at length, conference organizers hope to promote good clinical practice that comes from adopting technologies and practices that are backed by strong scientific evidence and that have been validated by ethical debate.

ASSISTED REPRODUCTION TECHNOLOGIES: WE CAN DO BETTER

IVF has changed little since Robert Edwards and Patrick Steptoe introduced the technique over 35 years ago. While there have been advances in the field, such as the ability to freeze sperm or eggs and test embryos for specific genetic abnormalities, the process remains much the same: Fertilize egg with sperm, transfer the resulting embryo into a woman’s uterus, and hope for a full-term pregnancy. The technique has brought five million babies into the world and fulfilled the longing of parents for children struggling with infertility.

But IVF has a downside. The drugs given to stimulate the ovaries to produce multiple eggs can overstimulate the ovaries and land women in the hospital, when the clinical condition is particularly risky. The procedure is stressful, primarily because it’s hard to predict whether it will work. Depending on the age of the eggs, the clinic, and the procedure used, any embryo transfer cycle has between a 25% and 40% chance of resulting in a baby. With the odds in favor of failure, doctors have typically transferred more than one embryo to boost the chance of success. While the rate of triplets and higher order births has come down drastically, the number of twins born from reproductive technologies remains at about 30% of all ART births in the U.S. A twin or multiple pregnancy has long been known to raise the risk of harm to both mother and babies. Maternal complications include increased rates of pre-eclampsia, gestational diabetes, and preterm labor. Risks for babies include low birth weight and prematurity as well as a higher risk of long-term disability and death as compared with singleton pregnancies.

WITH NEW TECHNOLOGIES, SUCCESS IS MORE LIKELY

At the conference, Christina Bergh from the Sahlgrenska University Hospital in Gothenburg, Sweden presented her 2004 paper that for the first time provided evidence that transferring a single embryo yields almost the same birth rate as transferring two women under age 36 (see page 23). Bergh’s paper was the first clinical paper presented and set the backdrop for the presentations that followed. It began with the question: How can we treat infertility and infertilt the least harm? The paper was among the most highly viewed over the last 10 years, primarily because it was published when rates of multiple births using ART were much higher than they are today. Since the paper was published, single embryo transfer (SET) has been widely adopted, particularly in Northern Europe. In Sweden, doctors use SET in 75% to 80% of all IVF cycles in women of any age, said Bergh. But there remain several countries in which it hasn’t caught on, she added, and this is likely because of differences in reimbursement prac-
analysis data with birth outcomes and showed that the DNA array technology could detect, with 99% accuracy, an abnormal embryo that was unlikely to develop into a baby. Another study showed that selecting embryos using this technology does indeed yield higher birth rates. The predictive power of the technology is so great that the Reproductive Medicine Associates of New Jersey fertility clinic of which Scott is the director now performs SET using the diagnostic technology in 70% of all IVF cycles, he said. The clinic also offers the diagnostic service to other clinics.

The technology isn’t easy to implement, however, and is expensive compared with other technologies on the market, challenger Carlos Simón pointed out, and no one has yet demonstrated that the array technology yields better clinical outcomes as compared with those alternatives that are cheaper and easier to use. Discussion also raised the point that array technologies may be rendered obsolete in the near future by next generation technologies that can sequence genomes faster and cheaper. In fact, there are already commercial systems available. During the meeting Renee Reijo Pera, a professor at Stanford University School of Medicine, presented her 2010 paper on noninvasive imaging of human embryos (see page 15). The paper showed that time-lapse image analysis of two-day old zygotes together with gene expression profiling could with 93% accuracy predict which embryos from abnormal ones are a boon to the field because they raise success rates. Scott’s clinic is seeing pregnancy rates of greater than 50%, he said, and preliminary data suggests that embryo selection is bringing preterm births rates down to a level on par with the general population.

Microarrays aren’t the only promising embryo selection technology available. During the meeting Tilly and his colleagues developed a diagnostic test that, like microarrays, allows researchers to screen embryos for specific traits—heriting certain traits based on sequencing specific parental genes. Adapting such tests to screen embryos for specific traits would be a simple task—the prospect of which has raised more than a few eyebrows. Nevertheless, technologies to parse normal embryos from abnormal ones are a boon to the field because they raise success rates. Scott’s clinic is seeing pregnancy rates of greater than 50%, he said, and preliminary data suggests that embryo selection is bringing preterm births rates down to a level on par with the general population.

EXPANDING THE BOUNDARIES OF TREATMENT

In the future, there may be no limit to the age at which a woman can have a child. genie founder and chair of biology at Northeastern University. His 2012 paper provided evidence that human ovarian tissue contains pools of ovarian stem cells that develop into human oocytes—confirming what researchers have found in other animals and overturning the long-held dogma that women are born with a fixed number of eggs that are never replenished (see page 10). Tilly first shocked the field in 2004 when he and his colleagues extracted ovarian stem cells from female mice for the first time.

In the recent paper, Tilly and his colleagues developed a more sensitive method to identify and collect mouse ovarian stem cells. Once the team confirmed that it had collected the correct cells by this new method, it repeated the technique with frozen ovaries donated from sex-reassignment patients. When cultured, the retrieved ovarian stem cells (OSC) turned into immature oocytes. The team then tagged the cells with green fluorescent protein to trace them and injected them into pieces of human ovarian tissue, which were then transplanted into mice. After about two weeks, the OSCs had differentiated into green-glowing cells that by all measures appeared to be human oocytes. “This was one of the most astonishing papers published in 2012,” said challenger Evers. “The Cobo study showed for the first time in a large number of patients that you can do it and have good outcomes.” Moreover, embryo cryopreservation if broadly implemented might reduce the need to put back more than one embryo. Evers added, because you can freeze several and put them back in subsequent cycles if a woman doesn’t become pregnant. It may even enhance pregnancy rates because the endometrial environment appears to be “more friendly” during normal cycles as compared to drug stimulated cycles, he added.}

Tilly said. If the research pans out, it would give women with ovarian cancer the chance to have children later in life. It would also open the possibility that women well into their 50’s and even older could bear biological children—the ethics of which “are not for me to decide,” concluded Tilly.

Vilella’s questions about the health of offspring raised another important point of debate: whether any of the procedures used in IVF, current or in the future, might unwittingly be harming the resulting offspring. At the meeting, Michael Skinner, director of the center for reproductive biology at Washington State University in Pullman, Washington, presented his serendipitous finding revealed in his 2005 paper on the epigenetic transgenerational effects of endocrine disruptor chemicals on rodents (see page 18). If a pregnant female rodent was exposed to endocrine disrupting chemicals during a window when the fetus’s sex cells were developing, the chemical exposure caused epigenetic alterations—changes in methylation patterns in the genome—in sperm. These alterations were passed down through at least four generations and reduced male fertility. Skinner has since tested several additional chemicals and found the same or similar effects on sperm, and that these epigenetic changes can cause disease in subsequent generations. In one study [Reprod. Toxicol. 34:708 (2012)], for example, Skinner showed that pregnant female rats exposed to the pesticide mixture DEET—commonly found in insect repellants—gave birth to pups with higher rates of disease that included pubertal abnormalities, testis disease, and ovarian disease. Disease susceptibility was predominantly passed via males and persisted for three generations.

A similar issue has been raised before in regard to IVF techniques. Could the egg or culture media be exerting an effect on the epigenetics of the developing embryo? To date, rigorous studies have not yet been done to answer this question. But the debate certainly generated enough interest and ideas for future studies.

WRAP UP

After the meeting, participants lauded the challenge and debate format. “From my perspective this type of format should be broadly applied,” said Tilly. “It’s an awesome idea,” Scott added. “We’re not prone to hyperbole. Some, however expressed a desire for more probing questions. “We were all being a little too nice,” Reijo Pera said. But everyone agreed that in contrast to large conferences where there is little time for discussion and questions, this one enabled much more opportunity to learn. Such feedback will be important in organizing the second Top Ten in reproductive medicine conference, which is already in the works. According to Simón, the next one will likely take place in four years—enough time to publish new papers and to measure whether the first conference had an impact on spreading ideas and improving the way research in reproductive medicine is done.
Several methods for performing genetic screens on embryos, both invasive and non-invasive, are in the research pipeline right now.

In vitro screening

Several methods for performing genetic screens on embryos, both invasive and non-invasive, are in the research pipeline right now. The 1960s through the 1970s was a period of great change in reproductive medicine. Knowledge about reproductive potential increased dramatically. Before in vitro fertilization (IVF), gynecology had little more to offer infertile couples than making a diagnosis and offering tender, loving care (1). When the efforts of the two pioneers, Robert G. Edwards and Patrick G. Steptoe, resulted in the birth of the first IVF baby in 1978, the world slowly started to understand the urgency that these two icons had caused in the medical field. Not without open antagonism from some, Edwards became acclaimed as the father of IVF and his discoveries were eventually recognized by the awarding of the Nobel Prize in Physiology or Medicine in 2010. By then, several millions of babies had been conceived through IVF and the term assisted reproductive technology (ART) was coined to include other procedures that improved infertility treatment. Edwards’ work laid the foundation for further exciting developments in reproductive medicine, such as preimplantation genetic diagnosis (PGD).

ART is focused on providing better gametes into close proximity in order to generate a viable embryo, which can then be placed in a receptive endometrium to generate a healthy, full-term pregnancy. The greatest progress has been made in improving embryo viability and increasing implantation rates which, unintentionally, has led to the main drawback—the unacceptable high incidence of multiple births. The rate of multiples has been substantially reduced by the introduction of elective single embryo transfer (eSET), one of the few ART developments that has been introduced based on robust clinical data (2). Regulatory initiatives for eSET approval have followed around the world. However, there is still much to be done before eSET is universally accepted.

An ART process begins with controlled ovarian stimulation (COS), the manipulation of the reproductive physiology to generate several mature oocytes simultaneously in a single cycle. In the early days of ART, this was a necessary functional step because the techniques for fertilization and culture were poor, so several eggs needed to be fertilized to obtain just a few viable embryos. Today, the procedures used in ART labs are much more sophisticated, so there is a diminished need for aggressive COS. The field is now moving towards more gentle and patient-friendly stimulation protocols which will produce only an adequate number of embryos, and no more (3).

In vitro screening

Several methods for performing genetic screens on embryos, both invasive and non-invasive, are in the research pipeline right now. After some initial controversy regarding the biopsy and screening of embryos for a limited number of chromosomes, the introduction of more accurate technologies that provide more detailed information on all chromosomes—such as single nucleotide polymorphism arrays—is looking promising, especially for use in older patients (4). In vitro embryo observation utilizing time-lapse technology (5), and the analysis of proteins and metabolites consumed and secreted by the developing embryo in culture, are other noninvasive methods that hold promise.

The same molecular techniques are applied during PGD in order to detect those embryos carrying particular disease-associated mutations. The list of diseases is steadily increasing and in the future it will become possible to diagnose multiple disorders simultaneously in a single embryo, shifting the frontiers in human health care. These advances will, however, also raise important ethical questions that will need to be addressed (6), such as how to contend with the partial expression or limited penetrance of certain diseases that might only manifest in later life.

Cryopreservation

Although the failure of embryo implantation was a concern in the early days of IVF and a valid justification for the use of multiple embryos, the problem was compounded by the poor performance of embryo cryopreservation techniques. However, vitrification—introduced as a method of cryopreservation in the 1980s—completely changed the field for both embryo and oocyte freezing (7). Today, survival rates after freezing/thawing of human oocytes and embryos are greater than 90%, bringing about new possibilities for ART, while also reducing the need for ethically questionable selective abortions.

Vitrification has also been applied as a means to preserve fertility in young women with cancer, allowing for the freezing of oocytes prior to chemotherapy or radiation treatment. Meanwhile, fertility preservation has more recently been introduced to avoid the deleterious effects of aging on the oocytes (‘social freezing’). Since age correlates positively with aneuploidies, an increasing number of women are requesting oocyte freezing before age 38 in an attempt to abrogate this effect (8).

Two other complications of COS deserve our attention: an altered endometrial receptivity that decreases the chance of implantation, and the development of ovarian hyperstimulation syndrome (OHSS), a rare but potentially life-threatening condition. A new two-step ART approach that involves the implantation of previously frozen embryos in subsequent natural cycles will potentially lead to a safer and more patient-friendly ART approach with fewer complications (9, 10).

The endometrial environment

Most endometrial attention in ART is focused on the embryo, but endometrial receptivity is an issue that is often neglected, predominantly because insufficient methods exist to ascertain the functional status of the uterine lining. In today’s ‘omics era, however, new tools are coming to the fore that will allow the best embryo(s) to be placed in a fully receptive endometrium (11). Despite numerous advances in reproductive technologies, we still have very limited possibilities for addressing the main cause of infertility, namely the woman’s age. The next decades will see research in developing methods to rejuvenate oocytes by exploring the cellular and molecular hallmarks of aging and attempting to at least partially reverse them (12). Similarly, the creation of gametes by cell reprogramming could be another source of healthy oocytes (and spermatozoa), an advance that will certainly change our perspective on infertility in the coming years (13, 14).

Most of these potential advancements in ART are still based on small, observational clinical ‘proof-of-concept’ studies. They need to be followed up with more comprehensive, multicenter randomized clinical trials. Subfertility couples belong to a vulnerable group and should not be exploited (15). We should provide them with dedicated care and evidence-based treatment. During the next decade, the medical establishment needs to provide more robust evidence for the value and integrity of the treatments currently offered to patients. As Robert Edwards, our very own Nobel Laureate stated: ‘the legacy to future generations is a matter of life’ (16).

REFERENCES

Germline Stem Cells in Adult Mammalian Ovaries

Cori A. Woods and Jonathan L. Tilly

INTRODUCTION

Until recently, a decades-old belief in the field of mammalian reproductive biology was that females are born with a finite endowment of oocytes, termed the 'ovarian reserve,' which is progressively depleted throughout life by either ovulation or atresia (1). Once exhausted, the ovaries cease to function. In women, this event heralds the onset of menopause (2). Although the traditional explanation for the cessation of ovarian function is clear, the mechanisms by which this paradigm is probably incorrect. In its place has emerged a model that aligns expanded research efforts with that observed in non-mammalian species, in that adult ovaries actively support formation of new oocytes and follicles (3–14). The underlying foundation of this major paradigm shift is rooted in the development of detailed methods for the isolation and characterization of a rare population of mitotically active germ cells from adult ovaries termed female germline stem cells (fGSCs) or oogonial stem cells (OSCs) (4, 6, 12). Once isolated, these cells can be stably propagated in vitro for months without loss of their ability to differentiate into oocytes following either defined culture in vitro or transplantation into ovarian tissue in vivo (4, 5, 7, 8, 14). In mice, oocytes formed from transgenic OSCs capable of undergoing meiotic prophase I and the metaphase-II stage of development, and these eggs can be fertilized to produce viable embryos and offspring (4, 7, 8, 12).

While intraovarian transplantation models clearly show that mammalian OSCs are capable of generating fully functional eggs, the role of these cells, if any, in adult ovaries under normal physiological conditions has not yet been reported. In non-mammalian vertebrates, such as the zebrafish (Danio rerio), genetic lineage-tracing approaches have been successfully employed to show that GSCs actively contribute to the adult oocyte pool used for reproduction (15). Development and analysis of similar genetic models in mice, which are currently under investigation in the Tilly laboratory, should provide a means to map the rates of new oocyte formation during postnatal life, and the contribution of these oocytes to offspring production. In addition, the ability to track a marked pool of oocytes formed at a specific point in life will facilitate studies of in vivo oocyte lifespan to determine how long a given oocyte, once generated, persists in adult ovaries. This information will help elucidate if the quality of eggs deteriorates in women with age simply because all of the oocytes have been sitting around for decades accumulating damage, or if the decline in egg quality is instead tied to a progressive impairment in the ability of OSCs to generate competent oocytes with age, as is the case in the aging Drosophila ovary (16).

CHARACTERIZATION OF MOUSE AND HUMAN OSCS

Although the beginnings of contemporary evidence for the existence of OSCs in postnatal ovaries date back nearly a decade (4, 8, 12–14), the recent development of protocols that allow for the enrichment or purification of OSCs from adult ovaries has greatly accelerated research in this area (4, 6, 8, 12). Building on earlier observations that mouse OSCs expose the C-terminus of the germ-cell-specific protein, Ddx4 (DEAD box polypeptide 4; also commonly referred to as Vasa or Mouse vasa homolog (Mvh)) on the outer cell surface (4–6), we developed and validated an antibody-based fluorescence-activated cell sorting (FACS) approach that purifies OSCs based on detection of this extracellular epitope of Ddx4 (extracellular Ddx4- or ecDdx4-positive cells) (6, 12, 13). The reason why Ddx4 is exposed on the surface of OSCs but is completely internalized in oocytes is unknown, but may be related to movement of the protein from a potentially sequestered transmembrane location to a more functionally active position in the cytoplasm as primitive germ cells undergo meiotic differentiation (13). Of note, similar OSC isolation strategies have been reported using antibodies against the externalized domain of the primitive germ cell marker, Lifem3 (interferon-induced transmembrane protein 3; also referred to as Fragsil) (6, 12).

Following isolation and expansion of OSCs in vitro, the cells can be genetically modified to express a traceable gene (such as green fluorescent protein, GFP) and followed to the recurrence of wild type mice where they actively undergo differentiation into oocytes that mature, ovulate, and fertilize to produce viable embryos and offspring (4, 7, 8, 12). Although this type of cell fate-tracking experiment is not feasible in humans, we have successfully employed the same model to establish germ-cell lineages in a variety of human OSCs, expressing GFP, in adult human ovarian tissue in an in vivo environment (6, 12). The ensuing observations that human OSCs form what appear to be meiotically arrested oocytes (positive for expression of YBX2, which is specifically expressed in germ cells at the diplotype stage of meiosis (17, 18) contained in follicles within one to two weeks of grafting not only provides definitive evidence that adult human ovaries are fully capable of supporting oogenesis and folliculogenesis, but also that oocyte and follicle formation from purified OSCs returned to their natural environment are events that can occur in a fairly rapid time frame (6, 12).

CONTROVERSIAL NEW FINDINGS?

Although independent verification of the existence of OSCs in adult mouse ovaries is now available from two independent laboratories (6, 8–12), several recent studies—both based on circumstantial negative findings with mice—claim otherwise despite the fact that neither study actually attempted to reproduce what we and others have done or to isolate OSCs from mouse ovaries using published protocols employed successfully by us and others. The first of these, from Liu and colleagues, relies entirely on a transgenic reporter mouse generated by crossing Ddx4-Cre mice with Rosa26YFP+ mice, on the assumption that OSCs could be specifically tracked due to Ddx4-driven Cre activation in these cells (as would be the case with all germ cells, irrespective of their differentiation status) (19). Unfortunately, an abundance of many key control experiments, discussed in detail recently (12), precludes any clear interpretation of the findings. In fact, in as-yet unpublished studies we have since evaluated oocytes from the same genetic mouse line, and combined this approach with our FACS-based protocol for OSC isolation, to highlight the erroneous nature of their primary conclusion that, in contrast to substantial evidence from us and others (3–4, 12, 20), mitotically active germ cells do not exist in postnatal mouse ovaries.

The second paper relies heavily on two key assumptions (21). The first is that oogenesis in embryonic and adult ovaries is accomplished through identical processes. In fetal ovaries, primordial germ cells proliferate and form cyst-like clusters prior to meiosis (22). Upon meiotic commitment, the cyst-like clusters associate with somatic cells, forming the ovigerous cords that will break down shortly after birth to produce the initial primordial follicle pool (23, 24). In this study, Lei and Spradling propose a model, with no supporting data, that relies on formation of germ cell cysts as an indispensable step in postnatal oogenesis as well. When they failed to observe evidence of cyst-like germ cell clusters in adult mouse ovaries, the authors concluded that oogenesis is not occurring (21). Another assumption relates to the “knee tracing model” used, in which both Cre-recombinase and estrogen receptor are expressed in essentially all cells of the mice under study. Short-term induction with Tamoxifen excites a stop-cassette located within a Rosa26-yellow fluorescent protein (YFP) transgene, yielding indiscriminate labeling in which all cell types are “marked” with YFP at very low frequency (1%–2%). Since all germ cells, including OSCs and oocytes, express YFP at equivalent frequencies, it is impossible to discern if any labeled oocytes originated from labeled OSCs or if any unlabeled oocytes originated from unlabeled OSCs. In fact, the authors acknowledge identification of “a few germ cells at a prefollicular state of development,” but do not discuss the meaning of such findings (22). Since oocytes are incapable of surviving in ovaries unless surrounded by granulosa cells as follicles, the rare “prefollicular” germ cells identified by Lei and Spradling are likely OSCs or their immediate progeny.

Whatever the case, assuming 2% of OSCs are YFP labeled in their...
model, one would expect the ratio of labeled and unlabeled oocytes comprising the primordial follicle pool to remain constant over time since a fixed proportion of labeled and unlabeled oocytes would be constantly entering the pool through de novo oogenesis from a chimeric population of OSCs and subsequently exiting the pool through follicle growth activation. This is exactly what Lei and Spradling observe (27). It bears mention that a toxicity model was also employed to assess if OSC activation and follicle renewal in postnatal ovaries occurs in response to damage. When such evidence was not produced, these negative findings were offered as further proof that OSCs do not exist. Surprisingly, however, the cytotoxic drug used was busulfan, which is a widely known GSC toxicant (23–25). Accordingly, it is unclear why one would expect to find damage-induced activation of a population of cells that are killed by the agent used to ‘activate’ them.

NEXT STEPS AND HUMAN HEALTH IMPLICATIONS

As work with laboratory animal models continues to fill in key gaps in our understanding of mammalian OSCs, the discovery of oocyte progenitor cells in human ovaries opens up many opportunities for their utilization, some from the perspective of the clinical management of infertility and others from that of basic biomedical discovery (8, 9). Given the remarkable similarities between mouse and human OSCs, and in particular the ability of OSCs from both species to participate in new oocyte and follicle formation when delivered back into adult ovarian tissue (8, 12), it would stand to reason that, as observed in mice (4, 7, 12), human OSCs also have the potential to generate developmentally competent eggs. However, because such work with human OSCs in vivo is not possible, strategies to mature human eggs derived from OSCs entirely ex vivo will be needed. In this regard, Telfer and McLaughlin have reported a multipotential cell culture system in which microthin human ovarian cortical strips are maintained under defined serum-free conditions to initiate primordial follicle growth activation (28, 29). Once the preantral stage of development is reached, the follicles are dissected out and subsequently reaggregated. When reimplanted into immunodeficient mice, oocytes can be aspirated for in vitro maturation to metaphase-II eggs. Should this methodology prove successful (30), it may provide an opportunity to test the developmental potential of human OSCs to form functional eggs (Fig. 1), given that human OSCs have already been shown to generate primordial oocytes contained in follicles in adult human ovarian cortical strips (8, 12). In addition to the ability to test the competency of oocytes derived from human OSCs, reaggregated follicles with cells and other support necessary for ensuring ex vivo development, the innate oocyte-forming capability of these cells is valuable for other reasons. During serial passage of pure OSCs in vitro, with no additional feeder cells) under defined conditions, a small subset of OSCs spontaneously initiate a differentiation process that results in the formation of what appear to oocytes (4, 12). Although these oocytes are not functionally competent, having never been associated with nor instructed by granulosa cells), they can be used as a powerful bioassay to rapidly decipher the factors and signaling pathways that guide oogenesis (Fig. 1). This can be achieved by assessing the rate of formation of in vitro-derived oocytes from a fixed number of OSCs exposed in different wells to various agents (12, 14). In addition to the scientific value of such knowledge, large-scale screening of cultured OSCs may facilitate identification and development of therapeutics to suspend or restore the dormant state of advancing maternal age or after exposure to toxic insults such as chemotherapy. Although more work is clearly needed to test these ideas, at this stage we believe it is appropriate to, at minimum, end further debate over whether mice are a relevant model for human ovarian biology and focus on what additional experiments are needed to more clearly define the regulation and function of these newly discovered cells in female reproductive biology.

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Acknowledgments: We thank Cooper Graphics (www.cooper247.
com) for preparation of the final Figure. Work conducted by the authors
was supported in part by grants from the National Institute of
Health (R37-AI011279, 2R15HD072280, P30-AG038909,
the Henry and Vivien Rosenfield Philanthropic Fund, and the Glenn Foun-
dation for Medical Research.

Competing Interests Statement: D.C.W. is a scientific consultant for
OvaScience, Inc. (Cambridge, MA); J.L.T. discloses interest in intellectual
property, U.S. Patent 7,955,846, and is a co-founder of OvaScience.

Figure 1. Bidirectional communication between oocyte and surrounding somatic cells. There are three major ways of oocyte-somatic cell communication in follicular development and oocyte maturation: (i) oocyte-secreted factors GDF9, BMP15, and GDF9/BMP15 heterodimer; (ii) granulosa cell-derived kit ligand and its receptor on the oocyte; and (iii) secondary messengers cAMP and cGMP.

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**OOCYTE-SECRETED FACTORS IN THE TRANSFORMING GROWTH FACTOR β (TGF-β) PATHWAY**

In follicular development, the mammalian oocyte modulates granulosa (directly) proliferation and differentiation rates through multiple oocyte-secreted factors (3). Among those factors, growth factor activation (9,10) and bone morphogenetic protein 15 (BMP15) are well known as two of the most important animal models to promote pro-oocyte growth. A novel study has been published as subsequent participation in the stages of folliculogenesis (4), GDF9 and BMP15 are close parallels in the TGF-β superfamily and signal through the conserved activin receptor P-Smad2/3 or P-Smad1/5/8 pathway, respectively, to stimulate proliferation and differentiation of granulosa cells. Animal models deficient in these two ligands have been used to study their role in follicular activation at the early stages of follicular development between poly- and mono-oovulatory mammals (Table 1). In mice, Gdf9 null females are sterile due to an arrest of follicular growth at the primary follicle stage (8). BMP15 null mice exhibit later defects in ovulation and fertilization rates, which lead to a reduced litter size (6). In sheep, homozygous BMP15 mutants exhibit a block in folliculogenesis at the primary follicle stage, resulting

This article was based on the original publication: J. J. Epig et al. Proc. Natl. Acad. Sci. U.S.A. 99, 2900 (2002). Changes were made to improve its fluidity and to clarify the facts. This change does not affect the authors’ conclusions. This change does not affect the scientific integrity of the study.
in sterility similar to that of Gdf9-null mice (7). A homozygous point mutation in sheep GDF9 also results in abnormalities at early stages of follicular development (8). In humans, although there is no direct evidence to prove that BMP15 and GDF9 are major causes of ovarian insufficiency, multiple studies have found that these two genes are associated with premature ovarian failure (9–11) or spontaneous dizygotic twinning (12). Therefore, these genetic data indicate that both GDF9 and BMP15 play important roles in the transition between primary and secondary follicles as well as in ovulation. However, which of the two proteins is the dominant regulator might differ according to varying protein activities and expression patterns among species.

To further resolve the functions of these two growth factors and their potential synergistic functions in later follicle developmental stages, recombinant BMP15 and GDF9 proteins were used to treat granulosa cells in vitro. In a recent study, our group purified human (h) and mouse (m) recombinant GDF9 and BMP15 homodimers and heterodimers to study their oocyte-secreted activities (13). We showed that mGDF9 homodimer was a potent trigger of the TGF-β signaling cascade and upregulated downstream molecules expansion (14). These results suggest that mGDF9 could induce the proliferation and differentiation of granulosa cells, while mBMP15 homodimer was inactive. By contrast, hGDF9 homodimer was inactive, and hBMP15 homodimer had a relatively low activity compared to mGDF9. In our studies, mGDF9 homodimers were the most biologically active in the follicular stages of oocytes.

NEGATIVE OOCYTE REGULATORS IN THE PHOSPHATIDYLINOSITOL 3-KINASE PATHWAY

Granulosa cell-derived kit ligand is a positive regulator, stimulating oocyte growth and somatic cell proliferation. After binding with kit ligand, the kit receptor mediates PI3K signaling cascades in the oocyte via many downstream effectors. Among these regulators, several key components of the PI3K pathway function as suppressors of follicular activation at the early stages of follicular development. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a major negative regulator. Mice lacking PTEN in the oocyte exhibit premature ovarian failure due to depletion of promin-
the sperm (1). These chromosomes are highly condensed, highly methylated, and organized around a protamine scaffold rather than a histone scaffold. Yet, it is clear from several studies that native reprogramming in the human oocyte-to-embryo transition proceeds in over 75 percent of embryonic blastomeres (3); moreover, advances in somatic cell nuclear transfer (SCNT) procedures have revealed the robust ability of human oocytes to reprogram somatic DNA (4).

**USE OF NONINVASIVE TIME-LAPSE IMAGING TO PREDICT DEVELOPMENTAL FATE**

Over the last decade, key studies in human embryo development have used time-lapse imaging of embryogenesis to elucidate the role of various cell cycle parameters and factors that may predict success or failure in the embryo reaching the blastocyst stage and beyond. Wong et al. (2015) reported the use of time-lapse microscopy and gene expression profiling at the single embryo and blastomere level to document the growth of human embryos from zygote to blastocyst (3). Key developmental features were extracted and algorithms generated to predict success and failure in development leading up to the blastocyst stage; morphological assessment was augmented by a comparison of gene expression in embryos that succeeded or failed to develop. These studies indicated that the characteristics of the first cytokinesis and the first two cleavage divisions could be used as markers for a positive outcome. Successful development was shown for the first time to be highly regulated, following strict timing in pre-implantation development even prior to EGA. In normal development, degradation of maternal mRNA was shown to precede cell autonomous EGA. In contrast, arrested embryos most often displayed aberrant cytokinesis during the first cleavage divisions prior to EGA, accompanied by equally aberrant gene expression in the embryos and individual blastomeres. Since the studies by Wong and colleagues suggested that human embryo fate could be predicted accurately prior to EGA, it was proposed that success and failure is often inherited. Embryos that begin life with defective maternal programs, or inherit defective paternal components, are likely to display aberrant cytokinesis, embryo fragmentation, and arrest of individual blastomeres or the entire embryo.

The methods and algorithms described by Wong et al. provided a platform for improved and earlier diagnosis of embryo potential to hopefully allow for the transfer of fewer embryos earlier in development during assisted reproduction procedures. Subsequent reports have documented that the parameters identified are able to provide predictive power beyond the blastocyst stage, to implantation, and that other parameters may be added for ease of use or to adapt the technology to clinics that differ in terms of cell culture media, patient base, or other characteristics (5).

**EXTENSION OF STUDIES TO UNDERSTANDING GENESIS OF ANEUPLOIDY**

Based on the results of Wong et al, we hypothesized that measurement and analysis of specific parameters in preimplantation human embryos could provide insight into fundamental characteristics of the embryo, including ploidy. We first sought to correlate normal and abnormal development by correlating continual imaging with genetic analysis of single embryos and embryonic blastomeres (6). Results indicated that euploid embryos could not be accurately distinguished from those with aneuploidies when only cell cycle parameters were analyzed. By adding additional confocal microscopy analysis (which included reconstruction of all blastomere karyotypes to the four-cell stage), however, we concluded that the complexity in human embryonic aneuploidy is not simply due to errors on the meiotic/mitotic spindle. Rather, results suggested that generation of aneuploidy may also occur during interphase and can be explained by the containment of a subset of missing chromosomes within cellular fragments, which bud off from embryonic blastomeres and may persist or become reabsorbed. We also noted that chromosome-containing fragments may arise from micronuclei, or embryonic structures distinct from primary nuclei, with encapsulated chromosome(s) detected only in the blastomeres of cleavage-stage human embryos (Fig. 1). These findings suggest that individual human blastomere behavior is diagnostic of chromosomal status and provides the first example of the use of non-invasive imaging and automated fragmentation tracking to distinguish euploid and aneuploid cells. These studies predict that a combination of time-lapse parameter analysis, along with assessment of blastomere fragmentation dynamics (Fig. 2), may reduce the inadvertent transfer of aneuploid embryos, with implications for decreasing miscarriage risk and improving in vitro fertilization success. Based on this work, we have offered a model of aneuploidy development during human embryogenesis that is currently being further examined (Fig. 3).

**SUMMARY**

Numerous basic and clinical laboratories are now investigating the use of noninvasive time-lapse imaging to provide reliable information regarding the development of human embryos to the blastocyst stage, implantation, and beyond. It is hoped that advances will enable the separation of those embryos that are most likely to develop into healthy infants from those that likely would result in miscarriage or still-birth due to severe birth defects.

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Figure 2. Automated tracking of cellular fragmentation for embryo assessment. Time sequence of cumulative segment lengths in pixels for each frame of the time-lapse image analysis for three embryos was observed. Embryo C3 with high fragmentation, embryo B2 with low fragmentation and embryo C1 with no fragmentation. Note that the embryos with high fragmentation exhibit much larger cumulative length of segments than that of embryos with low or no fragmentation. Image from (6).
Environmental Induced Epigenetic Transgenerational Inheritance of Disease

Michael K. Skinner

INTRODUCTION

We have investigated the effects of two environmental toxicants (vinclozolin and methoxychlor) following exposure of rats during fetal gonadal sex determination, and the impact on gonadal development and function (1, 2). A serendipitous observation was made when F1 generation animals were mistaken to breed to generate the F2 generation offspring: the vast majority of the testes in the F2 generation carried a spermatogenic cell defect that induced apoptosis. This prompted a multiple year study that demonstrated the phenomenon of environmentally induced epigenetic transgenerational inheritance of disease for the first time (3). This study showed an increase in apoptosis in testis spermatogenic cells in the F1, F2, F3, and F4 generations, as well as in outcrossed offspring, through non-Mendelian genetic inheritance, affecting 90% of the male population. The causative epigenetic transgenerational inheritance to occur, the external insult must act on a gestating female during fetal gonadal sex determination, influencing the epigenetic state of the germ cell defect that induced apoptosis. This phenomenon was transmitted transgenerationally (Figs. 1 and 2) (4–6). Several studies did not measure true environmental risk, but provided a good foundation for future analysis to determine the environmental factors including nutrition (7), toxicants (8), and plastics (BPH and phthalates) (9, 10), hydrocarbons (JPE jet fuel) (11, 12). All were found to promote the transgenerational inheritance of disease. Other toxicants (13, 14) were found to promote the transgenerational inheritance of disease as a result of exposure to various environmental factors including nutrition (15, stress (16, 17), and other toxicants (18). Combined observations suggest that epigenetic biomarkers for ancestral toxicant exposure and adult onset disease may exist.

EPIGENETIC TRANSGENERATIONAL INHERITANCE

The definition of epigenetic transgenerational inheritance is “germ-line mediated inheritance of epigenetic information between generations that leads to phenotypic variation in the absence of direct environmental influence” (6). This is in contrast to epigenetic inheritance that involves direct environmental germline or somatic cell exposure, and epigenetic responses during early development that influence phenotypes in later life. An example is the Agouti mouse.

GERMLINE EPIPHENOMAS AND EXPOSURE (TOXICANT) SPECIFICITY

The environmental compound (toxicant) administered in the initial generation carried a spermatogenic cell defect that induced apoptosis. This phenomenon was transmitted transgenerationally (Figs. 1 and 2) (3–5). Several studies described, below, support this hypothesis of the molecular etiology of epigenetic transgenerational inheritance.

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The environmental compound (toxicant) administered in the initial generation carried a spermatogenic cell defect that induced apoptosis. This phenomenon was transmitted transgenerationally (Figs. 1 and 2) (3–5). Several studies described, below, support this hypothesis of the molecular etiology of epigenetic transgenerational inheritance.

This article based on the original publication: M. D. Attar et al., Science 308, 1468 (2005), Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA skinner@wsu.edu

Environmental Induced Epigenetic Transgenerational Inheritance of Disease

Figure 1. Environmentally induced epigenetic transgenerational inheritance of disease. The environmental stress such as toxicants, nutrition, and stress influence a gestating female during the period of fetal gonadal sex determination to then affect disease incidence (percentage) in the F1, F2, F3, and F4 generations. The disease incidence for vinclozolin-treated males compared to control lineage animals is shown for each generation. The incidence of disease was related to spermatogenic cell apoptosis, prostate disease, kidney disease, testis disease, and immune abnormalities are presented. Modified from (20).

Role of Germ Line in Epigenetic Transgenerational Inheritance

Figure 2. Role of germine in epigenetic transgenerational inheritance. An environmental factor acts on the F0 generation gestating female (left) to influence the developing F1 generation fetus, resulting in epigenetic programming of the methylation state of the primordial germ cell DNA. This altered DNA methylation pattern becomes fixed, similar to an imprinted gene, and is transferred through the germline to subsequent generations. Somatic cells in the offspring in later generations also carry the altered epigenetic, generating an aberrant transcriptome, and promoting adult-onset disease. Modified from (6).

Figure 3. Venn diagram of transgenerational epimutations associated with different exposure groups showing a number of common epimutations in the F3 generation as a result of environmental exposure to vinclozolin, BPH, and phthalates at a differentially methylated region (DMR) for each exposure group. The DMRs carry epimutations that can transmit epigenetic information transgenerationally (4). The critical measurement is environmental compound (toxicant) specificity, which is determined by the number of common epimutations in the F3 generation.
transgenerational transcriptomes of 11 different tissues in male and female vinclozolin-treated versus control lineage rats (30). All tissues had a transgenerational transcriptome that was unique to the specific tissue, with negligible overlap between tissues. It is intriguing that a relatively small number of epimutations can produce such a large number of specific transcriptome changes (30). The identification of epigenetic control regions—areas of 2–5 megabases with an overall decrease in mDMS and mLMD and long noncoding RNA regions—may provide a clue (Fig. 4) (30), suggesting unique molecular regulatory mechanisms that will require further investigation.

To further elucidate the role of epimutations in adult onset disease, the molecular etiology of ovarian diseases were studied (33). Ovarian transcriptomes and transcriptome that suggested specific signaling pathways were affected. Similarly, somatic Sertoli cells in the testis were found in a separate study to have transgenerational alterations in their epigenomes and transcriptomes, affecting genes previously shown to be involved in male infertility (33).

IMPACT AND FUTURE STUDIES

Our original publication (3) described the phenomenon of environmentally induced epigenetic transgenerational inheritance of a disease. Subsequent publications confirmed and clarified the molecular and physiological parameters involved. The research shows existence of an epigenetic (i.e., epigenetic) form of transgenerational inheritance that impacts the etiology of certain diseases, as well as a potential molecular mechanism describing how environmental factors could directly influence gene expression and therefore disease (4, 5). Further studies clearly are needed to clarify the role of epigenetics and transgenerational inheritance in disease etiology, evolutionary biology, and other areas of cell and developmental biology. The specific next steps needed include investigation into why specific sites are more susceptible to becoming transgenerationally programmed and the mechanism by which this occurs, as well as the translation of this work from animals to humans. These and other studies will undoubtedly have significant impact on our understanding of normal biology and disease etiology.

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oviductal transport of embryos, since only CB1-deficient recipients further confirmed that maternal CB1 is critical for appropriate signaling affects oviductal embryo transport. Wild-type (WT) mice displayed on the Fallopian tube epithelium (cannabinoid, significantly decreased cilia oscillation frequency tubes. In addition, treatment with oleoylethanolamide, an endo- than those in normal pregnancies (AEA levels in ectopic Fallopian tubes were significantly higher than those in normal pregnancies). Our studies in mice stimulated research on ectopic pregnancy in women and many of the findings in humans were consistent with the results of the mouse studies. In women with ectopic pregnan- our studies not only provided a useful animal model to study ectopic pregnancy, but also draw more public attention to the adverse effects of maternal usage of marijuana and synthetic cannabinoids.REFERENCES


Acknowledgments: We thank Serenity Curtis for her careful editing of the manuscript. Work in the Day laboratory was funded in part by the National Institute on Drug Abuse and National Institute of Child Health and Human Development at the U.S. National Institutes of Health. X.S. was supported by a Larner Foundation postdoctoral fellowship in reproductive biology.

This article based on the original publication:
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The wide application of in vitro fertilization has caused a dramatic in- increase in multiple births, associated with adverse neonatal outcome, mainly as a result of the transfer of several embryos per cycle. Random- ized controlled trials have observed that the best outcomes were carried out to investigate pregnancy and live-birth rates after single (SET) or double embryo transfer (DET) as well as obstetrical outcomes. Results showed that the live-birth rate was significantly higher after DET than SET if only fresh cycles were compared. If one additional frozen/thawed SET was added to the SET group, live-birth rates did not differ substantially. Obstetrical outcomes were consid- erably improved with the SET strategy. We therefore conclude that SET is the more desirable option for a majority of patients.

INTRODUCTION
The rapid development of different in vitro fertilization (IVF) tech- niques has resulted in increasing pregnancy and live-birth rates per cycle. In parallel, a dramatic increase in multiple births has occurred. Numerous publications have demonstrated higher risks for an ad- verse outcome, including preterm birth (PTB), <37 weeks, low birth weight (LBW, <2,500 g), and perinatal mortality for children born after IVF, compared with children born after spontaneous concep- tion, mainly due to the high rate of multiple births following IVF (1–3). Also, for IVF singletons, increased rates of PTB and LBW were noted (4–5), likely caused by inherent characteristics of the infertile couple such as the mother’s age and nulliparity. An increase in the frequen- cy of neurological sequelae has also been found, strongly associated with PTB and multiple births (6). An increased rate of physical malfor- mations has been reported for children born following IVF (7–9).

The most important factor affecting the rate of multiple births is the number of embryos that have been transferred during IVF. Reducing the number of transferred embryos from three to two had little effect on live birth rates, but decreased the rate of multiple births; the observed twin rate was, however, still high (6). Data from large registry studies—many per- formed in Sweden—on obstetrical outcomes after IVF showed an in- creased rate of severe medical problems in IVF children, generating an intensive debate in Sweden among obstetricians, paediatricians, doctors in reproductive medicine, and politicians. There was a call for transfering only one embryo during IVF, a strategy supported by some reports that single embryo transfer results in satisfactory pregnancy rates (9).

The aim in the trial discussed here (10) was to investigate whether a similar live-birth rate could be achieved if two embryos were trans- ferred at one time—one fresh embryo, and if no live birth was de- tected, one additional frozen/thawed embryo—rather than the stand- ard practice of transferring two embryos on a single occasion, and whether this would decrease the multiple birth rate.

METHODS
Between 2000 and 2003, eleven clinics in Sweden, Denmark, and Norway participated in the trial in which 681 women under 36 years of age, performing their first or second IVF cycle and having at least two good quality embryos available for transfer, were randomly as- signed to two groups, SET and DET. The study was double blind, so neither the physician nor the patient knew whether one or two embryos were transferred. A new cycle was started in each patient. Patients needed in each group (330) was based on the assumptions that the true live- birth rate in both groups would be 0.30 and the probability is 0.80 that the upper limit of the 95% confidence interval (CI) for the difference in live-birth rates between the groups did not exceed 0.10 (α=0.05).

MAIN RESULTS
The results showed that the live-birth rate was 128/330 (38.8%) in the SET group and 142/331 (42.9%) in the DET group (95% CI for the difference: 0.3–11.6). Thus equivalence between the two groups could not be demonstrated, but the live birth rate did not differ sub- stantially between SET and DET. Moreover, the multiple birth rate was dramatically reduced in the SET compared to the DET group: 0.8% (1) vs. 3.3% (7) (p<0.001). Most important, the neonatal out- come was considerably better for children born to the SET group, with significantly lower rates of PTB (11.6% vs. 21.1%, p<0.002) and LBW (7.8% vs. 27.5%, p<0.0001). The SET group showed less severe neonatal complications demanding neonatal care in hospital (17.8% vs. 58.9%, p=0.003) and also a lower rate of complex mor- bidity (7.8% vs. 24.3%, p=0.0001) (1). A financial analysis indicated that the SET strategy was cost-effective; the incremental cost-effect- iveness-ratio (ICER) was €73,307 (7.8% vs. 24.3%, p=0.0001) (1). A financial analysis indicated that the SET strategy was cost-effective; the incremental cost-effectiveness-ratio (ICER) was €73,307 ($99,089) per additional delivery in the DET alternative.

FURTHER DEVELOPMENT
Several randomized trials and meta-analyses (12,13) have com- pared the delivery rates in SET and DET groups. The studies have shown significantly higher pregnancy and delivery rates after DET compared to SET when only fresh cycles were compared. Perform- ing an additional frozen/thawed SET in the SET group resulted in a delivery rate comparable with that in the DET group (10). The Scan- dinavian countries, particularly Sweden, have played a pioneering role in reducing multiple births by introducing SET on a large scale. In Sweden, this strategy has resulted in no change in delivery rates for fresh or frozen/thawed cycles, while the rate of multiple births has decreased dramatically, from 25% to around 5%. The overall SET rate is 70%–80% (Fig. 1). Delivery-and multiple birth rates after SET and DET comparisons according to above-mentioned IVF cycles are illustrated in Figure 2. A gradual increase in SET rates has been seen worldwide, including...
RATIONAL FOR NOT APPLYING SET

Fears among both patients and clinicians that SET will lower pregnancy and live-birth rates is probably the most common reason given for not applying SET more broadly. A focus on short-term higher success rates (pregnancy and live-birth rates) rather than optimal outcomes, i.e., the birth and health of a singleton, has slowed progress in this area. Despite the overwhelming data indicating increased risks associated with multiple births, there is still an ongoing debate whether, in particular, twins are a desired outcome for IVF. It has been claimed that the risks associated with IVF twins are dramatically exaggerated and that twins should be encouraged (17).

There are also financial variables for patients involved to be considered, and large differences exist in reimbursement systems for IVF in different countries, which influences utilization of SET. In countries where IVF is completely or partially covered by public funding, SET has been accepted more readily. If patients are self-funded, they might choose more embryos to be transferred in order to maximize the chance of becoming pregnant.

Other factors impacting SET acceptance include a lack of knowledge concerning the risks of multiple births, failure to consider cumulative live birth rates that include frozen/thawed cycles, differences in legislation between countries, and impediments stemming from cultural beliefs.

CONCLUDING REMARKS

The most important risk associated with IVF is the higher rate of multiple births, resulting in increased child morbidity and mortality. In addition to problems in the neonatal period, preterm birth and low birth weight may have long-term consequences for future health. According to the Barker hypothesis (18), these adverse outcomes may lead to increased risk of type 2 diabetes and cardiovascular diseases in adulthood.

The association between IVF and a poorer obstetric outcome for singleton has also been widely documented, but is quantitatively a much smaller problem. Maternal characteristics seem to be the largest contributors to these adverse outcomes (19), while the contribution of IVF-related factors is less clear.

Recent data from Sweden indicates that it is possible to have two consecutive singleton births in the same or similar number of fresh IVF cycles using the SET strategy as with the DET strategy by simply adding a small number of frozen/thawed cycles, while concurrently avoiding the risk of multiple births (20).

Current knowledge strongly supports SET as an IVF strategy that is safe and effective for mothers and children.

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Oocyte Vitrification: A Major Milestone in Assisted Reproduction

Ana Cobo

The cryopreservation of female gametes has been pursued since the onset of assisted reproductive technology (ART). After a lengthy period of failures and sporadic successes, the introduction of refined vitrification methods has meant a huge step forward in infertility practice. The very broad scope of this technology encompasses various new areas such as fertility preservation for social or oncological reasons, the possibility of creating egg banks for ovum donation, and the opportunity to avoid general anesthesia and to acclimate cumulus oocytes in low-responder patients. Even segmented infertility treatments can be offered by stimulating ovaries, vitrifying embryos, and then transferring them during a natural cycle. All of these options were not available just a few years ago, but are now being routinely applied in increasingly more infertility centers worldwide.

INTRODUCTION

The development of efficient cryopreservation techniques for female gametes has been a real challenge as both freezing and thawing expose oocytes to severe stress that can result in cell death. The introduction of improved vitrification methods has meant increasingly successful outcomes, where the most commonly applied methodology since the onset of ART—slow freezing—has led to limited success (1, 2). Among the reasons for failure resulting from slow freezing, chilling injury and ice formation are recognized as being the most deleterious (3). Chilling injury is avoided with vitrification because cooling occurs extremely rapidly and ice formation is circumvented very efficiently by using high concentrations of cryoprotectants (CPAs). Application of vitrification has been limited since it was first employed in embryology in the early 1980s (4) because the concentration of CPAs required for the earliest vitrification protocols can have dramatic toxic effects. Significant changes made to these protocols have reduced the concentration of CPAs to circumvent deleterious consequences to cells (5). The efficiency of modified protocols has been successfully tested clinically, which is an essential requisite for fertility preservation, ovum donations, or other clinical applications, and also to overcome certain clinical obstacles that ART posed, such as the absence of a partner’s semen sample on

Figure 1. Delivery rate, multiple-birth rate, and single embryo transfer rate in Sweden 2000–2011 (fresh cycles).

Figure 2. Percent delivery per SET and DET, percent multiple birth per SET and DET, and the overall SET rate according to age of the mother. National data from the Swedish Quality Registry for Assisted Reproduction (www.ucr.uu.se/qivf/) for 2011 in/2017 fresh IVF cycles.

This article based on the original publication: A. Cobo et al., Fertil. Steril. 89, 7567 (2008). Instituto Valenciano de Infertilidad, University of Valencia, Valencia, Spain acobo@iv.es.
the day of ovum collection. A comparison of embryo development using vitrified and fresh oocytes was performed (6) to provide valuable information about the further potential of vitrified oocytes, and to serve as a foundation for additional studies.

OOCYTE VITRIFICATION AND EMBRYO QUALITY

A prospective cohort study was used to perform a first of its kind analysis of the quality of embryos emerging from simultaneously generated vitrified and fresh donor oocytes (6). All of the metaphase II oocytes assigned to a single recipient were randomly allocated one of two groups: vitrified (oocytes were vitrified and then warmed for one hour before implantation) or fresh (maintained in culture at 37°C). Intracytoplasmatic sperm injection (ICSI) using the husband’s semen sample was performed simultaneously on both vitrified and fresh oocytes. A high overall survival rate was achieved (87%; N=224 of 231 oocytes). No significant differences in fertilization rates for day one (80.8% vs. 80.5%) and in the blastocyst stage (81.1% vs. 70.0%) were similar in both groups. Additionally, promising clinical outcomes were obtained following the transfer of embryos developed from vitrified oocytes (40.8% for the implantation rate and 47.8% for the ongoing pregnancy rate). These outcomes are in contrast to those previously reported by other authors using a slow freezing methodology (7). The widespread introduction of oocyte vitrification into clinical practice has been strengthened by these findings, which have demonstrated that both embryo developmental capability and implantation potential are essentially unaltered by oocyte vitrification.

CONTRIBUTION OF OOCYTE VITRIFICATION TO CURRENT CLINICAL PRACTICE

Our findings were further replicated by other authors with both donor and own oocytes (7). In a follow-up controlled, randomized clinical trial we used a refined methodology to compare clinical outcomes utilizing both cryopreserved and fresh oocytes in an ovum donation program (8). In this study, vitrified oocytes could not be shown to be inferior to fresh oocytes in terms of the ongoing pregnancy rate (odds ratio = 0.921, 95% CI 0.667 to 1.274). This finding definitively confirms our previous observations regarding the unimpaired potential of vitrified oocytes to develop into competent embryos. The substantial increase in ongoing pregnancies in a proportion comparable to that of fresh oocytes. Most of the difficulties posed by fresh donations, such as long waiting lists, the need for donors, financial cost, and the lack of guarantee, can be overcome by oocyte cryobanking. Stored oocytes are available anytime they are needed, significantly alleviating donation logistics.

The benefits of oocyte cryostorage have also been demonstrated in autosomal in vitro fertilization (IVF) cycles (8–10) leading to high cumulative pregnancy rates (11). Rierzi and coworkers have mirrored our findings on embryo quality and clinical outcomes in a prospective randomized clinical trial that embryo donation patients undergoing own-oocyte IVF cycles (8). The consistency and reliability of oocyte vitrification for this population was further demonstrated in a multicentric study (12). Additionally, initial findings regarding the number of oocytes vitrified, the patient’s age, and the developmental stage of the embryos at the time of transfer have been published and have proven useful for patient counseling (12). Oocyte vitrification has also been suggested to be an interesting therapeutic alternative for low responder- eers (13), improving outcomes for a group that has been very difficult to treat successfully, and may even save patients the disappointment of failure after IVF cycles with a poor response using fresh oocytes (14).

The extensive research carried out to date has laid the groundwork for the establishment of successful protocols for extremely ef- ficient oocyte cryopreservation. These preservation programs have provided a viable alternative that avoids the downside of an unfavorable uterine environment resulting from administration of exogenous gonadotrophins during controlled ovarian hyperstimulation (15–18). The research described here has fundamentally changed the clinical landscape and strongly supports the position that oo- cyte vitrification offers advantageous clinical results in diverse populations, opening up a wide range of possibilities in the field of ART.

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This article is based on the original publication:


It might seem logical that if test results are consistent among all the cells in an embryo, then the test is valid. However, embryonic mo- saicism is a real phenomenon impacting approximately one in five embryos. Moreover, the number of cells directly impacted by the assay in an embryo, some disagreement is expected. When does it happen? The problem with the assay or mosaicism? A number of investiga- tors have attributed these differences to mosaicism, but there is no scientific rationale to preferentially attribute them to either mosaicism or laboratory.

The critical impact that screening has on management of individual embryos, the challenges of mosaicism, the fact that the as- say may be required to work with only a single cell, and the complex- ity in demonstrating clinical benefit, validation of embryonic aneuploidy screening is a complex process requiring multiple studies at the basic laboratory and translational/clinical level. The study reviewed here describes the first step in the complex process of validating a tool—single nucleotide polymorphism (SNP) arrays—for embryonic aneuploidy screening, namely standardizing the assay.

TARGET DNA AMPLIFICATION

SNP array technology provides an opportunity to simultaneously in- vestigate the copy number of all 24 chromosomes. Unlike fluores- cence in situ hybridization (FISH)-based testing, arrays require the incorporation of DNA amplification for which a variety of methodolo- gies could be employed. Methods for whole genome amplification (WGA) were compared for performance on SNP arrays (2). Results demonstrated superior performance for PTC-based performance over evaluating copy number accuracy (most relevant to aneuploidy screening). A FUNDATIONAL STUDY OF SNP ARRAY SCREENING

This study was conducted in three phases (3). Single cells from a variety of cell lines with known chromosomal abnormalities were evaluated and data analysis settings were established to reach the highest level of consistency. This calibration was necessary to define the methodology. Next, the same cell lines were used to prepare and blind single cell samples for analysis. Blind analysis provided a rigorous test of the accuracy of the method on positive controls. Finally, multiple single cells from human embryos available for re- search were evaluated to demonstrate consistency of diagnoses in the relevant tissue type.

Analyses of Cell Lines

The SNP array approach analyses the relative intensity of binding for a large number of SNPs spread across the genome. Average intensi- ties are considered to have a copy number of two. Those with lower intensities are assigned copy numbers of one (monosomy) and those with higher intensities are assigned copy numbers of three (trisomy). Given the vast amount of data typically involved in generating the assay of an entire chromosome, the results on a single chromosome should
all the same. The reality is that there is not uniform intensity for the SNPs on a single chromosome and individual SNPs may be assigned differently. This unique opportunity by application of a Gaussian smoothing algorithm that evaluates the intensities amongst adjacent SNPs and averages them to enhance accuracy. However, the smoothing algorithms used for conventional karyotyping of the cell lines or clinical samples, where hundreds or millions of cells are available, do not function well following whole genome amplification of a single cell. The optimal smoothing distance was determined on cells with known karyotypes. It was important to validate smoothing on chromosomes that were monosomic and trisomic, and not just diploid.

Tolerances were established for the level of discordance amongst individual SNPs on a single chromosome. The upper limit of discordance, meaning the percentage of SNPs on a given chromosome that produce varying results, was established for each chromosome. If that level was exceeded, the assay was deemed “non-concurrent” and the result as non-concurrent rate per chromosome should be <0.1% and per cell should be <2%. These data were all generated on samples where the karyotype of the cell being tested was known. Functionally, this is starting with the answer and working backwards; a critical step in the process, but far from sufficient to validate the assay.

In the second phase, the prospective blinded evaluation, the testing algorithm was applied to blinded samples. The accuracy per chromosome was >96.9%. More importantly, the overall accuracy of single cell aneuploidy screening using this methodology is 98.6%.

Analyses of Single Cells from Arrested Embryos In the third phase, individual cells from arrested, cleavage-stage embryos were evaluated. Given the underlying mosaicism, there was no expectation that the results would be uniform. However, when discrepancies occurred, it was logical that they would typically involve reciprocal errors of the same chromosomes. For example, a trisomy X in one cell would be a monosomy X in another cell from the same embryo. Additionally, the level of non-concurrence of the SNP assignments on each chromosome should be similar to that observed using uniform and rigorous methods. The infertile couples were frequency matched to fertile couples according to age, number of prior cycles and infertility etiology.

The NIH Reproductive Medicine Network (RMN) conducted a study of semen parameters and fertility among fertile and infertile men, beginning with the 1999 edition of the WHO manuals, (3–5) and to confirm the geometric mean, (6) indicating that FISH analysis yielded ranges of values that delineated three groups—fertile, infertile, and subfertile. These results can be more meaningfully applied in clinical practice and research than previous measurements.

INTRODUCTION

During a standard infertility evaluation, both male and female partners are evaluated (4). This study provides a unique opportunity to test SNP array-based blastomere analysis in the first trimester of pregnancy, in an effort to shed light on those factors that contribute to euploid and aneuploid newborns. Although the presence or absence of a RRM on a particular chromosome is important, the properties of the RRM are less well understood. Consequently, this information is limited by more than just the inability to test all 24 chromosomes.

In addition, it serves as a standard when developing quantitative real-time (q)PCR (8) and next generation sequencing based methodologies (9). Finally, SNP arrays were used to demonstrate significantly reduced concurrence of CCS by array comparative genomic hybridization (aCGH). The advantage of aCGH is its ability to analyze multiple chromosomes in parallel. The use of aCGH may thereby provide a “global” measure of a cell’s aneuploidy.

REFERENCE


What is a “Normal” Semen Analysis? David S. Guzick

Reference values for semen characteristics have been based on the distribution of values in fertile men. In an effort to provide more meaningful reference values, in 2001 members of the National Institutes of Health’s (NIH) Reproductive Medicine Network (RMN) reported values for semen measurements based on a comparison of fertile and infertile men. Instead of a single value for each semen measurement that would be used to demonstrate that FISH was the “normal” data analysis yielded ranges of values that delineated three groups—fertile, infertile, and subfertile. These results can be more meaningfully applied in clinical practice and research than previous measurements.

METHODS

For this cross-sectional study, two semen samples from each of the male partners in 765 infertile couples and 696 fertile couples were evaluated using uniform and rigorous methods. The infertility couples were drawn from a randomized clinical trial in which the female partners all had normal results in an infertility evaluation (9). Fertile men (controls) were recruited from prenatal classes at the same hospitals in which the infertile couples were recruited. Within each of nine RMN sites, fertile couples were frequency matched to infertile couples according to the five-year age groups of both partners.

Technicians from each of the nine RMN sites attended training sessions in semen analysis at a central laboratory. Semen specimens were stained at the clinical sites and shipped to the central laboratory for assessment of sperm morphology by a single technician, trained...
by a factor of two to three in the likelihood of infertility when one sperm measurement was in the subfertile range can be compared with an increase by a factor of five to seven in the risk of infertility when two sperm measurements were subfertile, and an increase by a factor of 16 when all three measurements were subfertile.

The frequency distributions of fertile and infertile men with regard to the three sperm measurements are shown in Figure 1. Overall, the degree of overlap between fertile and infertile men is striking. Indeed, approximately equal proportions of fertile and infertile men had values in the indeterminate ranges of the three sperm measurements. There was an excess of infertile men with values in the subfertile ranges of these variables, and a corresponding excess of fertile men with values in the fertile ranges.

The area under receiver-operating-characteristic (ROC) curves (12), which assesses the level of discrimination between fertile and infertile men, was significantly greater for morphology (0.66) than for concentration (0.60) and motility (0.59). A single value for each semen measurement that would distinguish fertile from infertile men had values in the indeterminate ranges of the three sperm measurements. There was an excess of infertile men with values in the subfertile ranges of these variables, and a corresponding excess of fertile men with values in the fertile ranges.

by the developer of a strict morphology assessment (10). All data were then sent to a central site for data coordination and statistical analysis. Classification and regression tree (CART) analysis (11) was used to estimate thresholds for each sperm measurement that would discriminate between fertile and infertile men. Two thresholds were estimated for each sperm characteristic: an upper limit defined by classification and regression tree (CART) analysis. Arrows indicate the thresholds between subfertile and indeterminate and indeterminate and fertile ranges (green). Adapted from (8).

DISCUSSION AND UPDATE
A case can be made that the case-control methodology used in the RMN study is the best of an imperfect set of options. Follow up of couples who have discontinued contraception and the advancement of prospectively defining couples as fertile and infertile, but such an approach requires large samples, given the low incidence of infertility, and is complicated by the unknown extent of female infertility among the couples so defined as infertile. Reference values from such a methodology have not been proposed.

The WHO manual uses a statistical cut-point among fertile men, defined as the 5th percentile in the last edition. Such men are at the statistical tail of the normal distribution of fertile men, but they are still fertile. Using a population of fertile men to predict male infertility makes little sense, biologically or methodologically. Moreover, the databases from which cut-points were chosen in the first four editions were constrained by imprecise defined reference populations of fertile men, and there was variability in the standards and protocols among andrology laboratories (13). Reference values determined in the latest edition are based on a pooled database with improved laboratory consistency and a better characterized group of recent fathers. The 5th percentile of semen characteristics among these fertile men were: sperm concentration <15 million per milliliter, less than 32% motility, and less than 9% normal morphology. Table 1 also shows CART-defined thresholds that identify the indeterminate and fertile ranges, and associated odds ratios.

Table 2 shows the odds of infertility increase with an increasing number of sperm measurements in the subfertile range. An increase

close to the cut-point found in the RMN study that separated subfertile from indeterminate. Combining the advantages of the WHO manual, the RMN study identified a somewhat lower threshold for motility (32% vs. 40%). This is reflected in Figure 1, which shows no difference between fertile and infertile men in the range of 32%–42% motility. Additionally, Figure 1 shows a clear difference between fertile and infertile men in the range of 5%–8% normal morphology, which justifies an RMN-defined cut-point for morphology that is higher than the WHO manual.

Seminal characteristics are, biologically, continuous variables. Whether they be sperm measurements such as concentration, motility, and morphology, or sperm function tests such as zona binding assays, egg penetration tests, acrosome reaction testing, or others, no measurement has a clear cut-point that separates fertile from infertile. Thus, clinicians cannot convey with certainty to an infertile couple that a semen measurement below a given threshold value is responsible for their infertility, nor that a value above such a threshold excludes a male factor etiology. This is essentially a probabilistic matter. In the RMN study, to capture this idea, instead of a single value for each semen measurement that would distinguish between “normal” and “abnormal,” we defined the two values that best delineated three groups: fertile, indeterminate, and subfertile. Since these values have been defined by comparing fertile and infertile men, they identify ranges that can be meaningfully applied in clinical practice and research.

REFERENCES
Circulating Angiogenic Factors and the Risk of Preeclampsia

S. Ananth Karumanchi1,2 and Ravi Thadhani3

Preeclampsia remains a major cause of maternal and fetal mor- 
idity and mortality worldwide. We have previously suggested that  
abnormal vascular endothelial growth factor (VEGF) signaling due to excess  
circulating soluble fms-like tyrosine kinase 1 plays a causal role in  
the pathogenesis of preeclampsia. This article summarizes the key  
pathophysiological consequences of these angiogenic abnormalities  
and discusses our efforts to translate this knowledge into novel diag-  
nostic and therapeutic strategies.

INTRODUCTION

As a leading cause of premature birth and maternal and infant  
mortality worldwide, preeclampsia remains a tragically unmet pub-

lic health challenge. Preeclampsia and related hypertensive disor-

ders conservatively cover over 75,000 maternal and 500,000 infant  
deaths globally each year (www.preeclampsia.org) mostly in develop-

ing countries.

The mechanisms that initiate preeclampsia in humans have long  
been elusive, leading to its description in medical textbooks as an in-

ipathic “toxemia” of pregnancy whose definitive treatment remains  
delivery of the placenta. Nearly a decade ago, we proposed that el-

avated plasma soluble fms-like tyrosine kinase (sFlt1), an anti-an-
giogenic protein, were key pathophysiological characteristics  
related with preterm delivery, an observation that has now been  
confirmed by several groups (31–33). In addition, we demonstrated  
that women diagnosed with preeclampsia, but with a normal  
augmentation in maternal blood pressure, proteinuria, and uric acid,  
are more likely to have a normal pregnancy outcome (34). Ther-

eyapeutic studies

Human and animal studies as outlined above have strongly sug-
gested that targeting the sFlt1 pathway may be a viable strategy to  
prevent or treat preeclampsia. Below are some strategies that have  
been evaluated in either preclinical or early clinical studies.

Therapeutic Apheresis

sFlt1 has a large volume of distribution, and circulating plasma lev-

els represent less than 20% of the total body sFlt1 burden (35). We  
hypothesized that a selective adsorption column such as dextran  
sulfate (a polycationic derivative of dextran) could augment sFlt1  
removal. Dextran sulfate binds sFlt1 efficiently (36) and these columns  
have been used previously to bind apolipoprotein B-containing  
protein LDL in pregnant women with familial homozygous hypercho-

lesterolemia without adverse consequences to mother or fetus (37). In  
a proof-of-concept study, we demonstrated that a ~30% reduction  
in circulating sFlt1 levels using dextran sulfate apheresis may be  
sufficient to ameliorate preeclamptic signs and symptoms and pro-

tect the fetus from effects of maternal hypertension (38). A phase I study  
to test the safety of human relaxin in women with preeclampsia is ongoing (39).  
Combination therapies are under development, and these studies have  
led to new insights into basic biology of vascular and placental ho-

moeostasis in health and disease. The microvasculature of organs af-
tected in preeclampsia such as the glomeruli and hepatic sinusoidal  
vasculature are more permeable due to the presence of intracellu-
lar paracrine serotonin to neuropeptides. While it was previously  
known that VEGF induces endothelial fenestrae in culture (18), ex-
perimenetal data in VEGF knockout mice demonstrated that fenestral  
density is regulated by constitutive expression of VEGF (14). There-
fore it is not surprising that the microvascular damage in preeclamp-
sia is largely concentrated in vasculature that constitutively express  
VEGF and that loss of endothelial fenestration in preeclampsia is due to  
excess circulating sFlt1. While the placenta is the major source of  
sFlt1 production, recent studies have suggested that syncytiotrophoblast  
(denegrating syncytiothrophoblast tissue) in the placenta are the ma-

or site of sFlt1 production (19). These syncytiotrophoblast cells are secreted  
into the maternal circulation. Studies using au-

topsy material have confirmed that shed syncytiotrophoblast contribute to  
circulating sFlt1 in preeclampsia (20, 21).

It is likely that other synergistic anti-angiogenic proteins such as  
soluble endoglin and sepharose 3B may also contribute to the  
pathogenesis of preeclampsia (21, 22). Patients presenting with  
pre-eclampsia also have lower soluble endoglin and sFlt1 have a more  
severe and premature form of the disease (21, 23). Animals exposed to  
high soluble endoglin and high sFlt1 developed the most severe  
features of preeclampsia, including cerebral edema (21, 24). More  
research into the role of these synergistic factors in preeclampsia  
is needed.

BIOMARKER STUDIES IN PREECLAMPSIA

Although VEGF is secreted, it acts as a juxtacrine or autocrine  
growth factor locally, and circulating VEGF levels are relatively low  
during pregnancy (<10 pg/mL). Furthermore, measurement of  
VEGF in serum is compromised by platelet VEGF release during  
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This article based on the original publication:  
R.J. Levine et al., Nat. Biotechnol. 27, 72 (2009)  
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MA; Harvard Medical School; Massachusetts General Hospital, Harvard Medical School, Boston, MA  
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Figure 1: Schematic of Impaired VEGF Signaling During Preeclampsia.  
During normal pregnancy, vascular homeostasis is maintained by physiological levels of VEGF signaling in the vasculature. In preeclampsia, excess placental secretion of sFlt1 acts as a VEGF trap by binding and preventing its interaction with cell surface VEGF receptors (Flt1 and KDR).

Normal

Preeclampsia

sFlt1

sVEGFR

sFlt1:PlGF

VEGF

Figure 1: Schematic of Impaired VEGF Signaling During Preeclampsia. During normal pregnancy, vascular homeostasis is maintained by physiological levels of VEGF signaling in the vasculature. In preeclampsia, excess placental secretion of sFlt1 acts as a VEGF trap by binding and preventing its interaction with cell surface VEGF receptors (Flt1 and KDR).
that in the ensuing decade, these molecular discoveries will lead to improvement of care of patients suffering from preeclampsia and its devastating complications.

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Disclosures: SAK is co-inventor of multiple commercial patents related to diagnosis/treatment of preeclampsia that have been licensed to multiple companies. SAK has served as a consultant to Roche, Beckman Coulter, and Siemens Diagnostics, and has financial interest in Aggamin LLC. RT also discloses financial interest in Aggamin LLC. RT also discloses co-inventor on patents related to licensed biomarkers in preeclampsia. RT also discloses financial interest in Aggamin LLC. SAK is co-inventor of multiple commercial patents related to diagnosis/treatment of preeclampsia that have been licensed to multiple companies. SAK has served as a consultant to Roche, Beckman Coulter, and Siemens Diagnostics, and has financial interest in Aggamin LLC. RT also discloses financial interest in Aggamin LLC. RT also discloses co-inventor on patents related to licensed biomarkers in preeclampsia. RT also discloses financial interest in Aggamin LLC. SAK is co-inventor of multiple commercial patents related to diagnosis/treatment of preeclampsia that have been licensed to multiple companies. SAK has served as a consultant to Roche, Beckman Coulter, and Siemens Diagnostics, and has financial interest in Aggamin LLC. RT also discloses financial interest in Aggamin LLC. RT also discloses co-inventor on patents related to licensed biomarkers in preeclampsia. RT also discloses financial interest in Aggamin LLC.
WHO Type I: Anovulation-Hypothalamic Amenorrhea

Hypothalamic amenorrhea can arise from genetic conditions leading to failure of the GnRH neurons to develop or migrate to the hypothalamus or from disruption of genes relating to onset of puberty. There are also common acquired conditions associated with stress, excessive exercise, and loss of body fat/weight (i.e., anorexia nervosa or exercise-induced amenorrhea), which can cause hypothalamic shutdown and downstream loss of ovarian function. While treatment with gonadotropins effectively bypasses the hypothalamic GnRH pulse generator and directly stimulates follicular development, the factors leading to the hypothalamic failure remain in doubt. Cessation of activity and regain of weight should cure the condition, but no high-quality clinical trials have yet demonstrated this.

The study of Welt et al. (17) looked at the effects of recombinant leptin administration on ovarian function in women with hypothyroidism and amenorrhea. The intervention group was observed without treatment for one month, then administered recombinant leptin for up to three months. A control group received no treatment. Five of eight patients in the intervention group either ovulated or had some resumption of ovarian activity (Fig. 1). None of the control subjects or intervention subjects during the initial observation period showed any change. This provided evidence that peripheral fat status was critical to maintaining reproductive function and supported studies that a critical amount of fat mass is necessary to initiate menarche.

WHO Type II: Ovulatory Dysfunction-Polycystic Ovary Syndrome

A study of Legro et al. (18) occurred at a time of great debate as to the best treatment for polycystic ovary syndrome (PCOS), a heterogeneous condition of hyperandrogenism and chronic anovulation, with characteristic polycystic ovaries filled with preantral follicles. PCOS was viewed by some as a metabolic syndrome due to dimin-

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Figure 1. Follicular, ovarian, and endometrial ultrasonographic measurements at the beginning and end of the one-month baseline period and at their maximum during recombinant Leptin treatment. Each symbol represents one subject. Reprinted with permission from (18).

Figure 2. Regimens of ovarian stimulation and hormone replacement used to synchronize the development of ovarian follicles in the oocyte donor and endometrial cycle in the recipient. Reprinted with permission from (20).

Figure 3. The time course and quantitative change in circulating concentrations (Mean ± SE) of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E2), and progesterone (P) during the menstrual cycle of four normal women treated with a GnRH agonist for three days after menses onset (hatched box, left). Data were centered around the midcycle gonadotropin peak (day 0). Reprinted with permission from (20).
Primary ovarian insufficiency can be due to genetic conditions such as Turner’s Syndrome or single gene defects such as galactosemia deficiency or mutations acquired from exposure to radiation or alkylating agents during cancer treatment. Further, while Type III anovulation occurs relatively rarely, all women experience an age-related decline in ovarian function, with increasing rates of embryo aneuploidy and miscarriage, culminating in the complete loss of ovulation, pregnancy, and menses as they age. While preservation of fertility is a rapidly expanding preventive treatment option, there have been no real advances in restoring ovarian function in women with age-related ovarian insufficiency. A breakthrough occurred when it was shown that embryos created from donor oocytes could be placed in the uterus of a woman with ovarian insufficiency whose endometrium had been conditioned. A further extension of this therapy to women with advanced age and infertility (verified by careful monitoring of gonadotropins and sex steroids prior to treatment) were administered a GnRH agonist in the early follicular phase that resulted in a temporary increase in gonadotropin secretion, followed by a decrease in FSH levels and a shortened luteal phase (Fig. 3). While this was seen at the time as a potential contraceptive approach, the use of progestin agonist in combination to supplement the luteal phase in IVF cycles where a GnRH agonist had been administered, and was also used to treat women with suspected LPDs.

OVARILY Dysfunction – LUTEAL PHASE DEFECTS

Many women with infertility or recurrent pregnancy loss do not present with chronic or sporadic anovulation, but are suspected to have ovulatory dysfunction leading to the absence of functional corpora lutea and corresponding altered LH levels. Several ovulatory disorders occur within the context of what appears to be regular ovulatory cycles. These include extremely rare disorders such as luteinized unruptured follicle syndrome, empty follicle syndrome, and more common disorders such as luteal phase defects (LPDs). LPDs, originally described by Georgeanna Jones, are characterized by inadequate corpus luteum function following ovulation, as measured by a variety of parameters including inadequate rise in basal body temperature, secretion of progesterone or its metabolites, an out-of-phase endometrial biopsy, or a shortened luteal phase (23). Subsequent studies have found this disorder difficult to diagnose largely due to the occurrence of these “abnormalities” in normal, fertile populations (24, 25).

However, the proof of concept for LPD was demonstrated in a classic study by Sheehan et al. (26) in which normally ovulating women (verified by careful monitoring of gonadotropins and sex steroids prior to treatment) were administered a GnRH agonist in the early follicular phase that resulted in a temporary increase in gonadotropin secretion, followed by a decrease in FSH levels and a shortened luteal phase (Fig. 3). Although such observations are critical for subsequent therapies. Further, it underscored the importance of understanding of ovulatory dysfunction or the identification of novel approaches to restore reproductive function.

The study by Bhattacharya et al. (27) randomized couples with unexplained infertility to treatment with three different groups (with different types of intervention): empiric treatment with the ovulation induction agent clomiphene or metformin alone vs. combination therapy using a double blind crossover design and diabetes. These include extremely rare disorders such as luteinized unruptured follicle syndrome, empty follicle syndrome, and more common disorders such as luteal phase defects (LPDs). LPDs, originally described by Georgeanna Jones, are characterized by inadequate corpus luteum function following ovulation, as measured by a variety of parameters including inadequate rise in basal body temperature, secretion of progesterone or its metabolites, an out-of-phase endometrial biopsy, or a shortened luteal phase (23). Subsequent studies have found this disorder difficult to diagnose largely due to the occurrence of these “abnormalities” in normal, fertile populations (24, 25).

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UNEXPLAINED INFERTILITY – EMPIRIC OVULATION INDUCTION

Unexplained infertility is defined as infertility that presents both a great challenge for pregnancy and a higher risk of multiple pregnancies. The study by Bhattacharya et al. (27) randomized couples with unexplained infertility to treatment with three different groups (with different types of intervention): empiric treatment with the ovulation induction agent clomiphene or metformin alone vs. combination therapy using a double blind crossover design and diabetes. These include extremely rare disorders such as luteinized unruptured follicle syndrome, empty follicle syndrome, and more common disorders such as luteal phase defects (LPDs). LPDs, originally described by Georgeanna Jones, are characterized by inadequate corpus luteum function following ovulation, as measured by a variety of parameters including inadequate rise in basal body temperature, secretion of progesterone or its metabolites, an out-of-phase endometrial biopsy, or a shortened luteal phase (23). Subsequent studies have found this disorder difficult to diagnose largely due to the occurrence of these “abnormalities” in normal, fertile populations (24, 25).

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INTRODUCTION
Infertility impacts the lives of 8% to 12% of couples attempting to conceive for the first time. In roughly half of these cases a male factor is causative yet, surprisingly, in many assisted reproductive technology (ART) clinics the male is not clinically evaluated beyond a routine semen analysis. While most textbooks state that primary infertility in approximately 30% of infertile men is idiopathic, some experts speculate that 30% to 80% of all male infertility results from a (usually undiagnosed) genetic cause. In these patients, no explanation can be found for their impaired semen quality. In fact, this statistic reflects our poor understanding of the basic mechanisms regulating spermatogenesis, sperm maturation in the testis, and the molecular events required for fertilization and early embryonic development; hence, our inability to properly diagnose the cause of the infertility. Indeed, many of the commonly used diagnostic categories for male infertility are descriptive rather than mechanistically based. A diagnosis of non-obstructive azoospermia (NOA), testicular failure, cryptorchidism, or obstructed production provides no insight into the molecular cause of the infertility. In this review, we focus on the molecular defects that underlie the congenital/ genitourinary birth defects associated with infertility, endocrine dysfunction, and the epidemic of male reproductive failure.

STRUCTURAL AND NUMERICAL CHROMOSOME ABNORMALITIES ARE ACCOUNTED FOR A SIGNIFICANT PERCENTAGE OF MALE INFERTILITY
Karyotype abnormalities are present in about 6% of infertile men [reviewed in (1)]. These abnormalities can be numerical and affect only the sex chromosomes—for example, Klinefelter syndrome (46,XXY-46,XXXXY), which accounts for about 14% of all NOA’s—or structural, affecting the autosomes or the sex chromosomes, including chromosomal rearrangements, deletions, duplications, inversions, and translocations.

A well-recognized structural chromosome defect causing male infertility is the occurrence of Y chromosome microdeletions encompassing the azospermia factor (AZF) region, first described in 1995 (2). The Deleted in Azospermia (DAZ) gene was the first AZF spermatogenesis gene identified within a Y chromosome microdeletion. The AZF region is now further subdivided into smaller segments termed AZFA, AZFB, and AZFC. Sperm are unlikely to be found in men with deletions in AZF A or B, whereas men with AZFC deletions may still have azoospermia or severe oligospermia (2-4), indicating on a testis biopsy [reviewed in (1)]. For AZF C microdeletions, the rare variant having a major effect on spermatogenesis is seen in the 20-28 deletion, which accounts for about 6% of severe spermatogenic failure, whereas the more commonly occurring gr/gr deletion has a modest effect, doubling the risk of severe spermatogenic failure (4).

Upon homologous recombination and meiotic segregation, autosomal structural defects, such as Robertsonian translocations, can result in balanced or unbalanced translocations. Infertility can arise due to the production of unbalanced gametes (nullisomic or disomic) resulting in aneuploid embryos or chromosomally unbalanced offspring (5). Thus, before proceeding with ART to attempt to achieve a pregnancy, it is important to know if chromosome anomalies are present in the male patient.

ENDOCRINE PATHWAYS ARE CRITICAL FOR NORMAL FERTILITY IN MALES
Although endocrine defects account for only about 1% of all male infertility, the molecular abnormalities that underlie these conditions are increasingly evident. In short, any genetic defect affecting the hypothalamic-pituitary-gonadal axis, steroid hormone biosynthesis and metabolism, steroid hormone receptors and their signaling pathways, peptide hormones and their receptors and associated signaling pathways, or paracrine factors and cytokines and their respective signaling pathways can affect male fertility [reviewed in (6-8)].

The best example of the relative complexity of genetic defects that underlie a seemingly discrete infertility phenotype is revealed by the studies of hypogonadotropic hypogonadism by Crowley and others (9-11). Gene defects causing GnRH deficiency vary in relation to their site of action on the hypothalamus to the GnRH-receptor and the clinical phenotype observed. For patients with anosmia, neurodevelopmental gene functions that regulate GnRH neuronal migration or olfactory bulb/tract development are affected due to gene deficiencies, such as anosmic anosmia/olfactory impairment (KAL1 and NELF). In contrast, GnRH-deficient patients with normal anosmia may have defects in genes that encode members of the kisspeptin, neurokinin B, and GnRH signaling pathways such as KISS1, KISS1R, TacR1, TacR2, and GnRHR. Interestingly, defects in the prokinetin and FGF signaling pathways (FGF8, FGF11, PROK2/3) are variably present in patients and families with both anosmia and a normal sense of smell within the same pedigree [reviewed in (9)]. Despite the identification of numerous mono- and biologic, and genomic mutations in the genes above, a molecular cause for slightly less than half of isolated GnRH deficiency remains unknown and a topic of intense investigation. It is clear that even for hypogonadotropic hypo- spermatogenesis, considerable genetic complexity underlies the causal defects.

GENETIC AND GENOMIC DEFECTS IN MALE INFERTILITY
Using mouse models, investigators were surprised to learn that genes required for normal spermatogenesis function in the production and reproduction of sperm when deleted, cause infertility. One of the most unexpected findings was that loss or pharmacological blockade of testsis-expressed taste genes caused male infertility in the mouse (20). The targeted deletion of the C1R/C1S, KLK7, and K12 genes, which encode taste receptors and the gustation o-subunit GNT3A, lead to male sterility due to immotile sperm with poor morphology (detached or amorphous heads, tails flipped over heads) and reduced fertility for the C1R/C1S-/- males, indicating a potent role in spermogenesis and sperm maturation (20).

In 2008, Matzuk and Lamb summarized the gene defects in mouse models and human patients associated with male infertility [see supplement to (7)], and a large number of additional genetic defects have since been defined affecting germinilary origin fertility, disorders of sexual determination and differentiation, puberty, spermatogenesis, sperm function, and other aspects of male reproductive health. For example, the mice with the c.144delC mutation (CBAVD) that causes the severe asthenozoospermia (21). Unfortunately, mouse models are not informative, because unlike humans, the Ca2+ channel in the mouse is not sensitive to progesterone. Nevertheless, there are literally thousands of possible gene defects identified in mice and humans causing male infertility. In the clinic, however, just one gene is analyzed on a routine basis in males with congenital bilateral ab-""
42

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Figure 1. SHOX deletions and duplications in patients with Y chromosome microdeletions: co-existing genomic syndromes. Y chromosome microdeletions are usually diagnosed in a clinical genetics laboratory using a multiplex PCR approach. However, when array comparative genomic hybridization is employed, in addition to the SHOX gene, which is located in the pseudoautosomal region of the Y chromosome, GO homeobox-containing gene syndrome (38) (Fig.1). SHOX syndrome is the most common cause of short stature and results from mutations, microdeletions, or microduplications of the SHOX gene, which is located in the pseudoautosomal region of the sex chromosomes. SHOX is a dosage-sensitive gene that does not undergo a reactivation. While SHOX syndrome occurs in Turner and Klinefelter syndrome patients (deletion and duplication, respectively), the clinical spectrum varies. The associated Madelung deformity of the forearm and mesomorphic disproportion of the limbs develops over time and appears during the second decade of life (or perhaps never). There are a number of other clinical indications (among them scoliosis, microgryia, and muscular hypotrophy of the calves) that are found in some, but not all, SHOX syndrome patients [reviewed in (37)]. The presence of SHOX deletion or duplication in a subset of men with Y chromosome microdeletions suggests that this co-existing genomic syndrome could be inherited by the male offspring of men conceived by ICSI, although to date there have been no reports of SHOX syndrome in ICSI conceived offspring.

There may be other associated health issues for the NOA male that are clinically unappreciated. Our studies show a 2.9-fold increased risk of cancer in NOA patients (38). Walsh and colleagues (39) evaluated data on 22,562 partners of infertile couples and showed that the risk of testis cancer was nearly threefold higher in infertile men compared with normal controls (38). Jacobson et al. similarly evaluated more than 32,000 men who had their semen analysed over a 30-year period and found a 1.6-fold increased risk of testis cancer in men with reduced sperm counts (40). There was also an increased incidence of cancers of the pancreas and other digestive organs in these men. Walsh and colleagues performed an analysis of their patient cohort and showed that those with male factor infertility were 2.5 times more likely to be diagnosed with high-grade prostate cancer (39, 41).

Indeed, a comprehensive male infertility evaluation identified a number of significant (and previously undiagnosed) medical pathologies. In a landmark paper by Honig et al., significant medical pathologies were uncovered in 1.1% of 1,236 patients presenting to a male infertility clinic (42). Ten patients (0.8%) had tumors (six testis, three brain, and one spinal cord) and their findings point to the need for increased awareness of tumors in men presenting to an ART clinic. It is believed that there are common etiologic factors for infertility and cancer development, such as deficient DNA repair mechanisms (38, 40, 43).

CONCLUSIONS

We have witnessed an exponential increase in advances in our understanding of Y chromosome microdeletions and the control of spermatogenesis. As a result of breackpoint hotspots, as well as homologous recombination errors due to amiploci associated with palindromic structures present on the human Y chromosome, Y chromosome microdeletions may occur (35). These rearrangements can even occur due to amiploci on the short (Yp) and long (Yq) arms of the Y chromosome through intrachromosomal non-allelic homologous recombination (34). These structural Y chromosome defects affect the male specific Y chromosome and, although not all have physical implications of the Y about 25% of men with NOA due to Y chromosome microdeletions have a co-existing genomic syndrome, SHOX (short stature homeobox-containing gene) syndrome (38) (Fig.1). SHOX syndrome is the most common cause of short stature and results from mutations, microdeletions, or microduplications of the SHOX gene, which is located in the pseudoautosomal region of the sex chromosomes. SHOX is a dosage-sensitive gene that does not undergo a reactivation. While SHOX syndrome occurs in Turner and Klinefelter syndrome patients (deletion and duplication, respectively), the clinical spectrum varies. The associated Madelung deformity of the forearm and mesomorphic disproportion of the limbs develops over time and appears during the second decade of life (or perhaps never). There are a number of other clinical indications (among them scoliosis, microgryia, and muscular hypotrophy of the calves) that are found in some, but not all, SHOX syndrome patients [reviewed in (37)]. The presence of SHOX deletion or duplication in a subset of men with Y chromosome microdeletions suggests that this co-existing genomic syndrome could be inherited by the male offspring of men conceived by ICSI, although to date there have been no reports of SHOX syndrome in ICSI conceived offspring.

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Molecular Interplay in Successful Implantation

Jeeyeon Cha1*, Felipe Vilella2*, Sudhansu K. Dey1**, and Carlos Simón2**

ABSTRACT

Embryo implantation in the maternal womb is a fundamental event in mammalian procreation. The phases between the prereceptive and receptive phases are key in preparing the uterus to receive the embryo. Specific expression of molecules and their potential functions critical for uterine receptivity, implantation, and decidualization are discussed. Interplay of ovarian P4/estrogen-dependent and glucocorticoid-inducible gene expression is integral to the process. Recent studies have focused on endocannabinoids (eCBs) and their receptors. eCBs are lipid mediators expressed in the placenta,滋养 and protect the fetus. Implantation is brought into contact with the embryonic vascular system, establishing a functional placenta. Maternal resources, filtered across the placental barrier, nourish and protect the fetus. Implantation is the first significant encounter between the mother and embryo, and is crucial for a successful pregnancy.

MOLECULAR INSIGHTS AND CLINICAL IMPLICATIONS OF ENDOMETRIAL RECEPTIVITY

The molecular events between the prereceptive and receptive phases last approximately 24 hours (beginning day four of pregnancy or pseudopregnancy) in mice and three days in humans during the mid-luteal phase of the menstrual cycle (1–3). Understanding the ‘molecular clock’ at play during the WOI could help unravel the molecular processes responsible for endometrial receptivity, implantation, and decidualization in mice and humans.

INTRODUCTION

Implantation requires an effective bidirectional communication between the receptive endometrium and an implantation-competent blastocyst. The duration of the window of implantation (WOI) to achieve optimal pregnancy outcome is short-lived. Blastocyst attachment to the uterine lining (luminal epithelium) initiates the implantation process, which is followed by localized stromal cell proliferation and differentiation to generate specialized decidual cells at the site of implantation, a process called decidualization. Appropriate decidualization directly pertains, in which the maternal circulation is brought into contact with the embryonic vascular system, establishing a functional placenta. Understanding the “molecular clock” at play during the WOI could help unravel the molecular processes responsible for endometrial receptivity, implantation, and decidualization in mice and humans.

MOLECULAR IN IVESTIGATION

The molecular interplay between the prereceptive and receptive phases are crucial roles in regulating the WOI. Progesterone receptors (PR-A and PR-B) and estrogen receptors (ERα and ERβ) are expressed in the uterus. Similarly, luteinized mural corpus luteum also expresses aromatase enzyme. Dual activation of the WOI is necessary. ERα-mediated PR activity and has profound effects during pregnancy (7, 8). In the absence of Fkbp52, P4 signaling and responsiveness are significantly diminished, resulting in implantation failure. Depending on the genetic background of the mice, functional P4 resistance can be overcome by exogenous P4 administration (9). FKBP52 is also expressed in human endometrium (10).

EMBRYO-UTERINE INTERACTIONS

ER and PR signaling during implantation are executed by juxta- tin, paracrine, and autocrine factors orchestrated by various growth factors, cytokines, lipid mediators, homeobox transcription factors, and morphogens. Mouse models have improved our mecha- nistic understanding of several factors critical for uterine receptiv- ity and implantation (1; also see reviews 1 and 2). Among the growth factors, heparin-binding EGF-like growth factor (HB-EGF) stands out as a major player in mediating embryo-uterine interac- tions during the attachment reaction in both mice and humans (Fig. 2; 11–14). Membrane-anchored HB-EGF expressed in the luminal epithelium functions as a juxta- tin mediator for adhesion of the blastocyst to the endometrium. PR-A and PR-B are infertile in mice and humans are also mediated by L-selectin ligands and their receptors displayed on the luminal epithelium and blastocyst cell surface, respectively (15). Leukemia inhibitory factor (LIF), a member of the interleukin (IL) family of cytokines, is expressed in mouse uterine glands early on day four of pregnancy (day of uterine receptivity) and in the stroma surrounding the blastocyst upon attachment late on day four (16, 17). This factor is essential for uterine receptivity and implan- tation via binding to its receptor, LIFR, and co-receptor, gp130, to activate STAT3 signaling. LIF-gp130/STAT3 signaling is critical to implantation since uterine deletion of the genes encoding gp130 or Stat3 has been shown to cause implantation failure (16, 18). More recently, interleukin 6 family of cytokines, especially interleukin-6, has been shown to be critical for uterine receptivity. IL-6 directly upregulates IL-6 family-related genes (19). Co-workers (20, 21), a critical enzyme for prostaglandin (PG) biosynthesis, is expressed in the uterus at the site of implantation (21–23). Cox2-derived PGs are thought to participate in implantation since ablation of the Ptgs2 (Cox2) gene leads to infertility (21–23). PGs also influence vascular permeability and decidualization in the stromal bed (24). Cox2-derived prostacyclin (PGI2) activates PPARβ, which appears to influence implantation as Pparβ−/− mice show delayed implantation and compromised pregnancy outcome (25). Cytochrome phospholipase A2 (cPLA2), encoded by Pla2g4a, which generates arachidonic acid for Cox2-generated PG synthesis, is expressed in the uterus similarly to Cox2. Its critical role in implantation is evidenced by deferred implantation and related adverse effects in Pla2g4a−/− mice, compromising pregnancy outcome (26).

Figure 1. Signaling network for uterine receptivity and implantation. Hybrid cartoon, based on mouse and human studies, portraying compartment- and cell-type specific expression of molecules and their potential functions critical for uterine receptivity, implantation, and decidualization. Interplay of ovarian P4/estrogen-dependent (ERα) and glucocorticoid (GR)-dependent (NR3C1) molecules in the prereceptive uterine in specific compartments contribute to the establishment of implantation site. a. Endocrine/paracrine factors, and morphogens. Mouse models have improved our mecha- nistic understanding of several factors critical for uterine receptivity and implantation (1; also see reviews 1 and 2). Among the growth factors, heparin-binding EGF-like growth factor (HB-EGF) stands out as a major player in mediating embryo-uterine interac- tions during the attachment reaction in both mice and humans (Fig. 2; 11–14). Membrane-anchored HB-EGF expressed in the luminal epithelium functions as a juxta- tin mediator for adhesion of the blastocyst to the endometrium. PR-A and PR-B are infertile and have multiple ovarian and uter- ine defects, although PR-B−/− mice contain normal fetuses (5, 6), suggesting that PR-A is the key player in implantation. FKBP52, an immunophilin co-chaperone for steroid hormone nuclear receptors, optimizes PR activity and has profound effects during pregnancy (7, 8). In the absence of Fkbp52, P4 signaling and responsiveness are significantly diminished, resulting in implantation failure. Depending on the genetic background of the mice, functional P4 resistance can be overcome by exogenous P4 administration (9). FKBP52 is also expressed in human endometrium (10).

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RESULTS

Waves of epithelial cell shedding, angiogenesis, and remodeling are required for implantation, and they are tightly regulated by local and systemic signals. The importance of these waves for successful implantation has been extensively studied, and they are crucial for the establishment of a stable implantation site. The key players in these waves include various growth factors, cytokines, and matrix metalloproteinases (MMPs).

GROWTH FACTORS

Growth factors, such as fibroblast growth factor (FGF) and EGF, play a critical role in the regulation of epithelial cell proliferation and differentiation. For example, FGF-2 is known to stimulate epithelial cell proliferation and differentiation, while EGF promotes the differentiation of epithelial cells into glandular structures.

ANGIOGENESIS

Angiogenesis is a complex process that involves the sprouting of new blood vessels from pre-existing ones. It is critical for the establishment of a stable implantation site, as the developing blastocyst requires an adequate blood supply to support its growth. The key players in angiogenesis include vascular endothelial growth factor (VEGF) and its receptors.

REMODELING

Remodeling of the extracellular matrix (ECM) is essential for the establishment of a stable implantation site. The ECM is a complex network of proteins and glycoproteins that provides structural support and facilitates cell-matrix interactions. The key players in ECM remodeling include various proteases, such as MMPs.

CONCLUSION

In conclusion, successful implantation requires a coordinated and tightly regulated process of cell proliferation, differentiation, and remodeling. The key players in these processes include various growth factors, cytokines, and proteases, and their proper regulation is crucial for the establishment of a stable implantation site.
of the uterus from receptive to nonreceptive states requires special attention since the molecular interplay required remains largely unknown and may be critical to extend the WOI during IVF. The complexities of pregnancy necessitate continued basic and clinical research, since aberrant implantation leads to compromised pregnancy outcomes and may even impact the long term health of offspring.

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