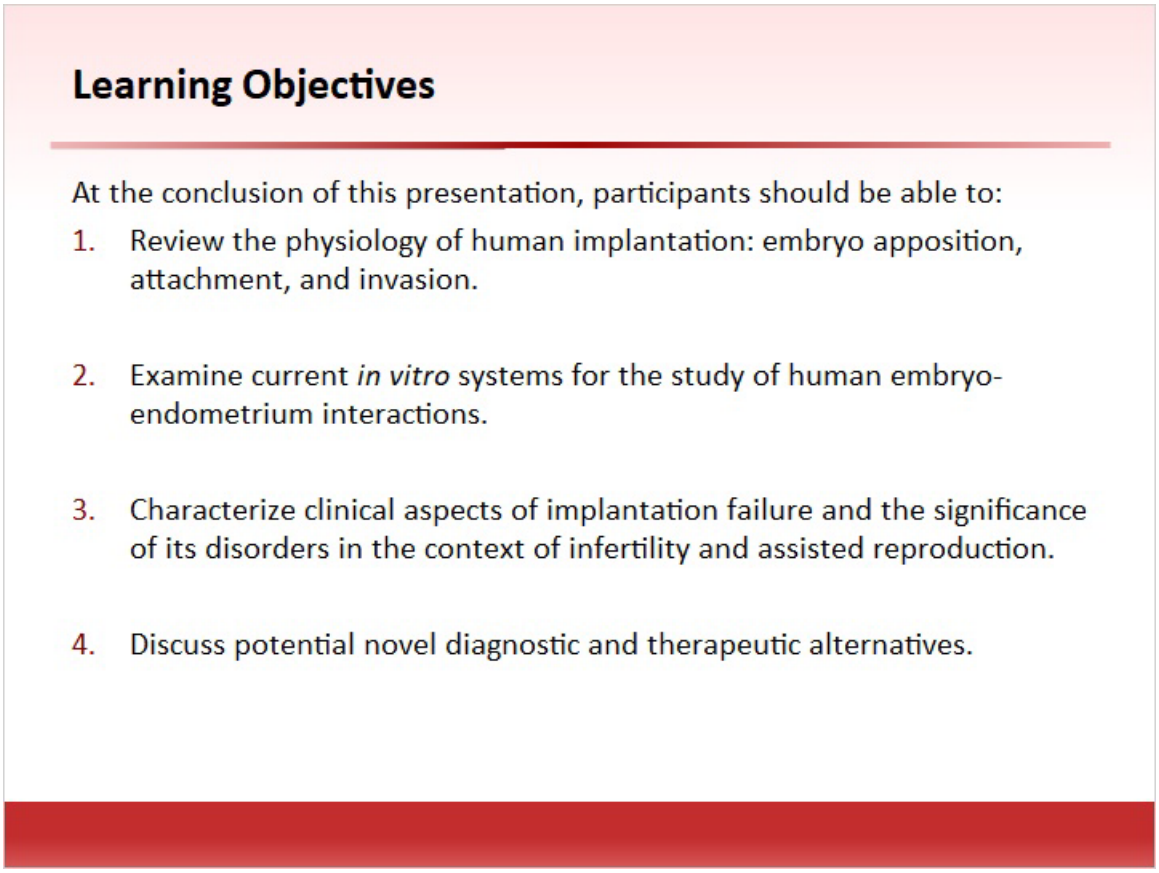
LABCC100 Lesson 5

1.1 Implantation:

Notes:

Welcome to the American Society for Reproductive Medicine's eLearning modules. The subject of this presentation is Implantation: Apposition and Invasion.

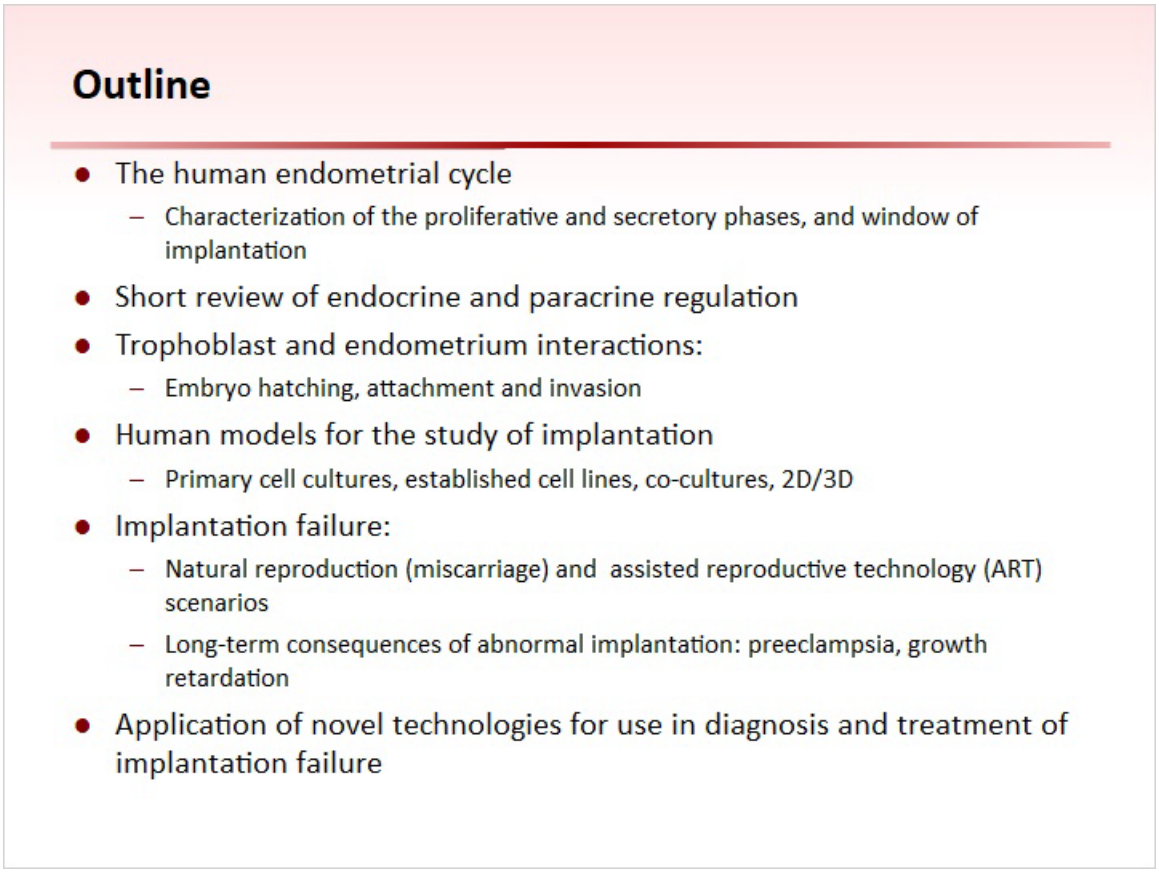
1.2 Learning Objectives



Notes:

At the conclusion of this presentation, participants should be able to:
Review the physiology of human implantation: embryo apposition, attachment, and invasion.
Examine current *in vitro* systems for the study of human embryo-endometrium interactions.
Characterize clinical aspects of implantation failure and the significance of its disorders in the context of infertility and assisted reproduction.
Discuss potential novel diagnostic and therapeutic alternatives.

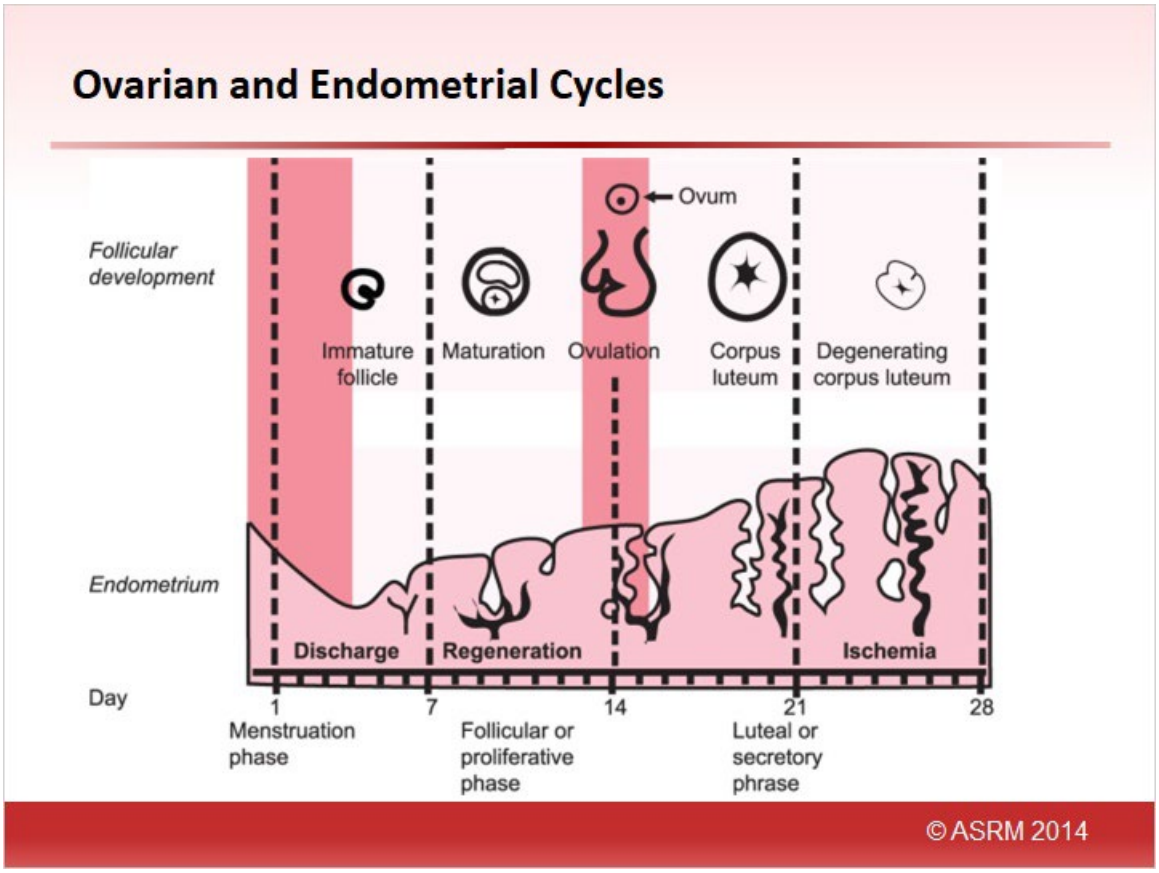
1.3 Outline



Notes:

The following topics will be examined in this presentation with varying degrees of depth:
The human endometrial cycle; endocrine and paracrine regulation;
Trophoblast and endometrium interactions;
Human models for the study of implantation;
Implantation failure in natural and assisted reproductive technology (ART) scenarios; and
Application of novel technologies in diagnosis and treatment of implantation failure.

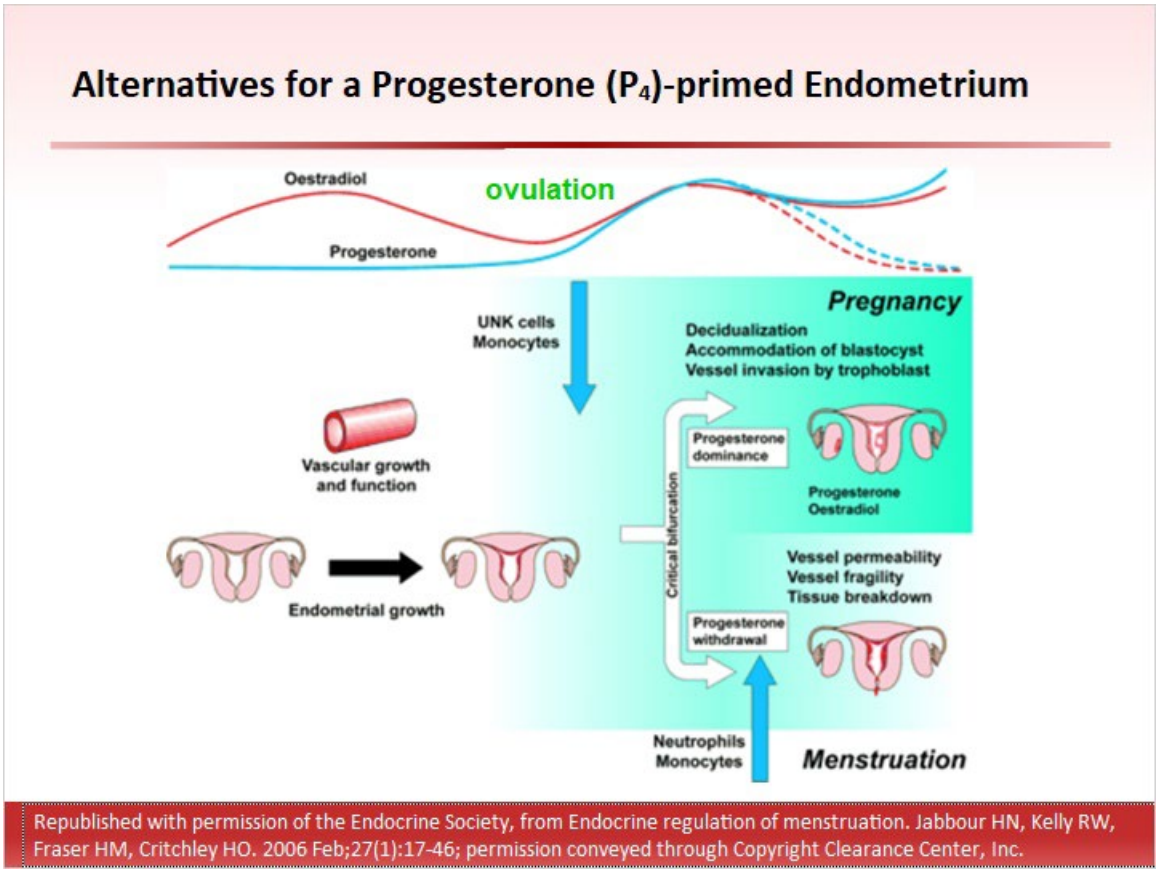
1.4 Ovarian and Endometrial Cycles



Notes:

The ovarian cycle and resulting concomitant endometrial cycle occur under regulation of the ovarian sex steroids estrogen (the primary driver of the proliferative phase) and progesterone (critical in the secretory phase). Note the endometrial proliferation and secretion, and remarkable angiogenesis.

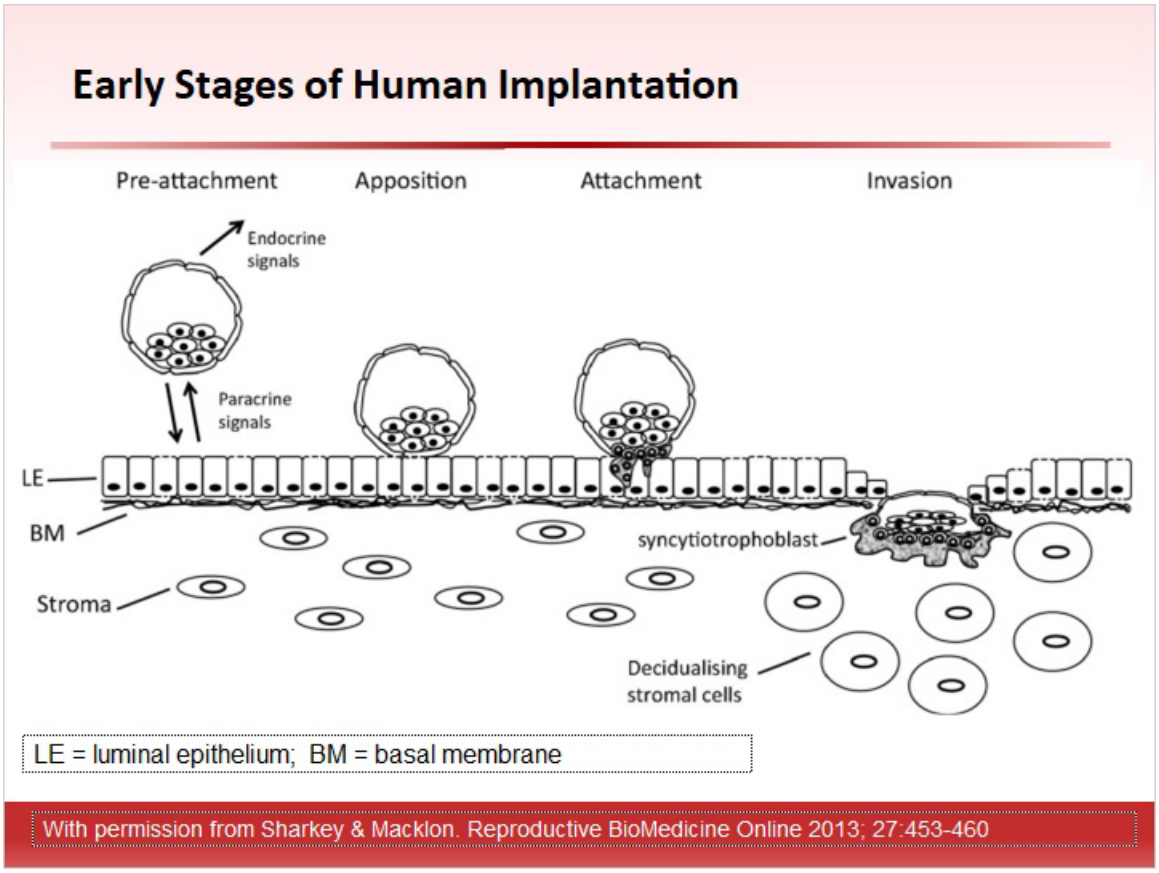
1.5 Alternatives for a Progesterone (P4)-primed Endometrium



Notes:

Following ovulation, and in the absence of pregnancy, the endometrium is programmed to undergo menstruation upon progesterone withdrawal due to corpus luteum demise. If pregnancy ensues, the corpus luteum is rescued by hCG and the endometrium continues decidualization to accommodate the blastocyst under progesterone dominance.

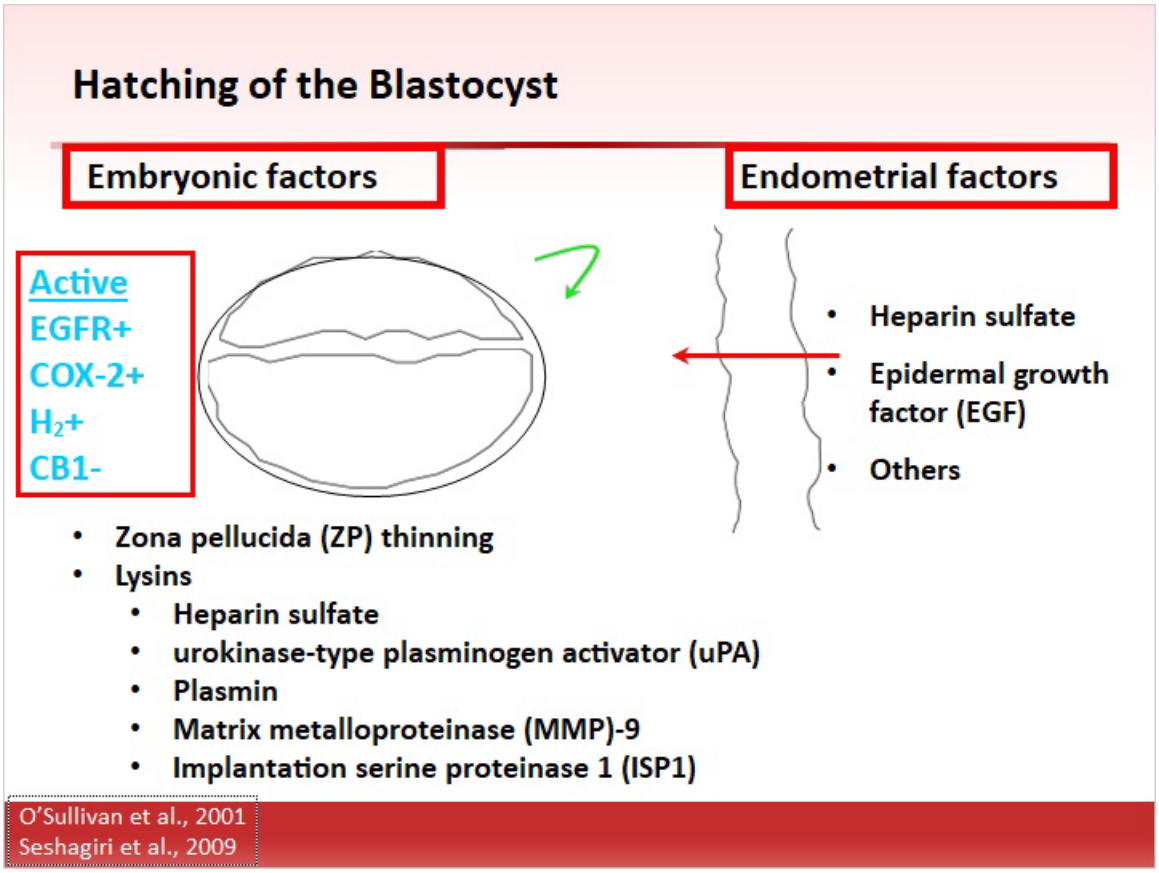
1.6 Early Stages of Human Implantation



Notes:

This schematic drawing shows the steps of blastocyst implantation. Note that the blastocyst has extensive and reciprocal paracrine communication with the endometrium before, during, and after attachment to the epithelium.

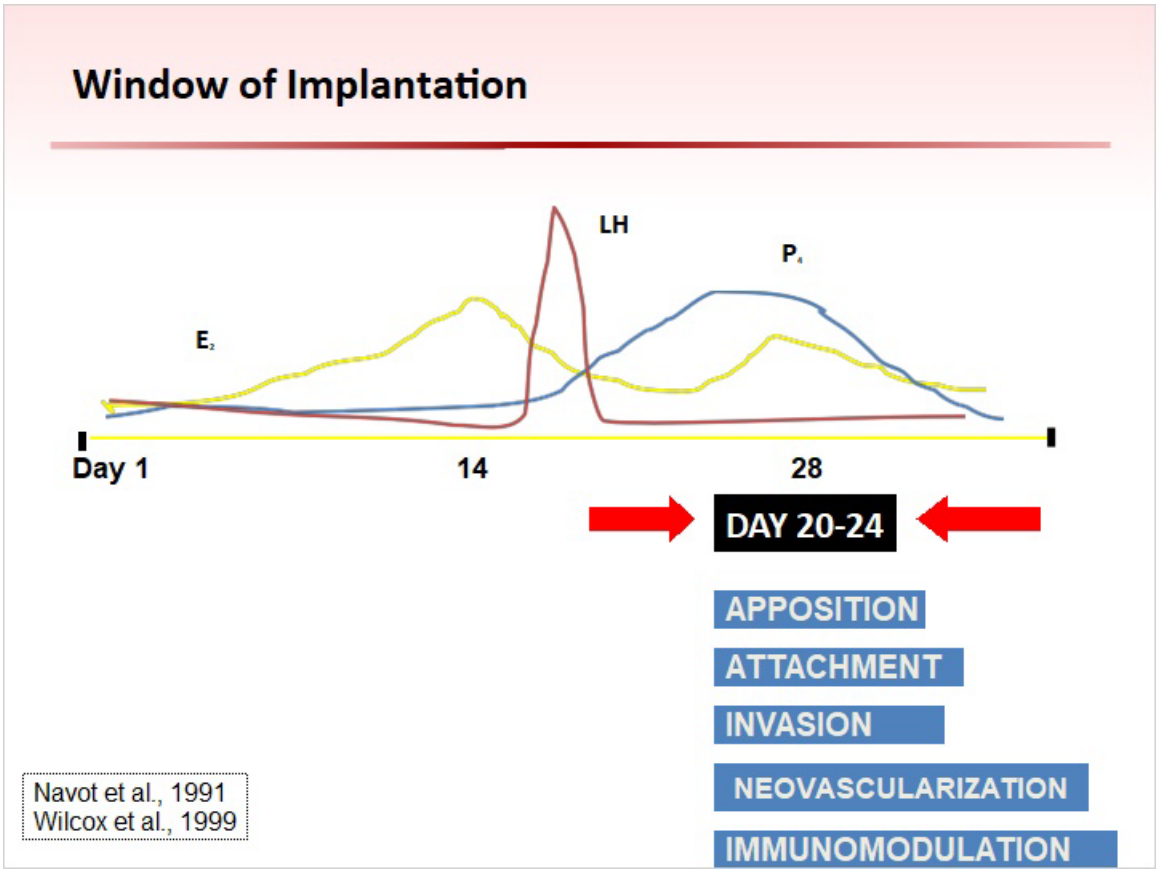
1.7 Hatching of the Blastocyst



Notes:

Prior to implantation, the blastocyst must hatch from its acellular glycoprotein coat, the zona pellucida. Blastocyst hatching is believed to be regulated by both dynamic cellular components such as actin-based trophectodermal projections, and a variety of autocrine and paracrine molecules such as growth factors, cytokines and proteases originating from the blastocyst and also probably of endometrial origin. New in vitro fertilization data from time-lapse videocinematography also demonstrate blastocyst contraction and zona rupture as captured *in vitro*.

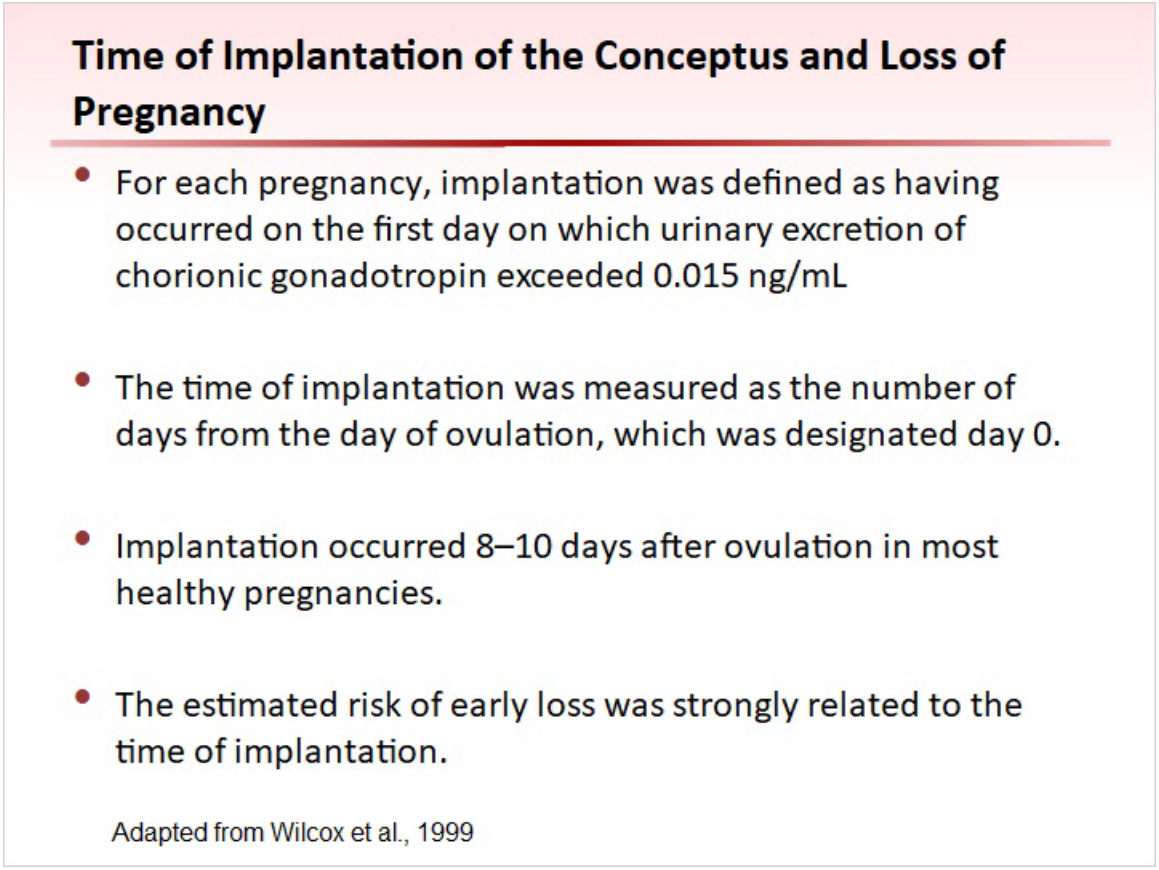
1.8 Window of Implantation



Notes:

The endometrium is receptive to embryonic implantation during a defined window that is temporally and spatially restricted. Under the influence of ovarian steroid hormones a "window of implantation" is established. In humans, this window extends from day 20 to 24 of the cycle, which in ART reflects the "embryo transfer window" (Navot et al, 1991; Wilcox et al, 1999). In the pre-genomic era, in a one to one approach, several molecular markers of the window of uterine receptivity have been postulated. Special attention has been given to integrins, leukemia inhibitory factor (LIF) and glycodelins, although their clinical significance is controversial. Realistically one can view the implantation process as a condition of equilibrium in the up- and down-regulation of a number of genes. Microarray technologies allow us to investigate multiple genes simultaneously in a more integrated fashion. Following blastocyst apposition, attachment, and invasion, other sequential blastocyst/endometrial factors regulate in a fine fashion the controlled embryonic invasion of stroma and vessels, where the processes of neovascularization (angiogenesis), and immunomodulation take place to favor physiologic (limited) invasion. Synchronism between embryo and endometrial development is critical for implantation.

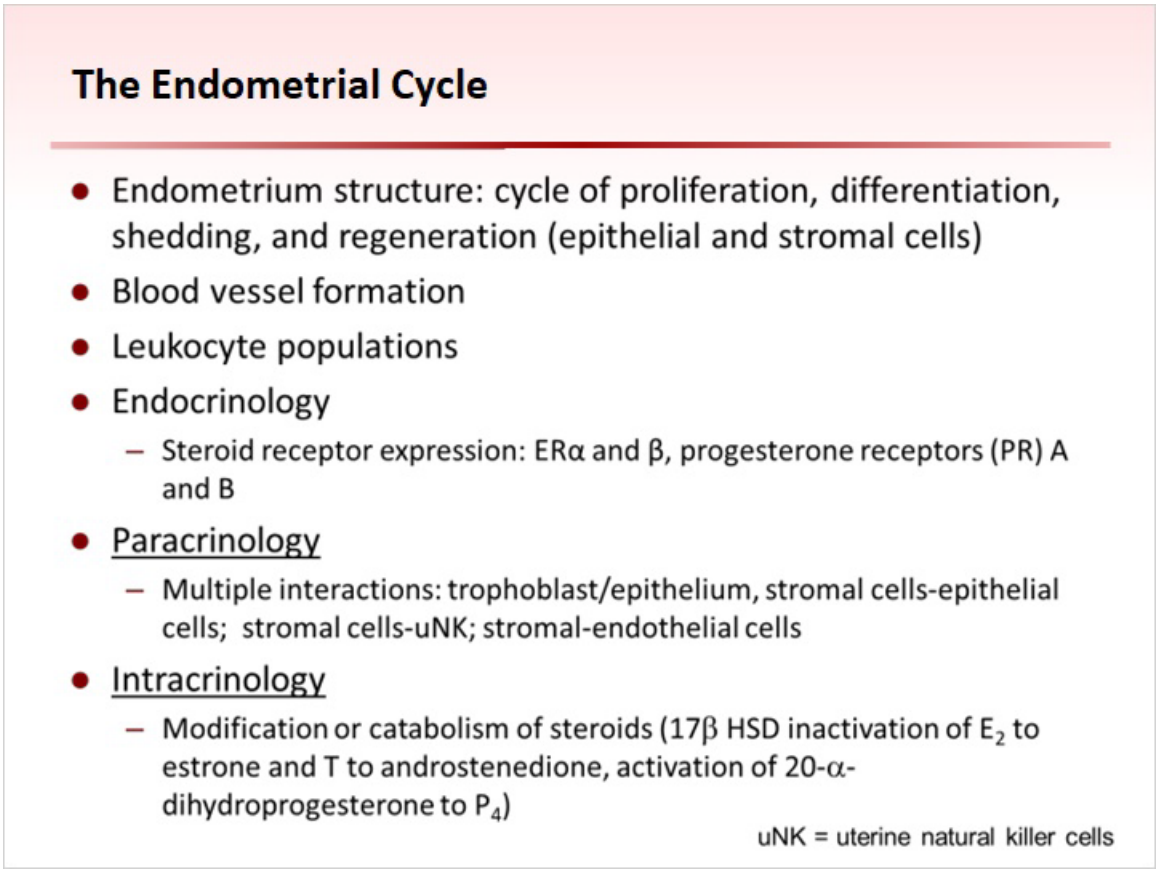
1.9 Time of Implantation of the Conceptus and Loss of Pregnancy



Notes:

This was a prospective study presenting data on implantation from a large sample of healthy women who conceived naturally (Wilcox et al., 1999). Early loss was least likely when implantation occurred by the 9th day. The proportion ending in early loss increased when implantation occurred later. The study suggested that a refractory period after the time of uterine receptivity may provide a natural mechanism by which impaired embryos are eliminated.

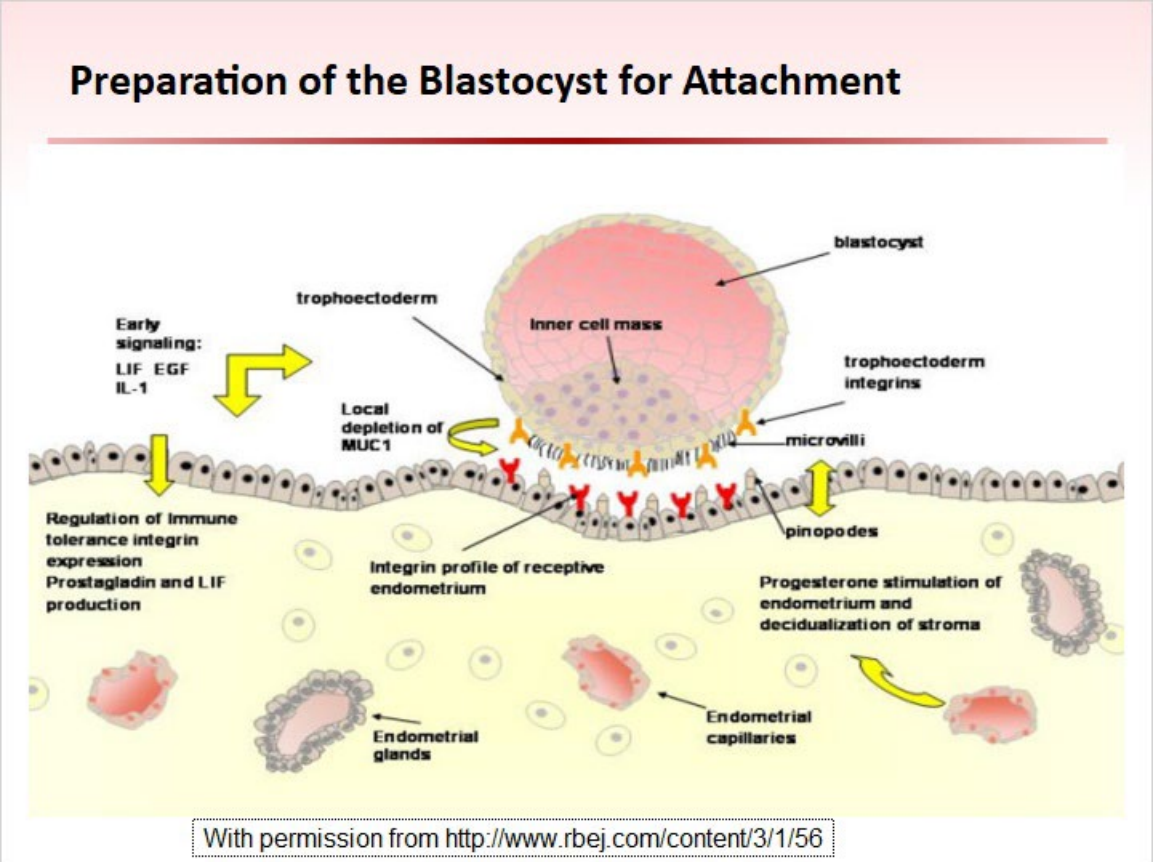
1.10 The Endometrial Cycle



Notes:

There are many critical participants in the endometrium that determine proliferation and secretion, and regulated controlled trophoblast invasion and absence of rejection. The most important processes are listed here.

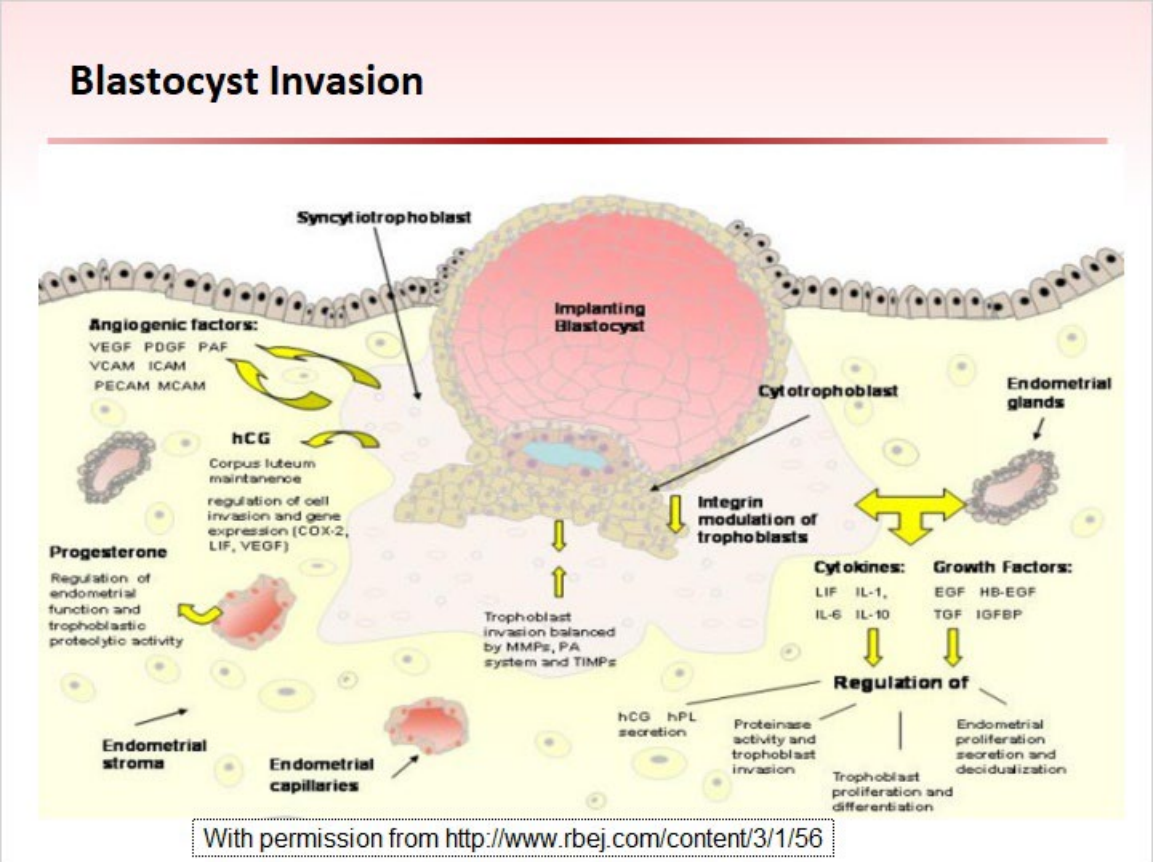
1.11 Preparation of the Blastocyst for Attachment



Notes:

This is a schematic representation of a blastocyst approaching the receptive endometrium, defined by the integrin profile and appearance of pinopods. Early signaling between the blastocyst and the endometrium precedes the attachment. Other important adhesion molecules may also play a role, including L-selectin and its ligand. Note apposition of the inner cell mass to the epithelium. Although the endometrial physiology is mainly regulated by estrogen and progesterone, indirect actions are established through numerous intermediary molecules acting in various compartments. In the human, the sequence of events leading to the establishment of the window of implantation normally occurs in every ovulatory cycle, independently of the presence of a blastocyst.

1.12 Blastocyst Invasion

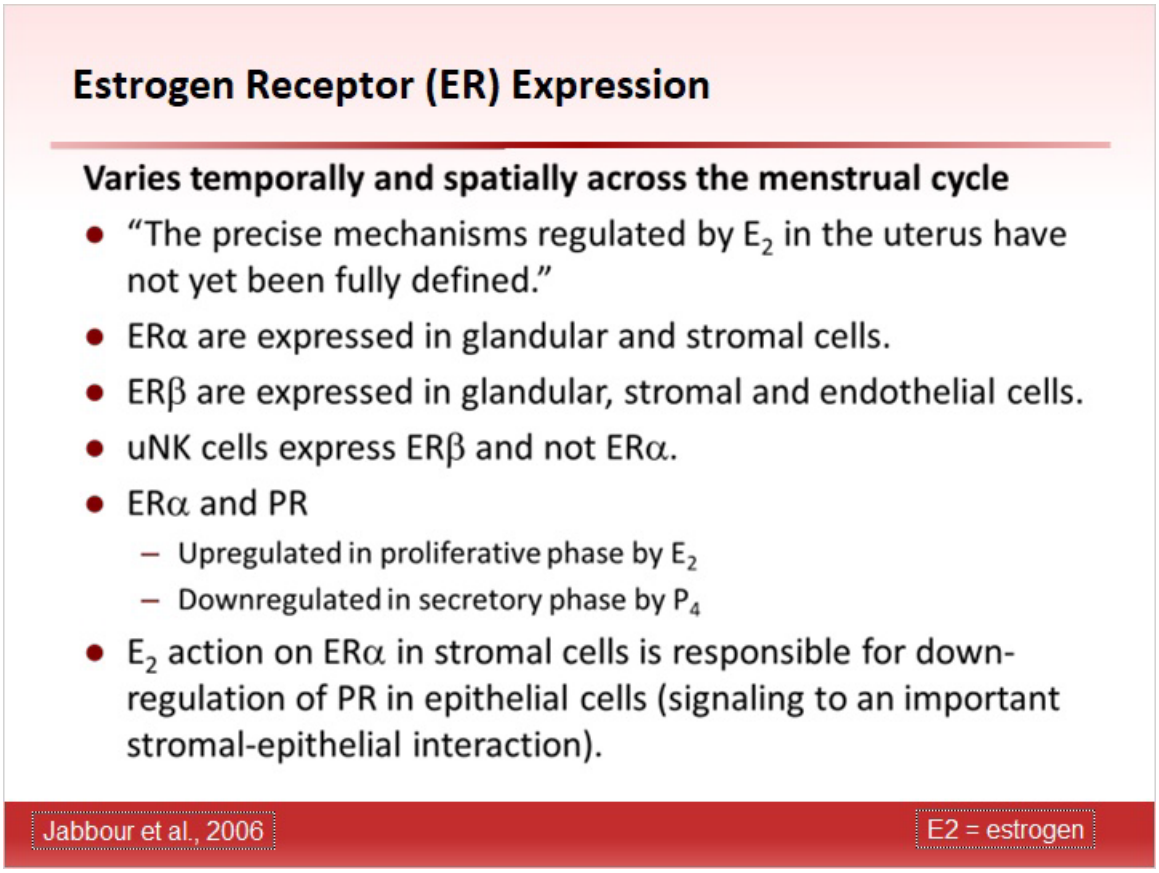


With permission from http://www.rbej.com/content/3/1/56

Notes:

Here is a schematic representation of an implanting blastocyst, highlighting interactions between trophoblastic and endometrial cells, including integrins, growth factors, cytokines, hormones and proteases.

1.13 Estrogen Receptor (ER) Expression

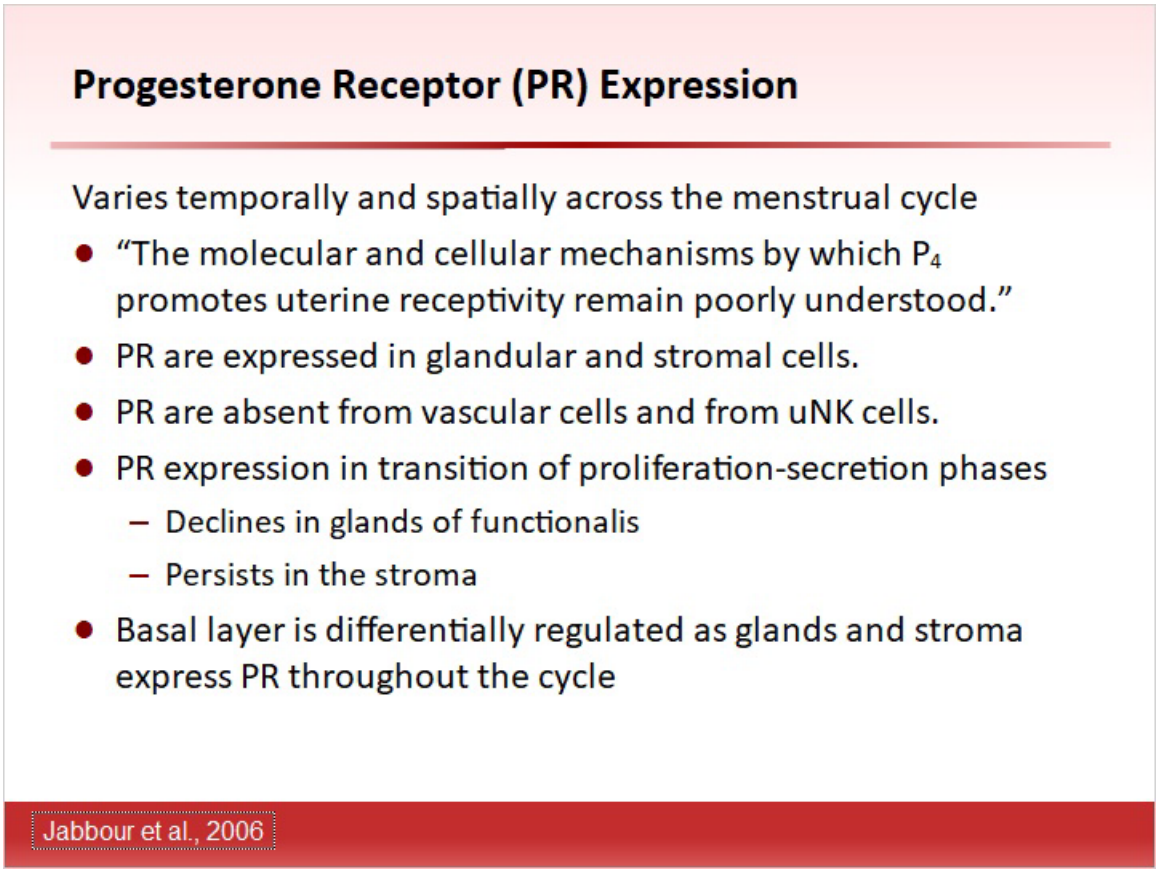


Notes:

In the human endometrium, estrogen receptor (ER) expression varies in significance and temporal-spatial variation. However, precise mechanisms have not yet been fully defined. ERα are expressed in glandular and stromal cells. ERβ are expressed in glandular, stromal and endothelial cells.

uNK cells express ERβ and not ERα. ERα and progesterone receptors are upregulated in the proliferative phase by E_2 and downregulated in the secretory phase by progesterone. E_2 action on the ERα in the stromal cells is responsible for down-regulation of the progesterone receptors in the epithelial cells (signaling to an important stromal-epithelial interaction).

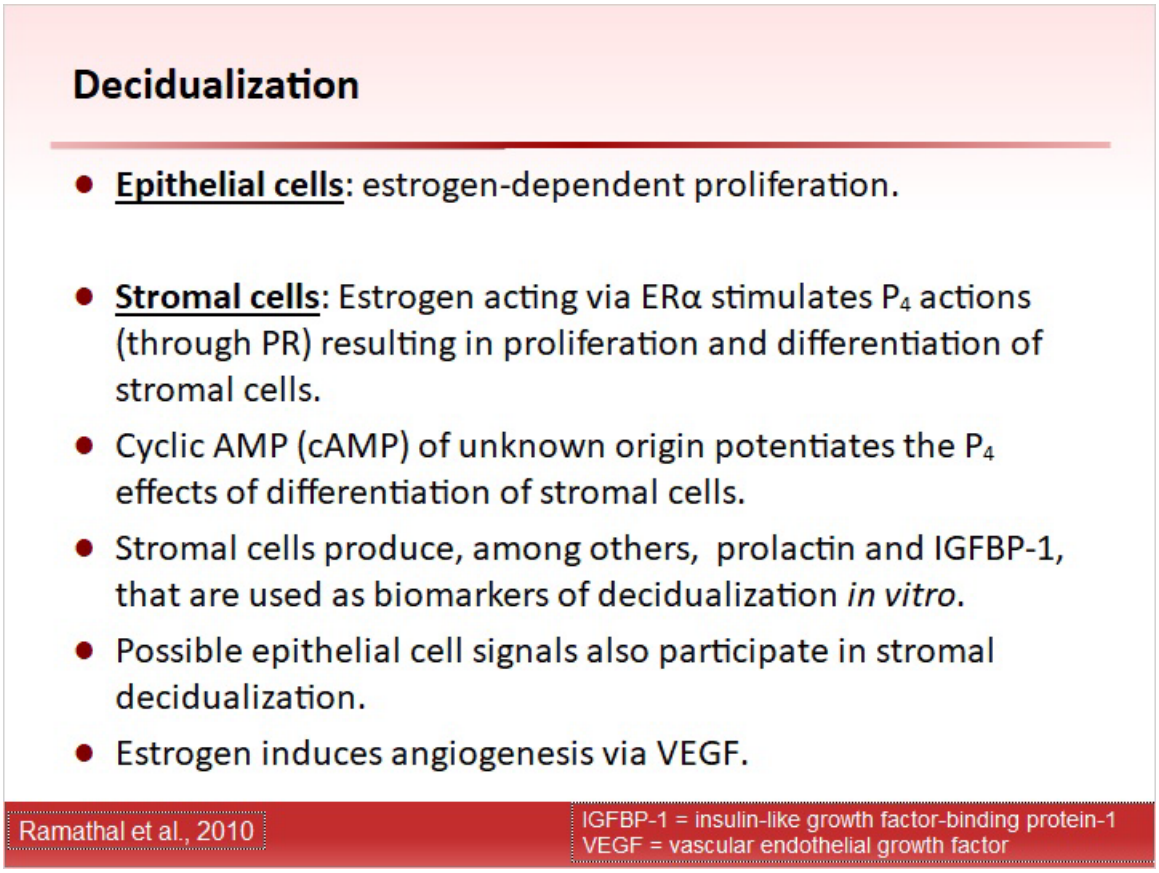
1.14 Progesterone Receptor (PR) Expression



Notes:

Likewise, progesterone receptor expression varies temporally and spatially across the menstrual cycle and molecular and cellular mechanisms by which progesterone promotes uterine receptivity remain poorly understood. Progesterone receptors are expressed in glandular and stromal cells but are absent from vascular cells and from uNK cells. Progesterone receptor expression declines in the glands of functionalis in the transition of proliferation-secretion phases but persists in the stroma. The basal layer is differentially regulated as glands and stroma express progesterone receptors throughout the cycle.

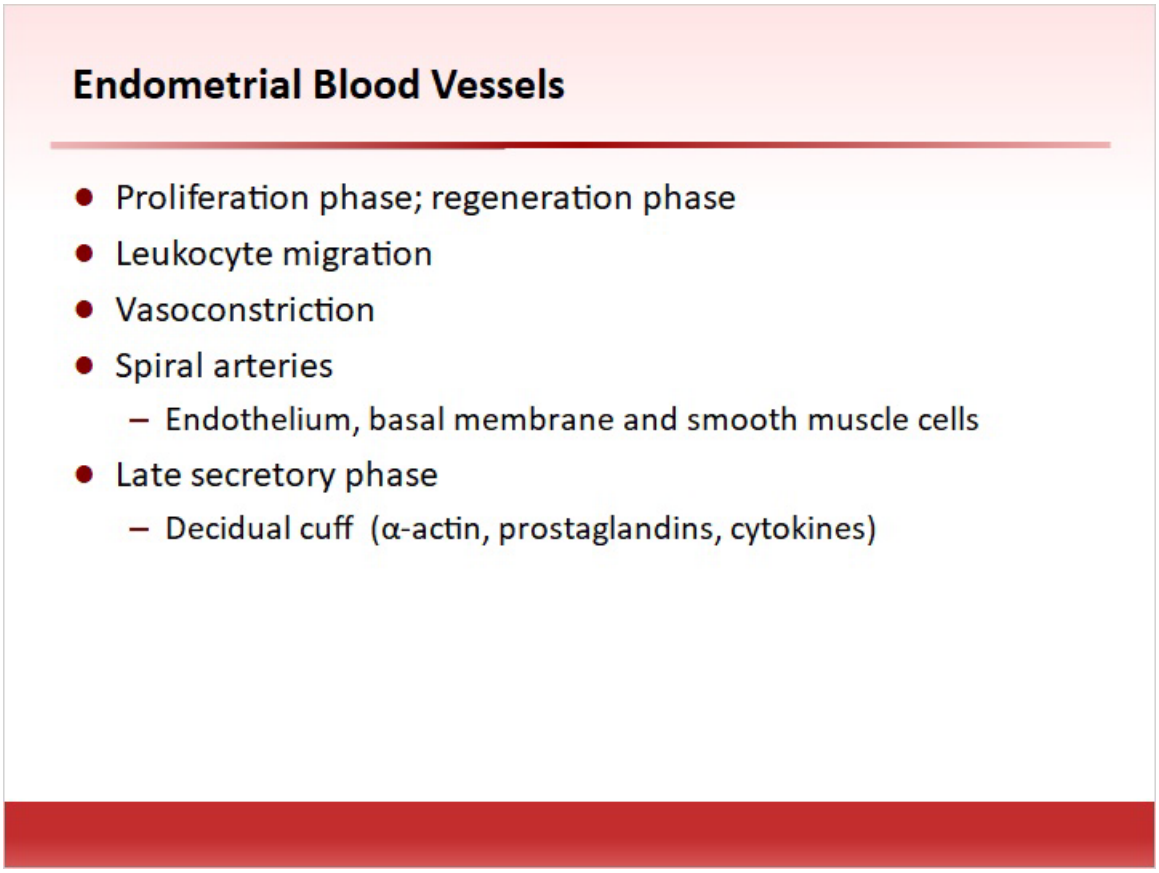
1.15 Decidualization



Notes:

Decidualization of the stroma is critical for establishment of the receptive stage. The process is dependent upon estrogen priming and progesterone and also the presence of cyclic AMP (cAMP) *in vitro*.

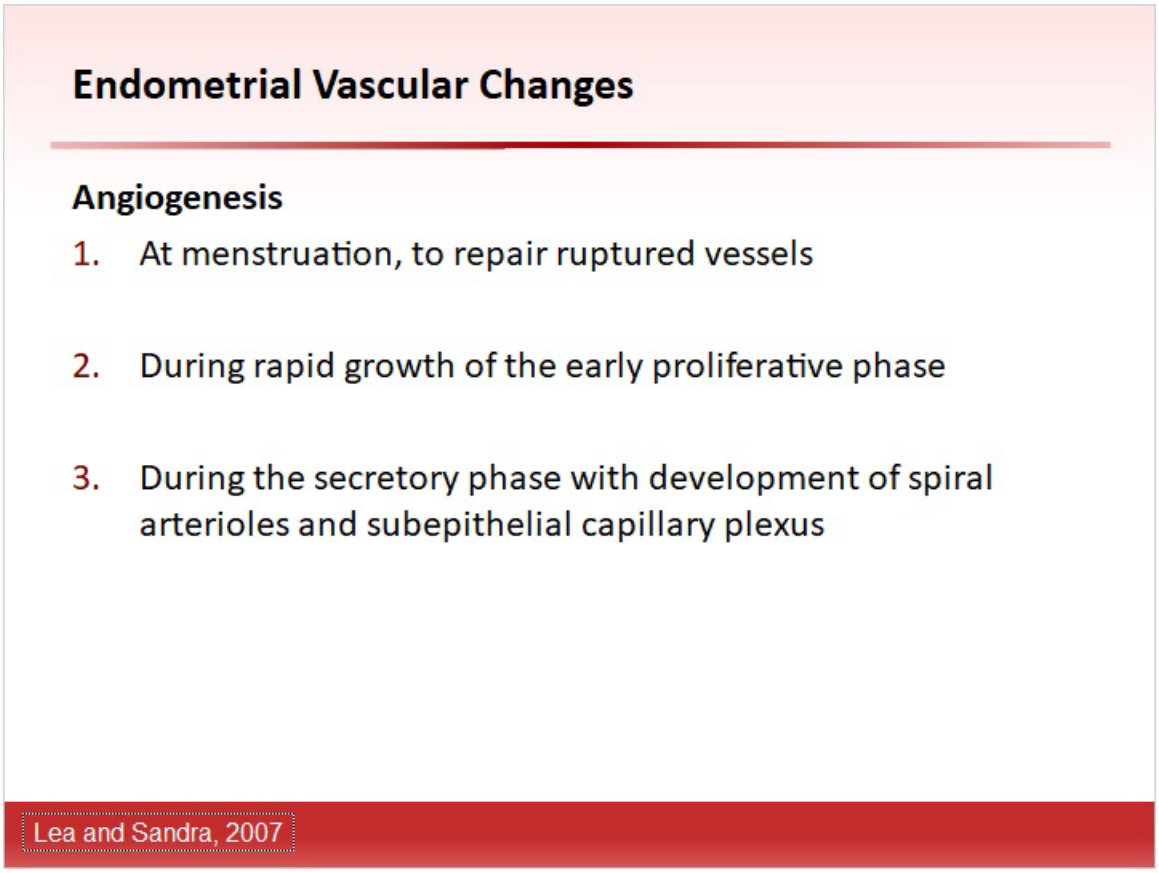
1.16 Endometrial Blood Vessels



Notes:

In addition to epithelial and stromal cell proliferation and differentiation, major changes occur in endometrial blood vessels and in leukocyte populations during the cycle.

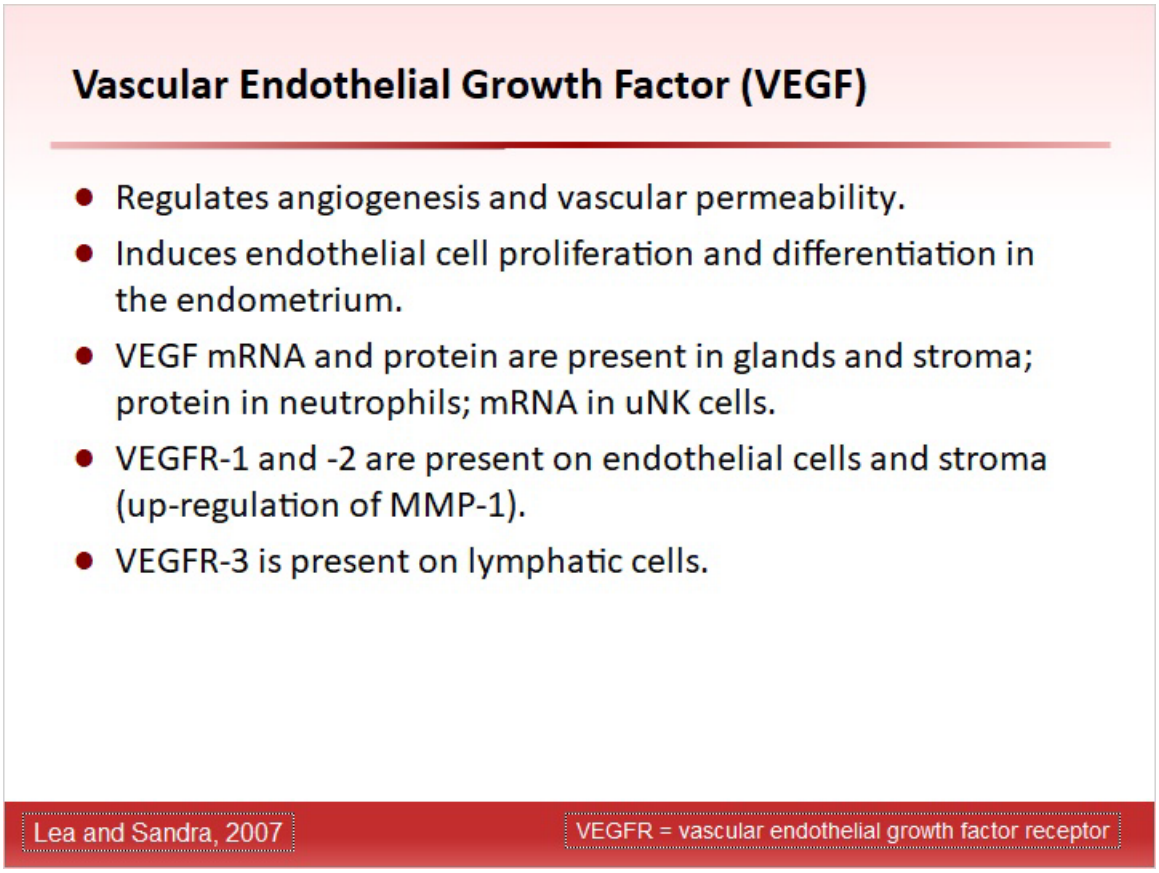
1.17 Endometrial Vascular Changes



Notes:

There are three stages of angiogenesis as noted here, under fine tuning by angiogenic factors: at menstruation, during rapid growth of the early proliferative phase and during the secretory phase.

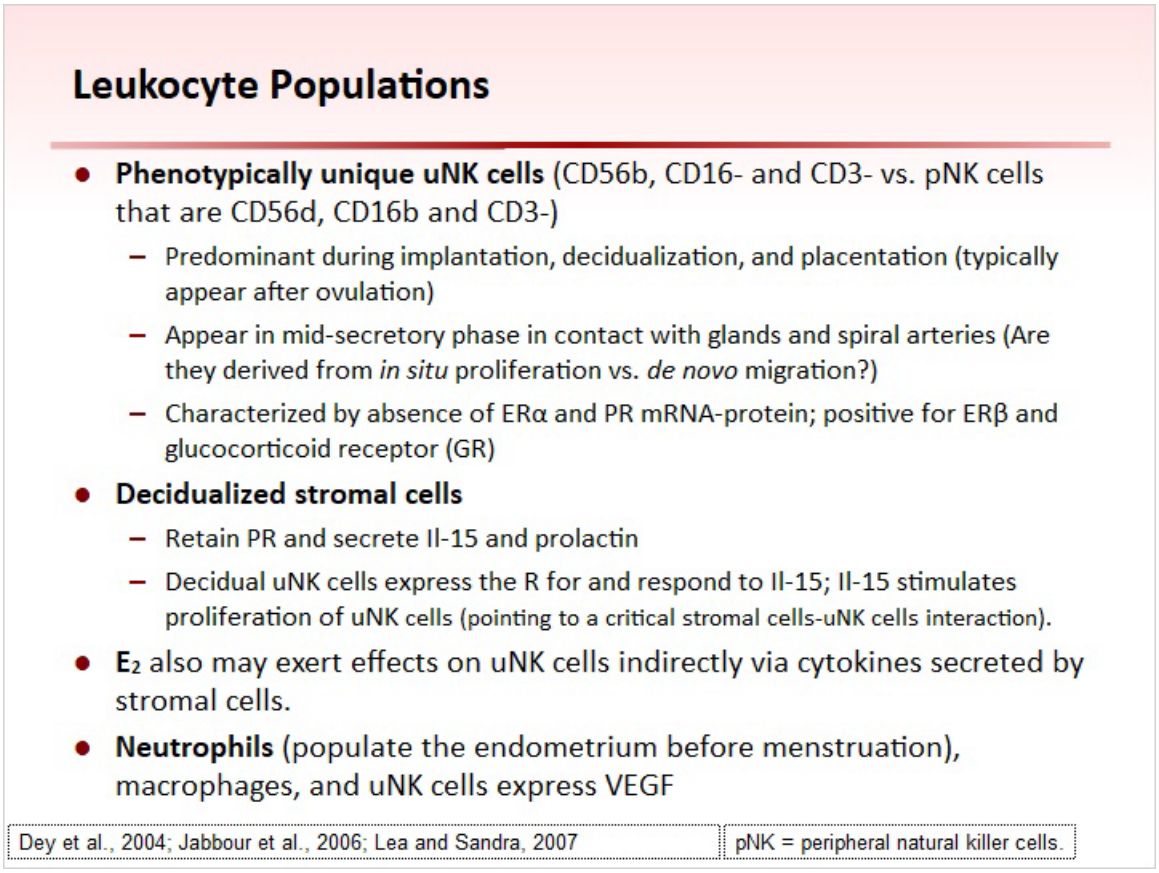
1.18 Vascular Endothelial Growth Factor (VEGF)



Notes:

VEGF and angiopoietins are major regulators of vessel formation, maintenance, stabilization, and regression. VEGF and its receptors (VEGFR) play a significant role in endometrial angiogenesis, and also participate in the regulation of other endometrial functions.

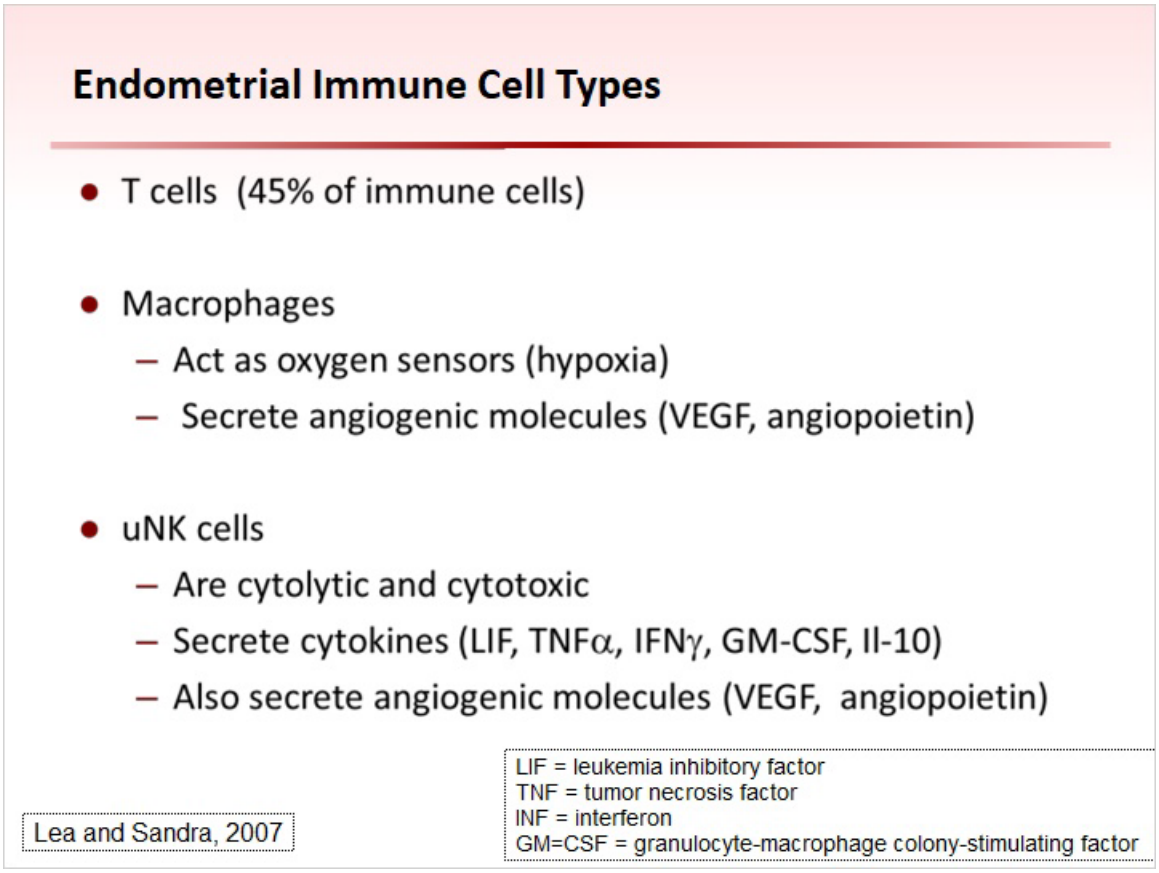
1.19 Leukocyte Populations



Notes:

Leukocyte populations also vary during the endometrial cycle. They express different receptors and participate in regulation of multiple endometrial functions. pNK: peripheral natural killer cells.

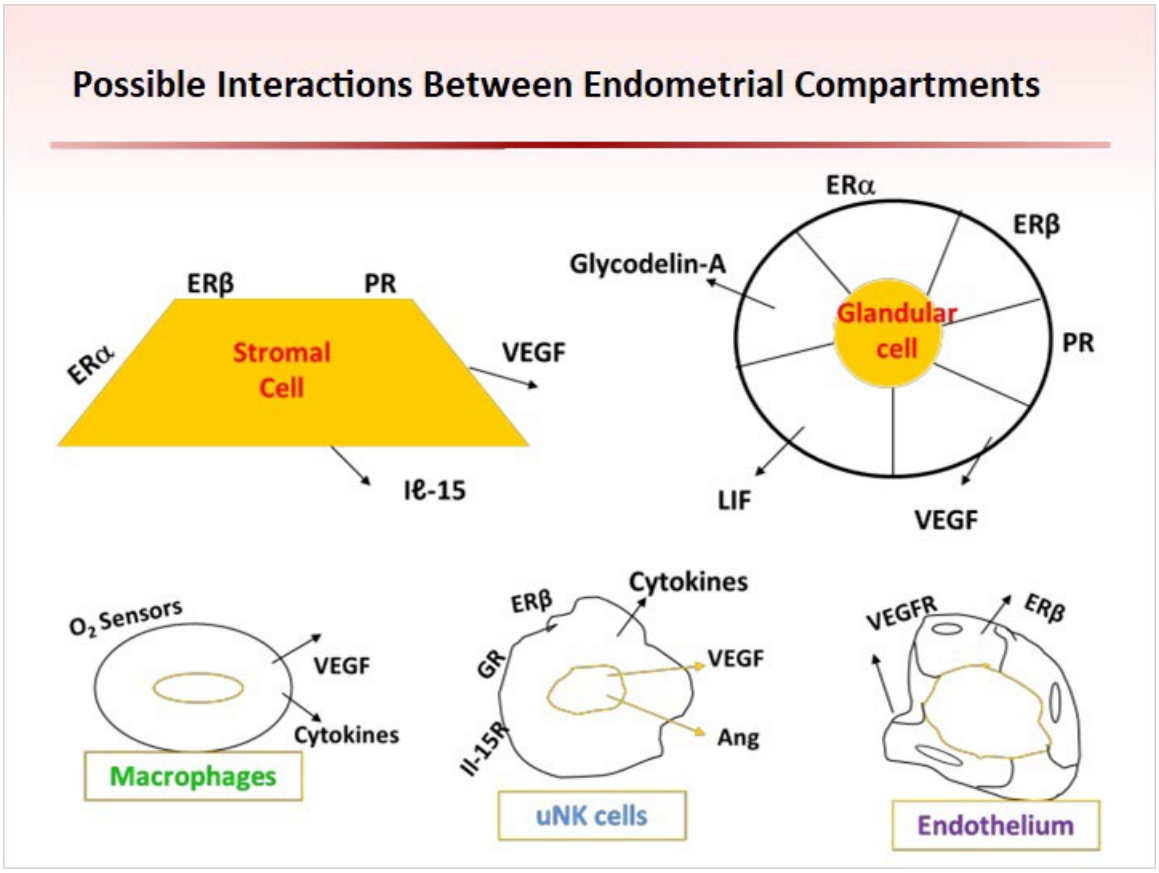
1.20 Endometrial Immune Cell Types



Notes:

The endometrium has a variety of immune cells with many key functions as shown here. The balance of these activities support implantation through a physiological inflammatory response and avoids rejection.

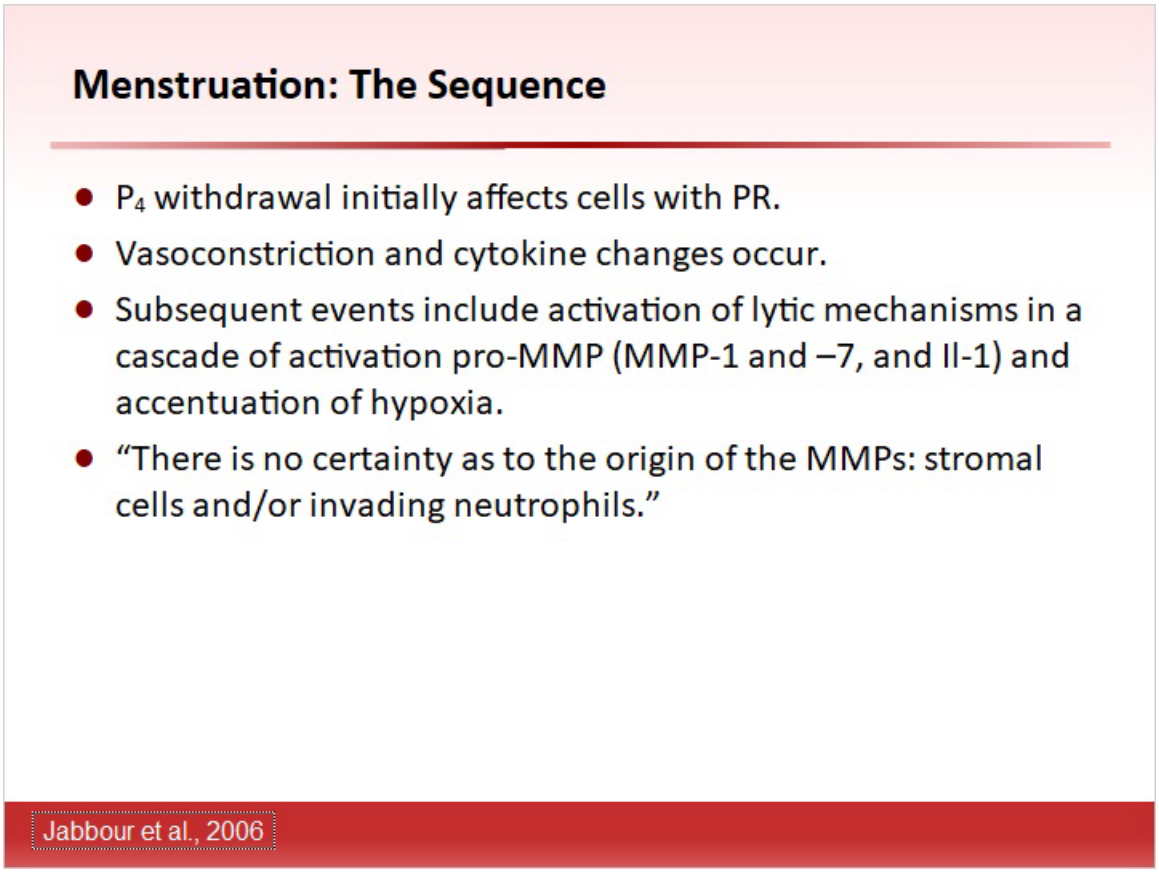
1.21 Possible Interactions Between Endometrial Compartments



Notes:

A schematic representation of paracrine relationships among the various cell types of the endometrium is shown here. LIF and some interleukins are critical for decidualization and stromal cell function during and after implantation.

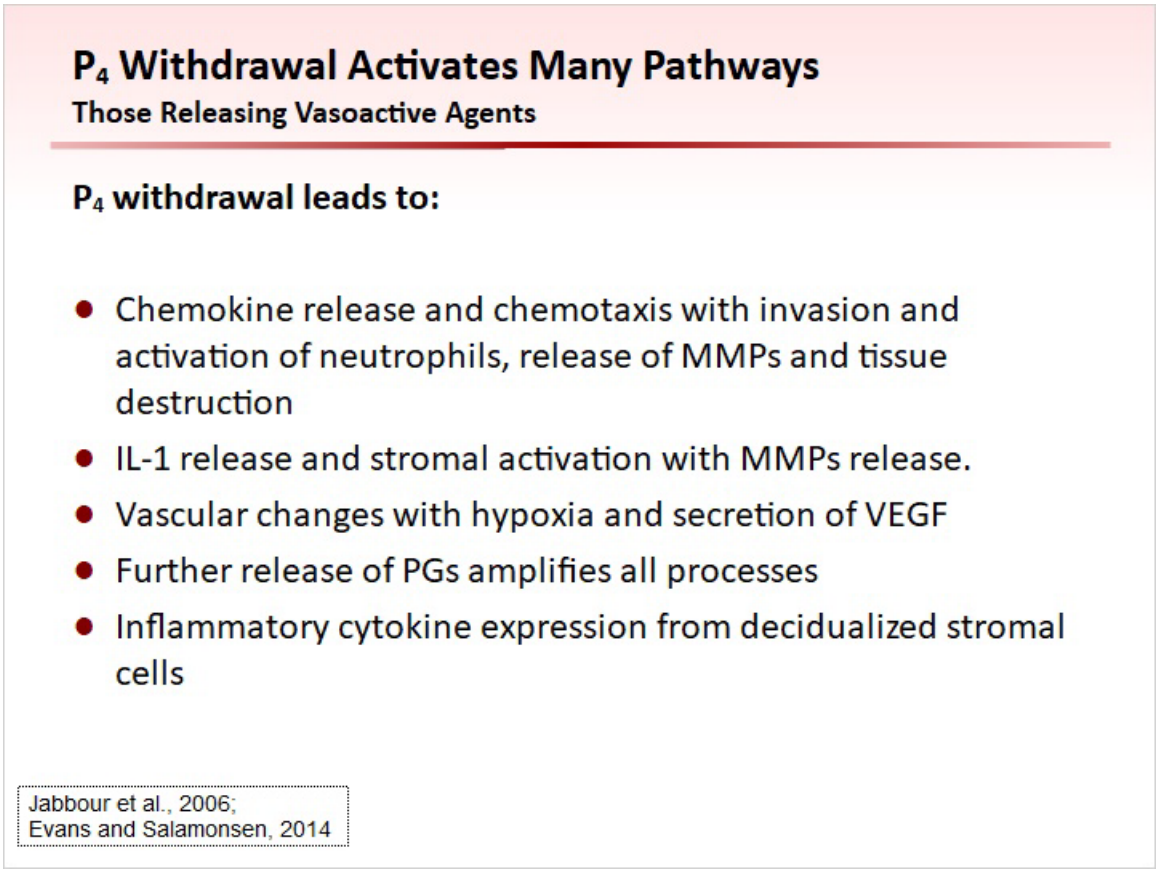
1.22 Menstruation: The Sequence



Notes:

In the absence of pregnancy, the endometrium enters the menstruation phase as a result of progesterone withdrawal. Progesterone withdrawal initially affects cells with progesterone receptors. Vasoconstriction and cytokine changes occur. Subsequent events include activation of lytic mechanisms in a cascade of activation pro-MMP (MMP-1 and -7, and Il-1) and accentuation of hypoxia. "There is no certainty as to the origin of the MMPs: stromal cells and/or invading neutrophils."

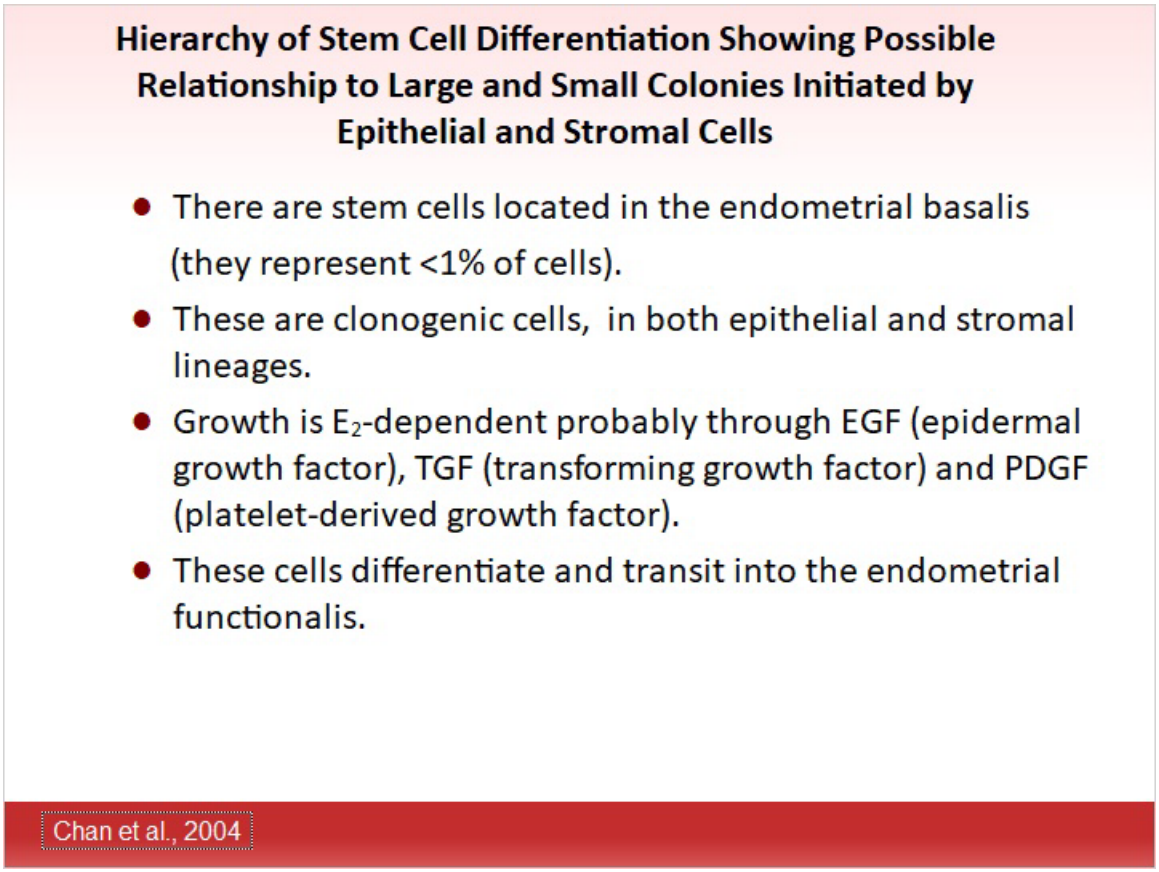
1.23 P4 Withdrawal Activates Many Pathways



Notes:

There is a cascade of events following progesterone withdrawal leading to menstruation.

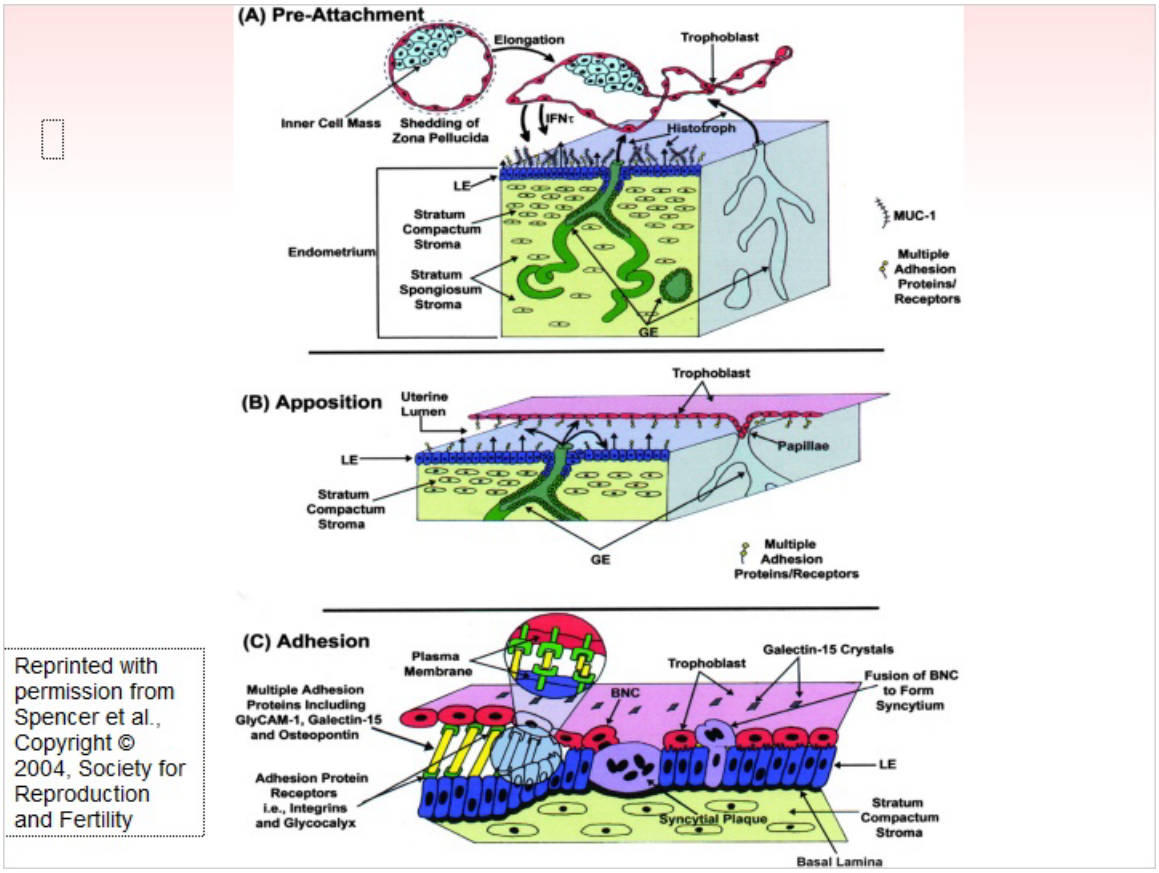
1.24 Hierarchy of Stem Cell Differentiation Showing Possible Relationship to Large and Small Colonies Initiated by



Notes:

It has been postulated that stem cells present in the endometrial basalis may be at least partly responsible for initiating the regeneration process after menstruation.

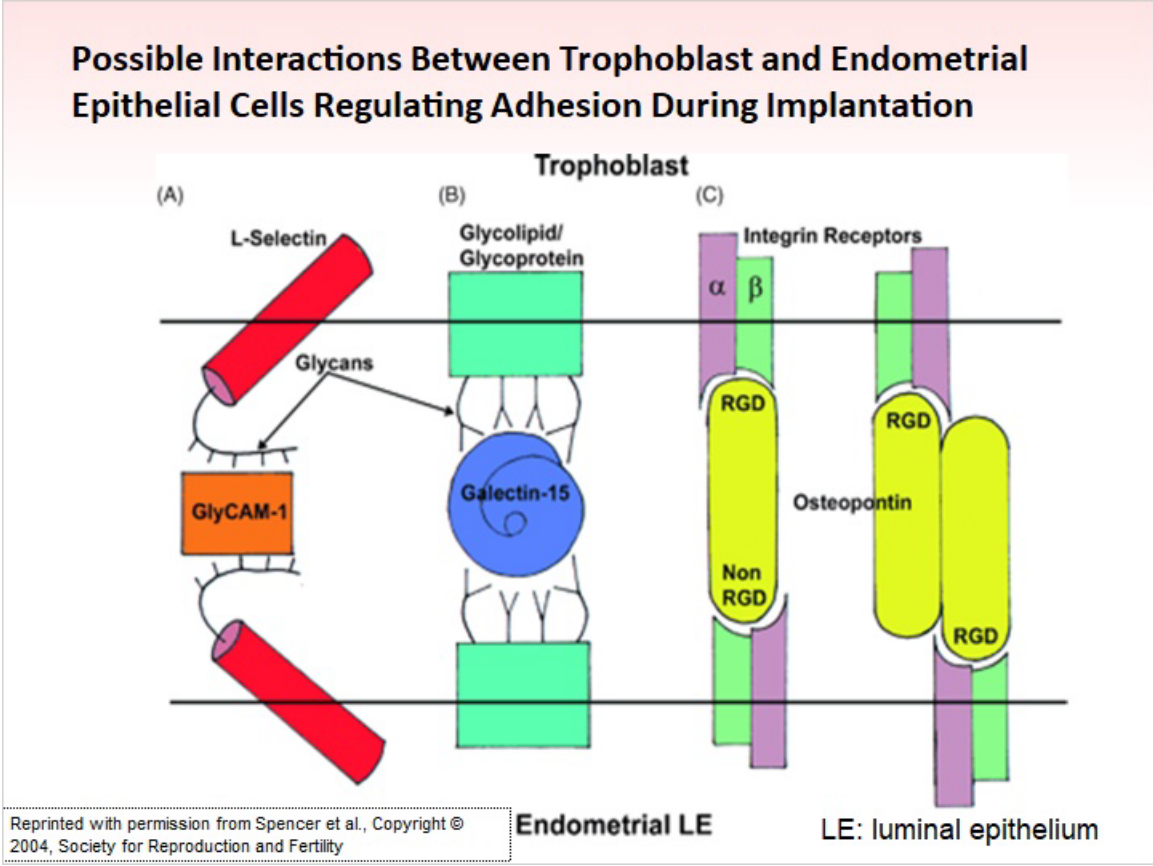
1.25 Implantation in domestic animals



Notes:

The noninvasive and protracted nature of implantation in domestic animals provides valuable opportunities to investigate fundamental processes of implantation that are shared among all mammals. This is a detailed schematic diagram of apposition and adhesion phases of blastocyst implantation in sheep (Spencer et al., 2004). Endometrial invasion does not occur in domestic ruminants; thus, definitive implantation is achieved by adhesion of the mononuclear trophoblast cells to the endometrial luminal epithelium (LE) and formation of syncytia by the fusion of trophoblast binucleate cells with the LE.

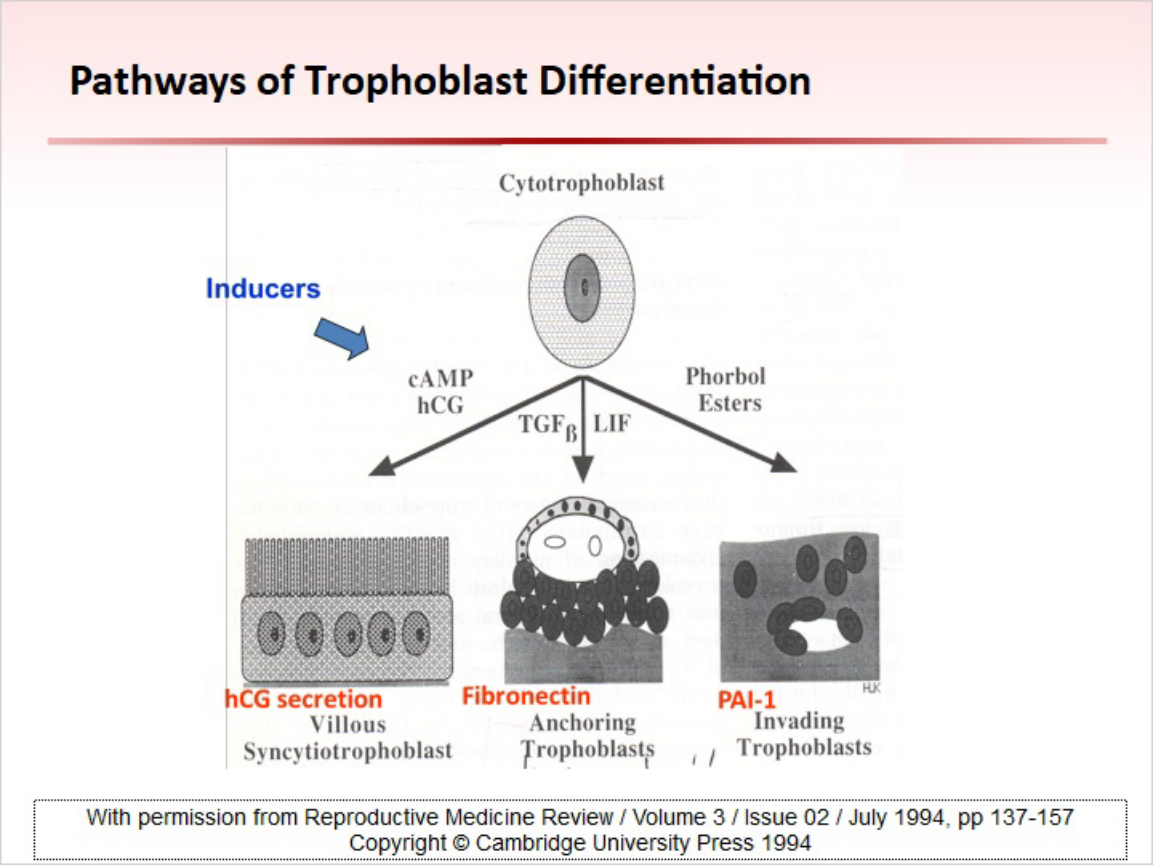
1.26 Possible Interactions Between Trophoblast and Endometrial Epithelial Cells Regulating Adhesion During Implantation



Notes:

Some of the critical molecules (receptor/ligand interactions) responsible for trophoblast adhesion in sheep are shown here (Spencer et al., 2004). HB-EGF and EGF receptor have also been proposed as an embryo receptor within the endometrium.

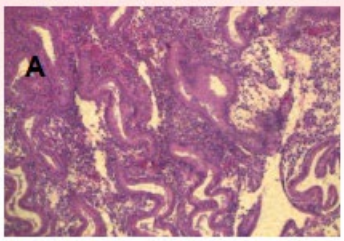
1.27 Pathways of Trophoblast Differentiation

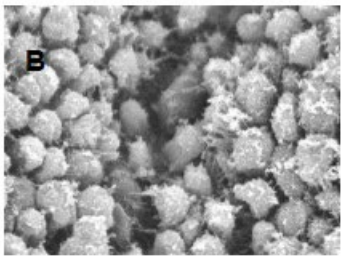


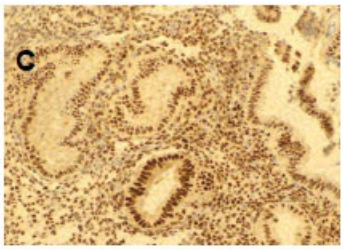
Notes:

In the human, invasion of stroma and vessels follows embryonic apposition and attachment, in synchrony with cytotrophoblast differentiation into three cell types with different functional properties and secretory mechanisms (modified from Kliman, 1994).

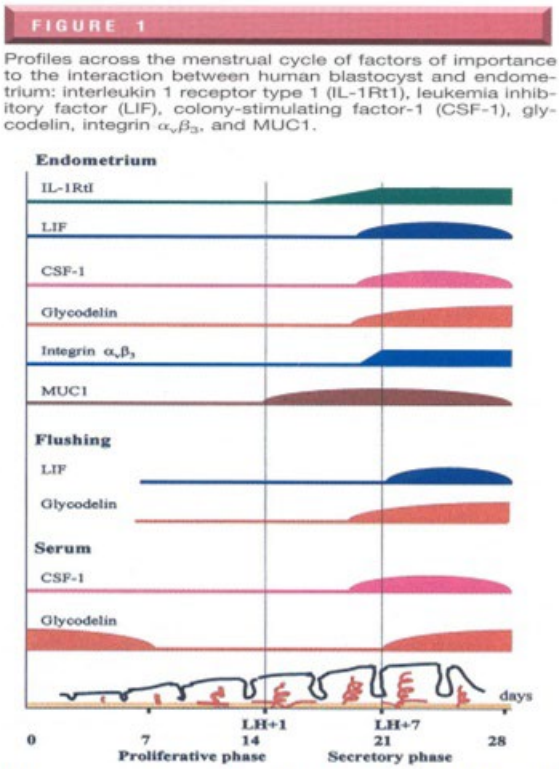
1.28 Potential Markers of Endometrial Receptivity







Photos courtesy of Drs. Oehninger, Mirkin and Nikas

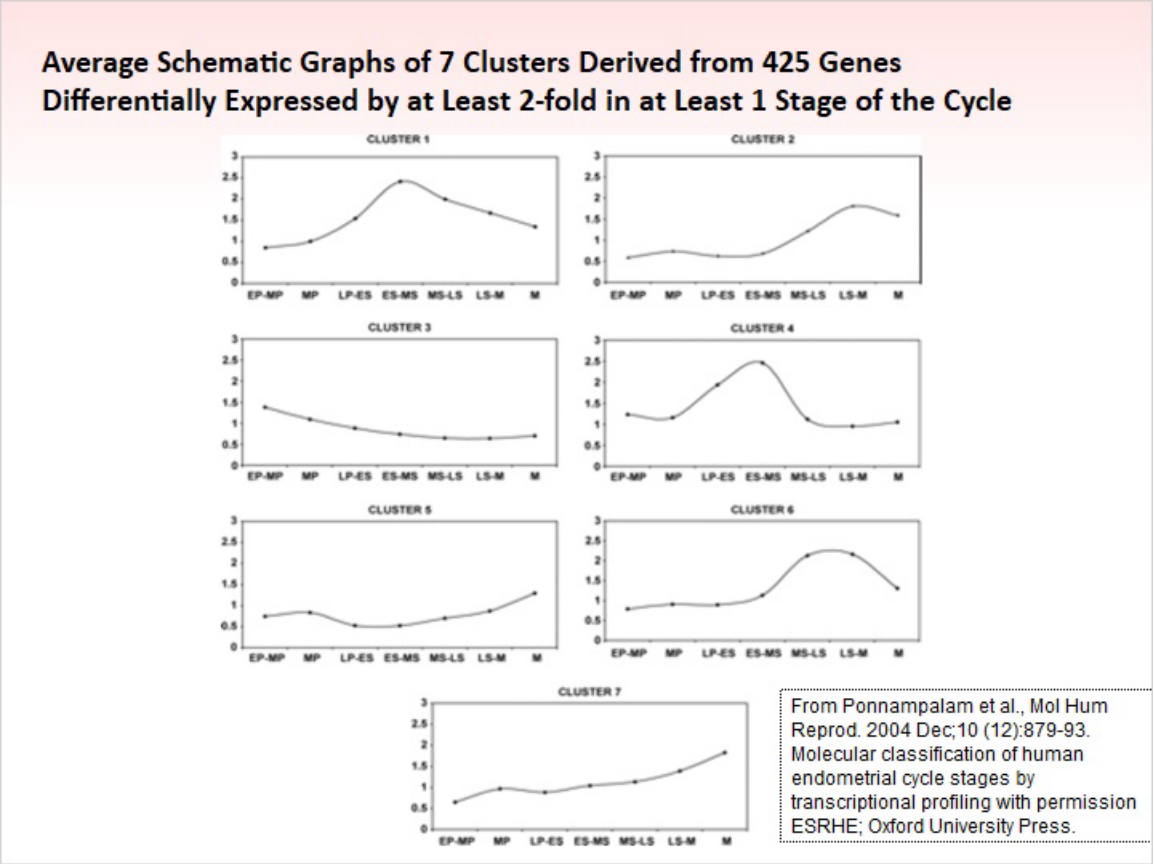


With permission from Lindhard et al., Fertil Steril. 2002 Aug;78(2):221-33

Notes:

Potential markers of endometrial receptivity are shown in the left panels. **A.** Endometrial histology. **B.** Scanning microscopy (showing pinopods). **C.** Immunohistochemistry. These techniques have been used unsuccessfully to ascertain endometrial receptive stage in the clinic. Definitive conclusions about the clinical value of these factors measured by immunohistochemistry and/or in endometrial secretions for the assessment of endometrial function and prognosis for pregnancy after ART cannot be drawn at present.

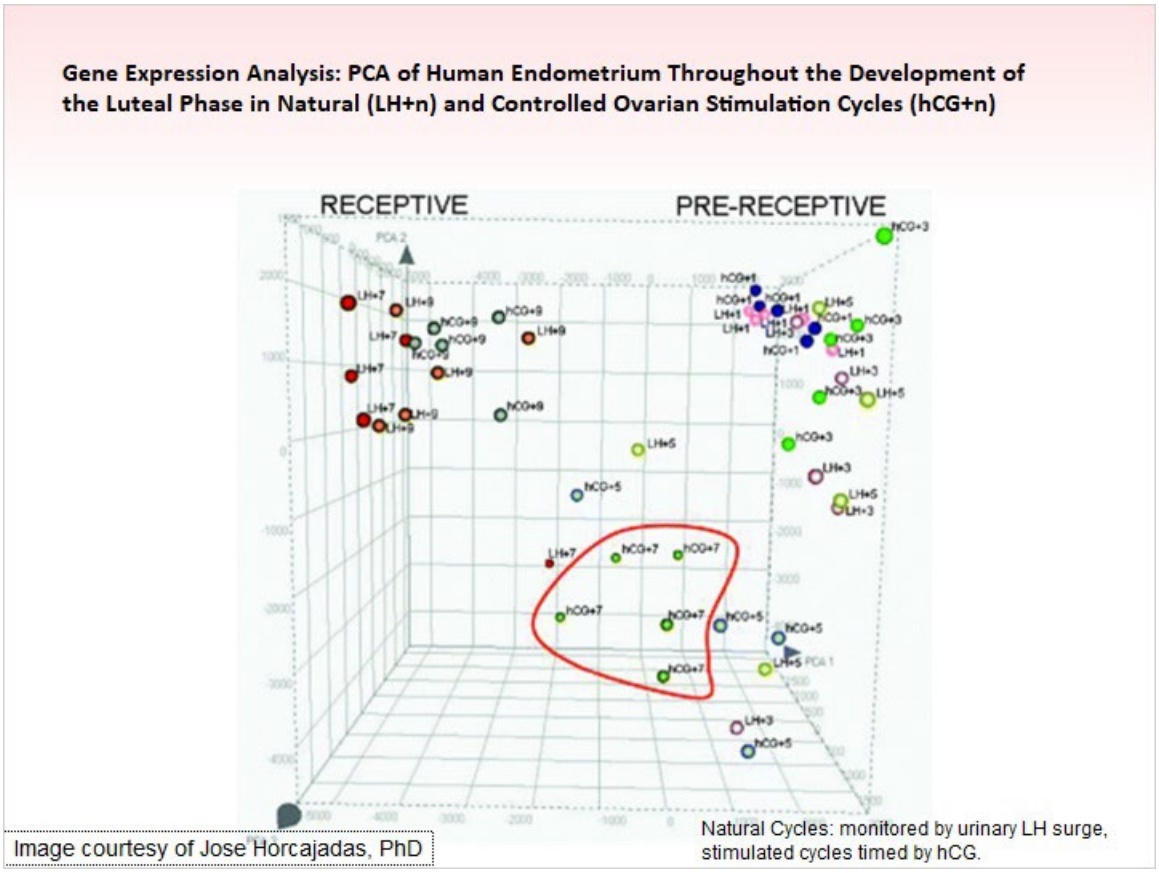
1.29 Average Schematic Graphs of 7 Clusters Derived from 425 Genes Differentially Expressed by at Least 2-fold in at Least 1 Stage of the Cycle



Notes:

A more novel approach was presented by Ponnampalan et al., (2004) defining a molecular classification of human endometrial cycle stages (early, mid-late proliferative and secretory phases, and menstruation) by transcriptional profiling (microarray technology and polymerase chain reaction (PCR) validation).

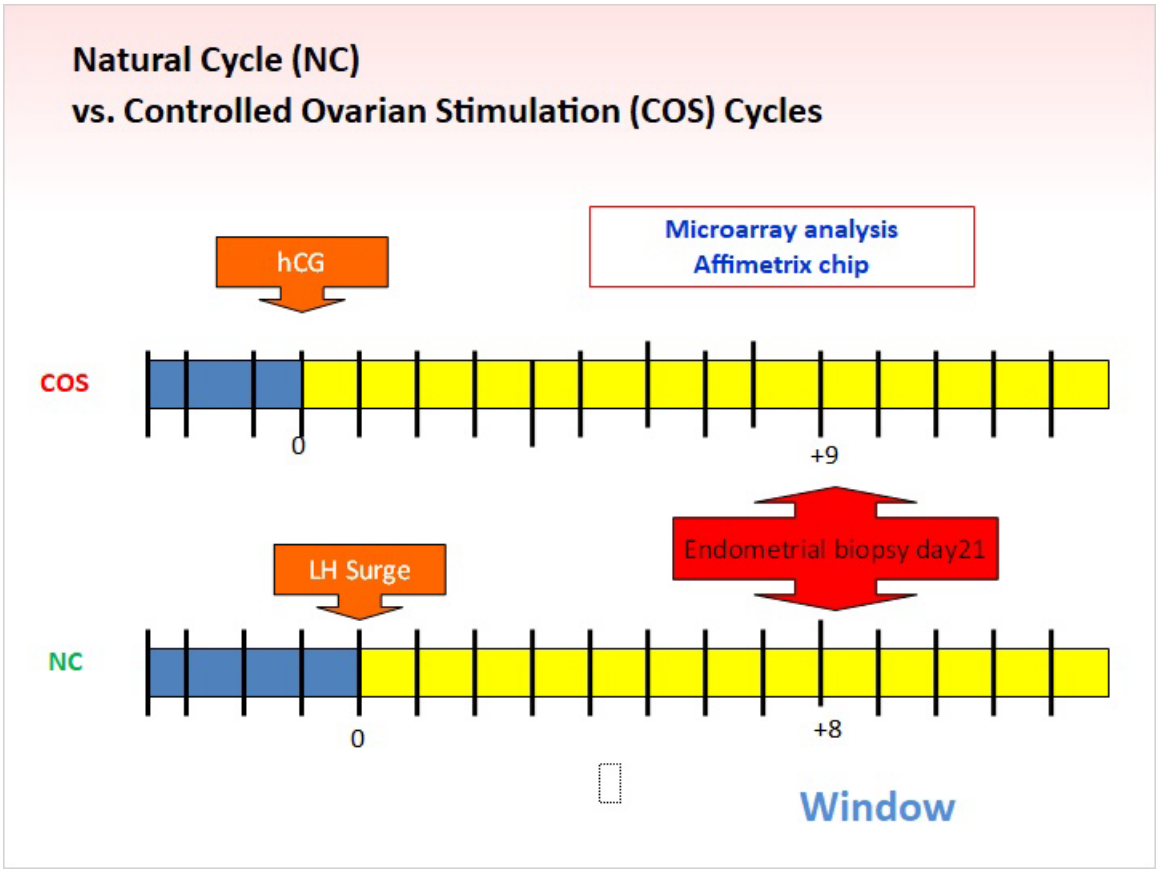
1.30 Gene Expression Analysis: PCA of Human Endometrium Throughout the Development of the Luteal Phase in Natural (LH+n) and Controlled Ovarian Stimulation Cycles (hCG+n)



Notes:

Using a similar approach followed by principal component analysis (PCA) Talbi et al.,(2006) extended identification of gene expression profiles in the different phases of the menstrual cycle. This may allow better defining of the factors that delineate the window of implantation and thereafter the factors that can be causative of failure to implant. Controlled ovarian stimulation induces a functional genomic delay of the endometrium with potential clinical implications (Horcajadas et al., 2007). Attempts have been made to characterize the prereceptive and receptive phases of the human endometrium. Natural cycles: monitored by urinary LH surge, stimulated cycles timed by hCG.

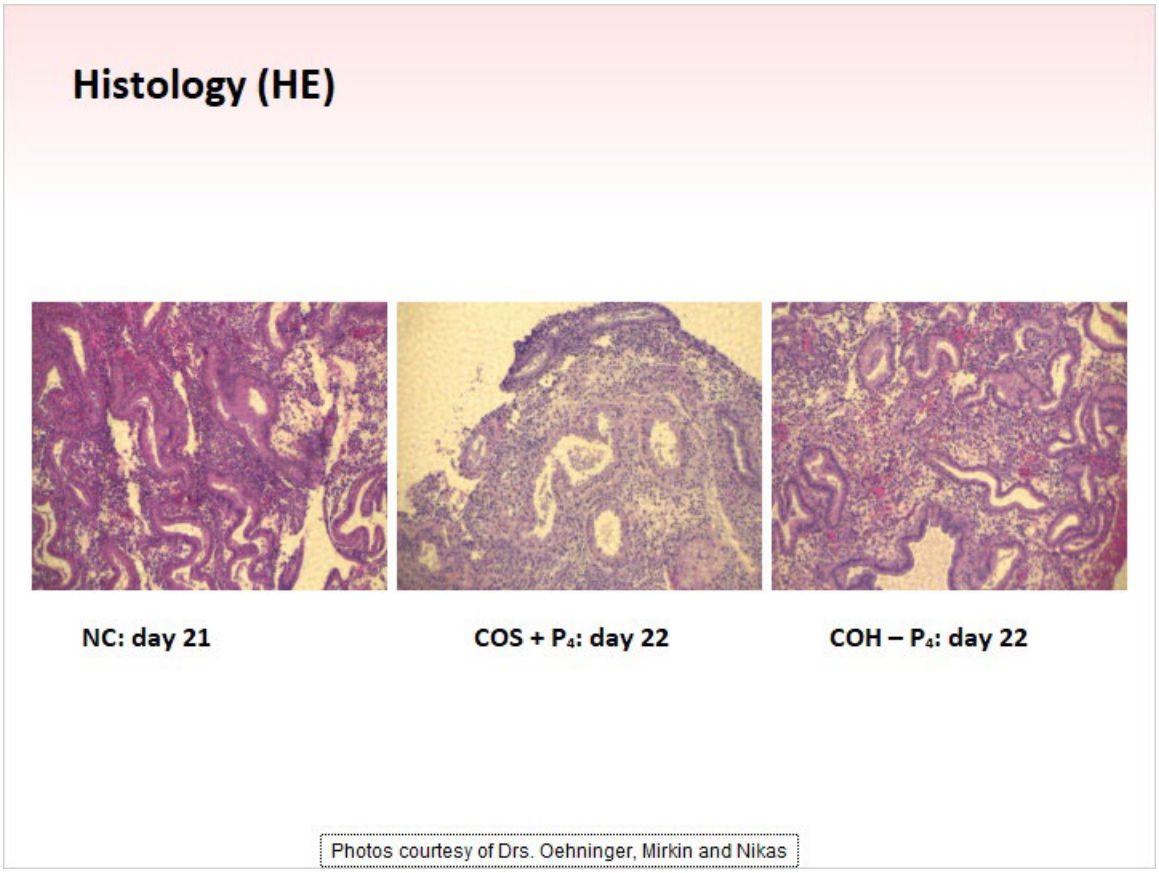
1.31 Natural Cycle (NC)



Notes:

Here is an analysis of gene expression in endometrial biopsies from control subjects in natural cycles (NC) and in oocyte donors undergoing controlled ovarian stimulation. Biopsies were performed on estimated cycle day 21 to identify genes relevant to embryo implantation (Mirkin et al., 2004, 2005). Controlled ovarian stimulation resulted in advanced histological dating (H-E) when compared with natural cycles (day 22 or 23 *vs.* day 21, respectively). Most frequently, the advancement was due to a stromal effect with variable, but low, degrees of glandular-stromal dyssynchrony.

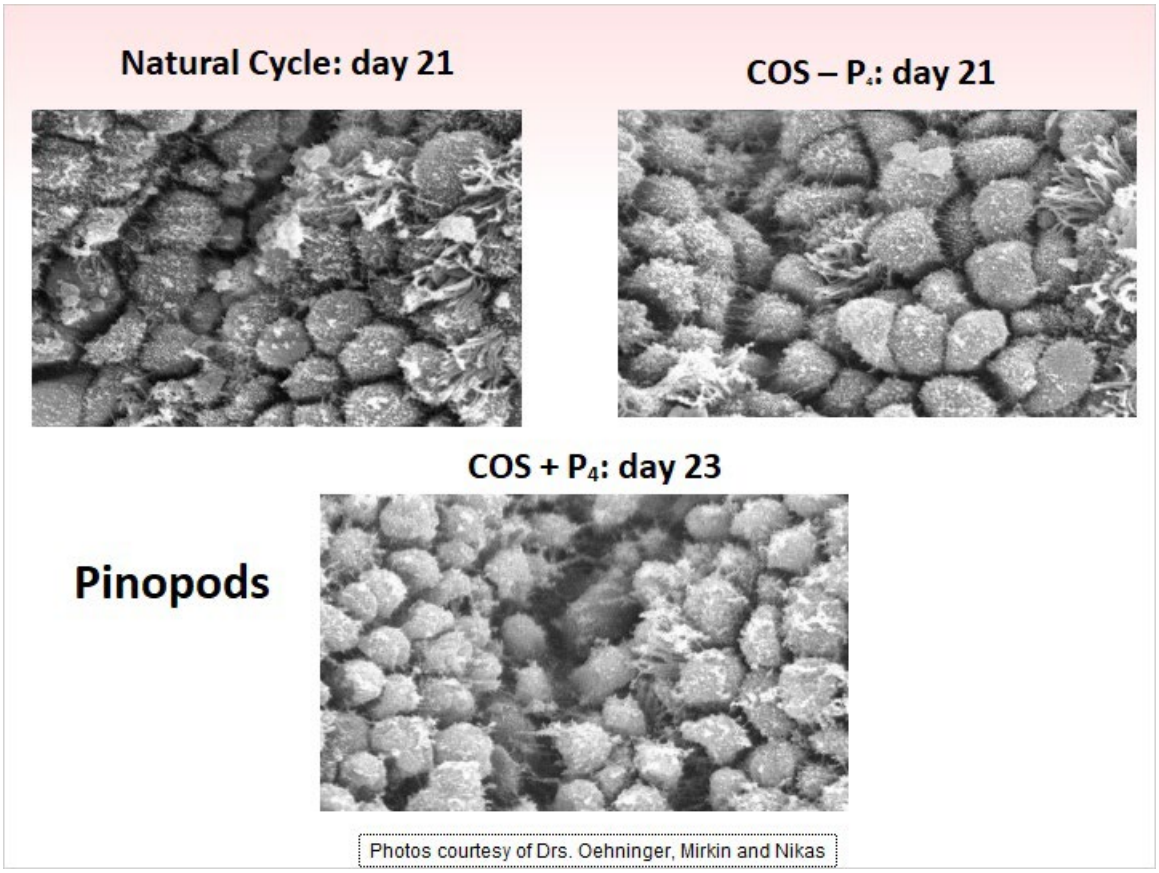
1.32 Histology (HE)



Notes:

This histology confirmed the natural cycle phase was day 21, whereas controlled ovarian stimulation cycles with or without progesterone supplementation were advanced on day 22 (Mirkin et al., 2004, 2005).

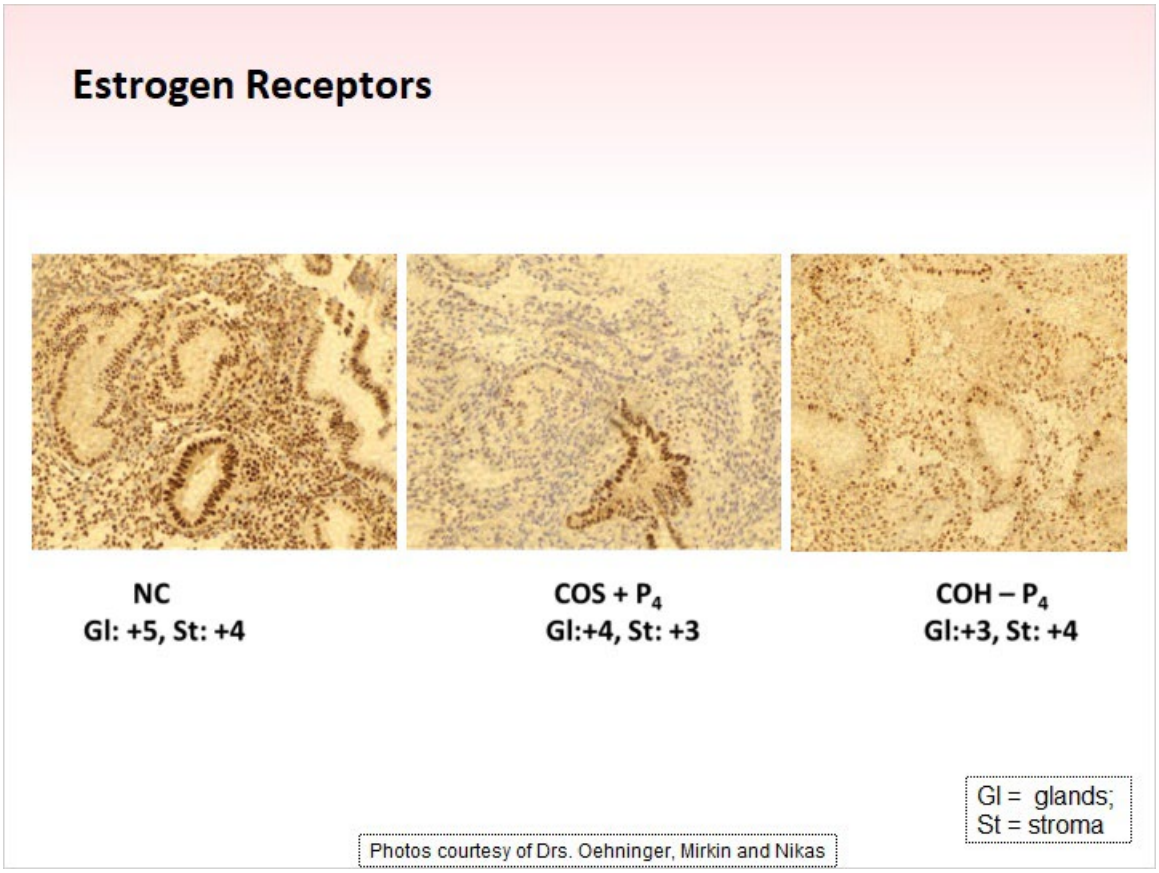
1.33 Pinopods



Notes:

This scanning microscopy confirmed pinopod pattern of expression (Psychoyos, 1994) as day 21 on natural cycle and controlled ovarian stimulation cycles without progesterone supplementation, but controlled ovarian stimulation with progesterone supplementation were advanced to day 23 (Mirkin et al., 2004, 2005). It should be noted that in prospective and well-designed studies pinopods have not been confirmed as reliable markers of implantation (Quinn and Casper, 2009).

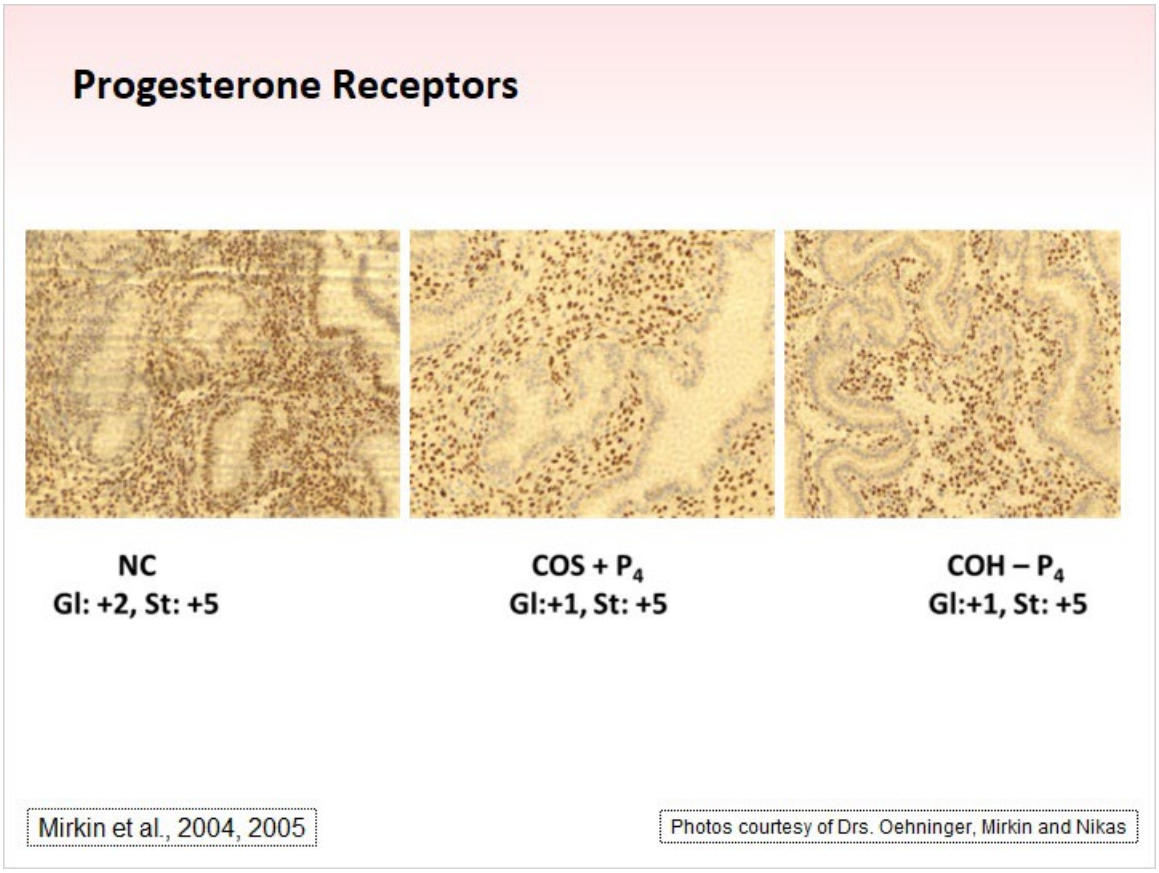
1.34 Estrogen Receptors



Notes:

Nuclear ER and PR expression were evaluated in glandular and stromal endometrial compartments. Staining was evaluated semiquantitatively by using a grading system for glands and stroma, ranging from 0 (no expression, to +5, maximal expression). Estrogen receptors by immunohistochemistry also showed advancement [NC: natural cycle] (Mirkin et al., 2004, 2005).

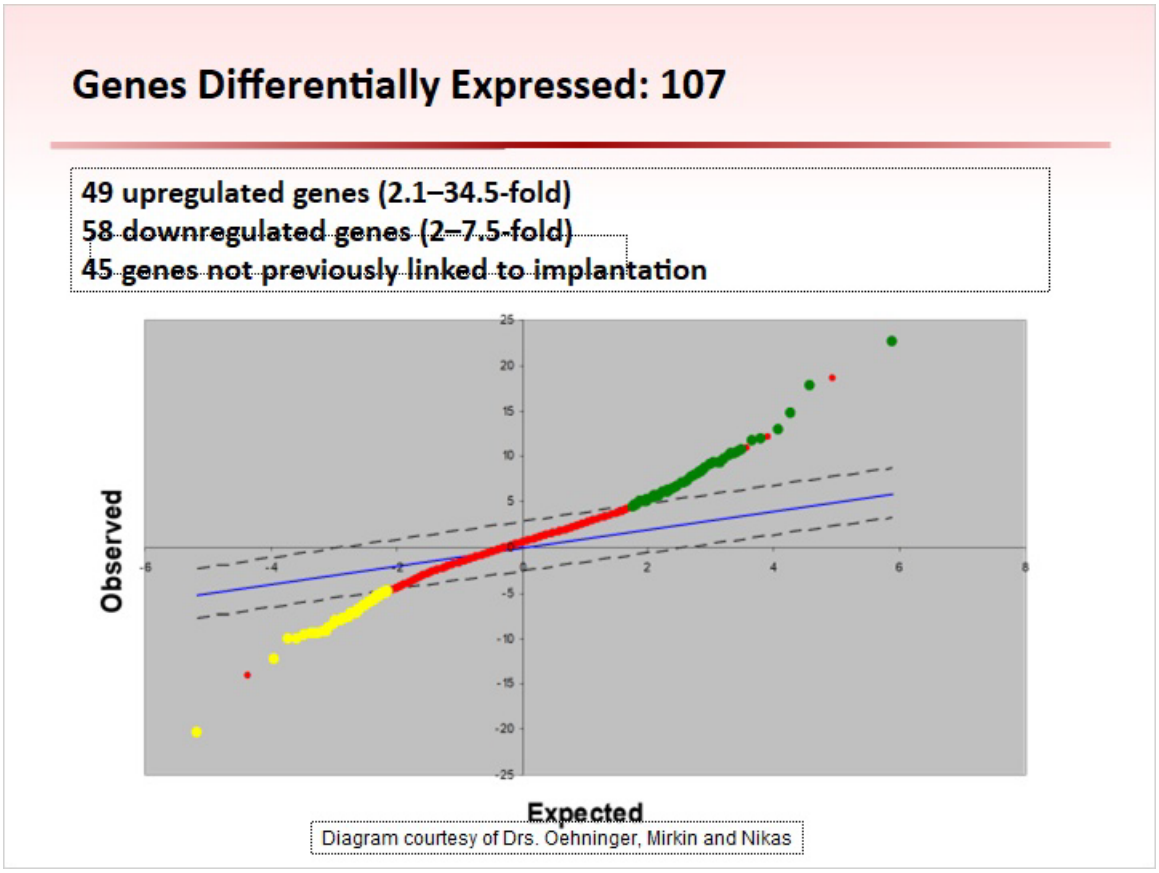
1.35 Progesterone Receptors



Notes:

Progesterone receptors by immunohistochemistry also showed advancement (Mirkin et al., 2004, 2005).

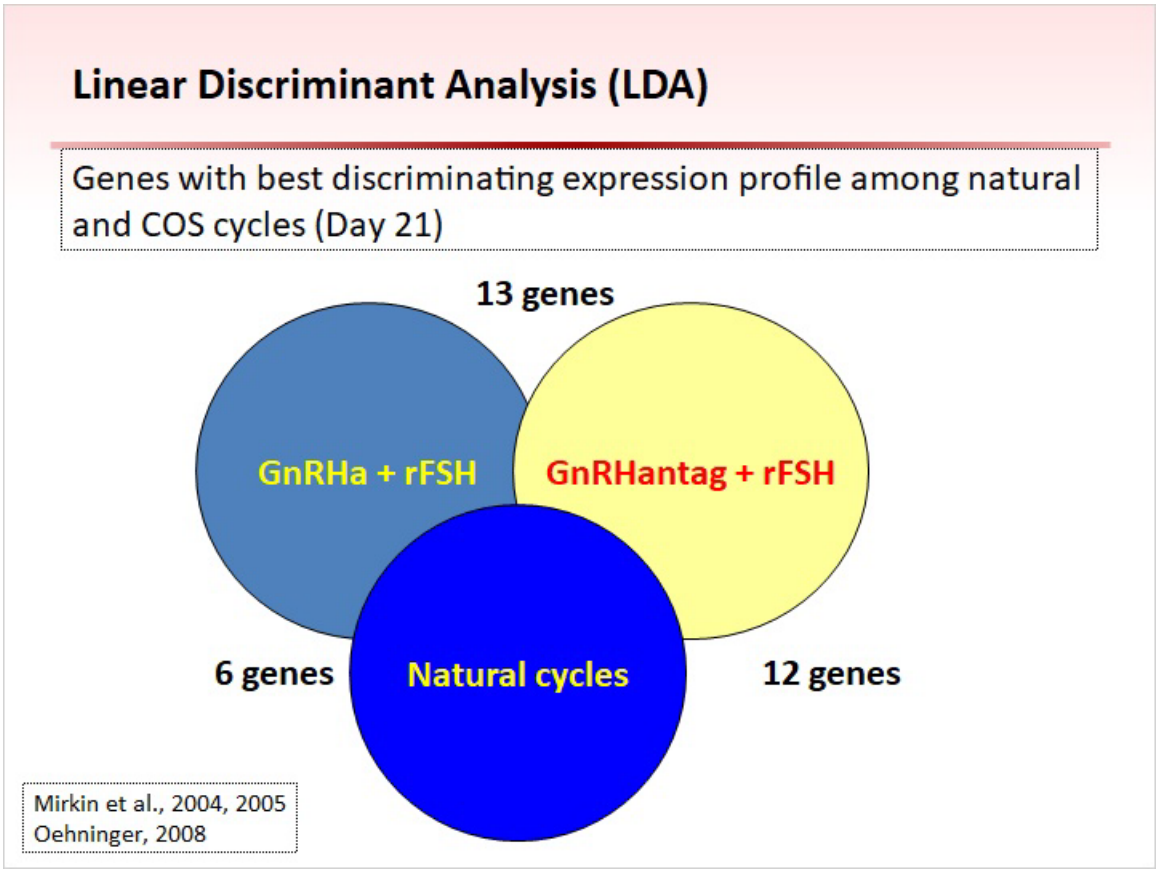
1.36 Genes Differentially Expressed: 107



Notes:

This microarray analysis demonstrated 107 genes differentially expressed between natural cycles and controlled ovarian stimulation.

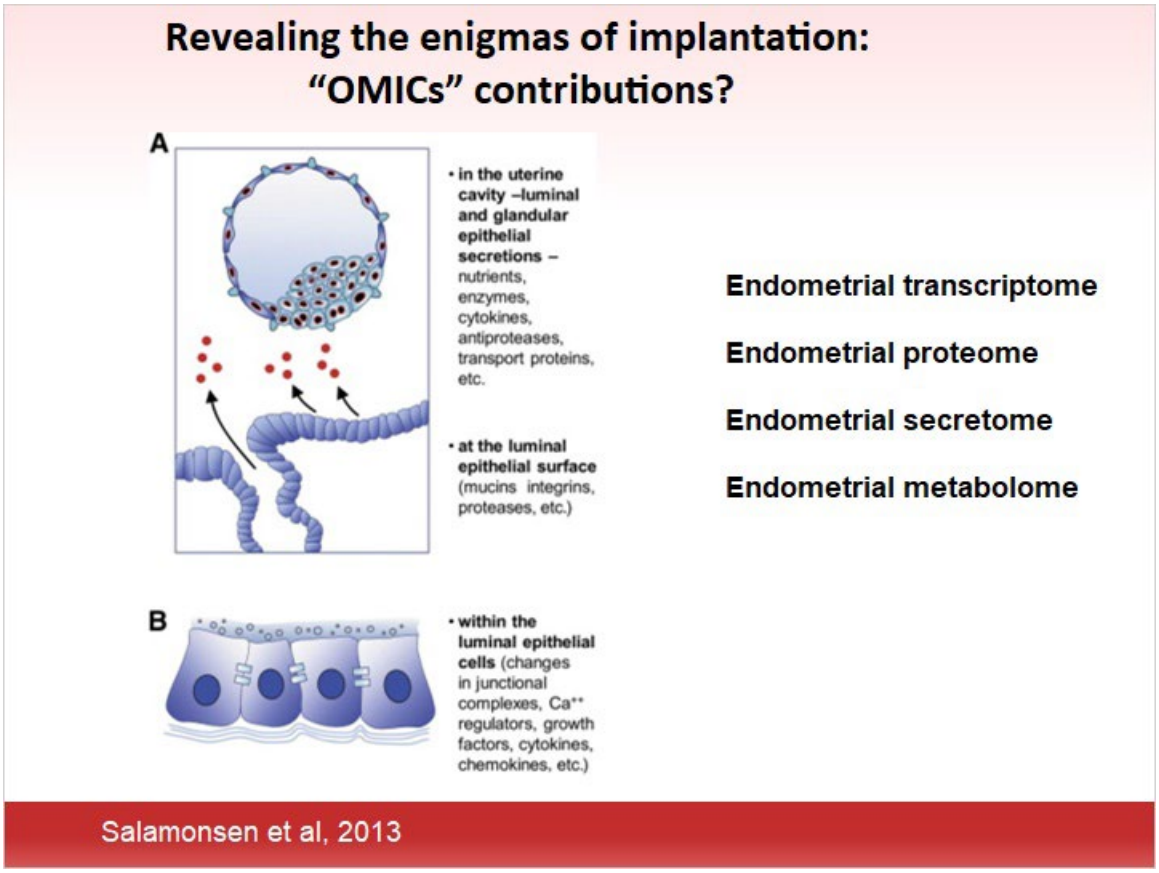
1.37 Linear Discriminant Analysis (LDA)



Notes:

A further analysis of natural cycles and gonadotropin-stimulated cycles stimulated with GnRH agonist or GnRH antagonist adjuvant therapies also demonstrated gene variations. Nevertheless, although these results confirmed that ovarian stimulation results in histopathologic changes and variations in gene expression when compared with natural cycles, the enigma is still present: What is the true impact of hyperestrogenism and of variable degrees of embryo-endometrium developmental asynchrony in the presence of high clinical embryo implantation rates in IVF? More work is needed to characterize the temporal patterns of gene expression and protein profiles. Working on whole endometrial tissue and/or isolated endometrial compartments might unveil patterns that are critical for the establishment of the window of implantation.

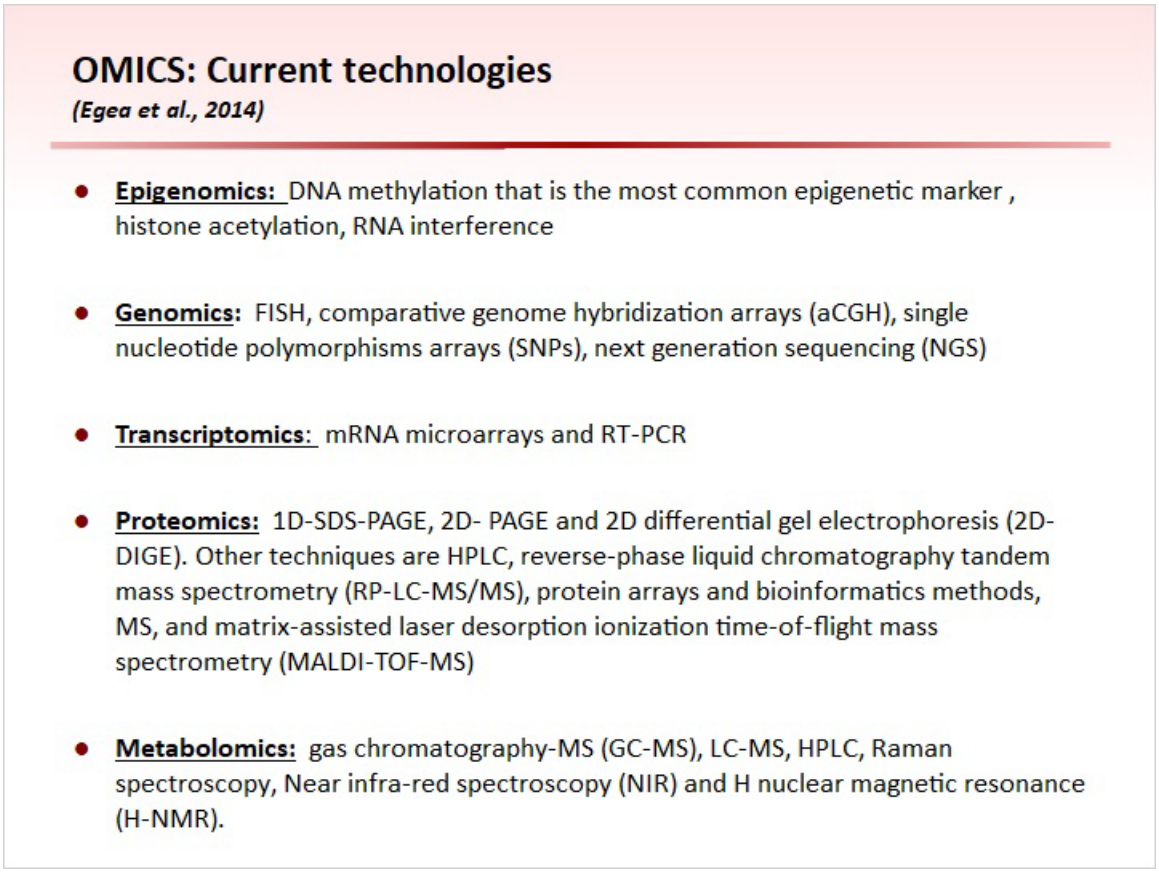
1.38 Revealing the enigmas of implantation: "OMICs" contributions?



Notes:

It is expected that results of the novel "OMICs" technologies may unveil some of the enigmas of implantation. At the level of the endometrium these techniques may include analysis of the transcriptome (mRNA molecular fingerprinting), proteome and metabolome, and also the secretome examined through the proteome of uterine fluid lavage.

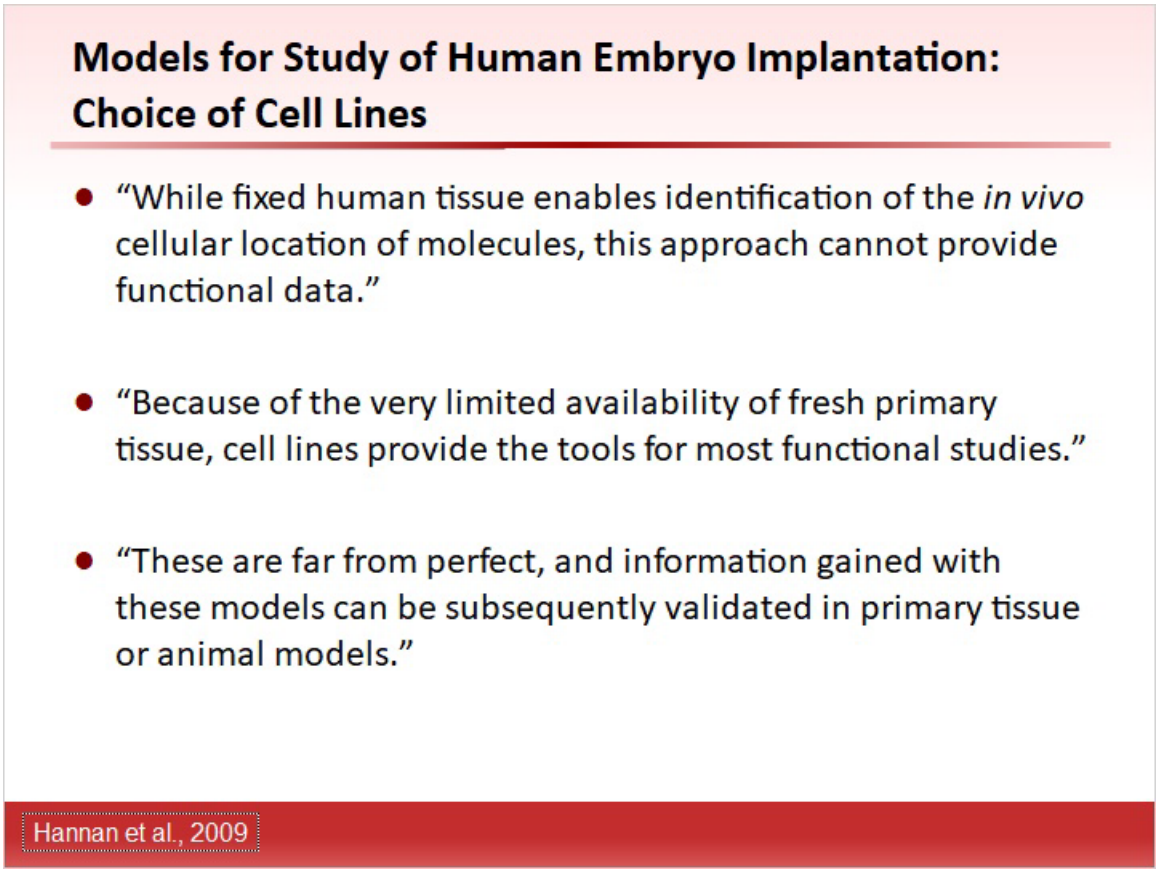
1.39 OMICS: Current technologies



Notes:

Shown here are current OMICs technologies and a description of general techniques that could also be applied to identify endometrial profiles predictive of implantation and/or the state of receptivity. Once the results from these techniques are obtained, they are frequently validated by using another technique. For instance, in transcriptomics, mRNA microarrays can be validated by RT-qPCR, in proteomics 2D-DIGE results have been validated by immunostaining, the western blot and enzyme-linked immunosorbent assay (from Rivera-Egea et al., 2014). For transcriptomics, an endometrial receptivity assay was recently developed, based on a microarray for 238 genes obtained from timed endometrial biopsies (Díaz-Gimeno et al., 2011). It has been suggested that results of the gene expression profiles in various patient populations can result in identification of “receptive” or “non-receptive” endometrial patterns, and that a clinical algorithm for embryo transfer personalization may be developed (Garrido-Gomez, 2013). These preliminary results need to be validated by further studies. The search for molecular markers for implantation through “OMICs techniques” continues. The window of implantation appears to be the most relevant time to study gene and protein expression profiles as a way to establish biomarkers of a receptive endometrium.

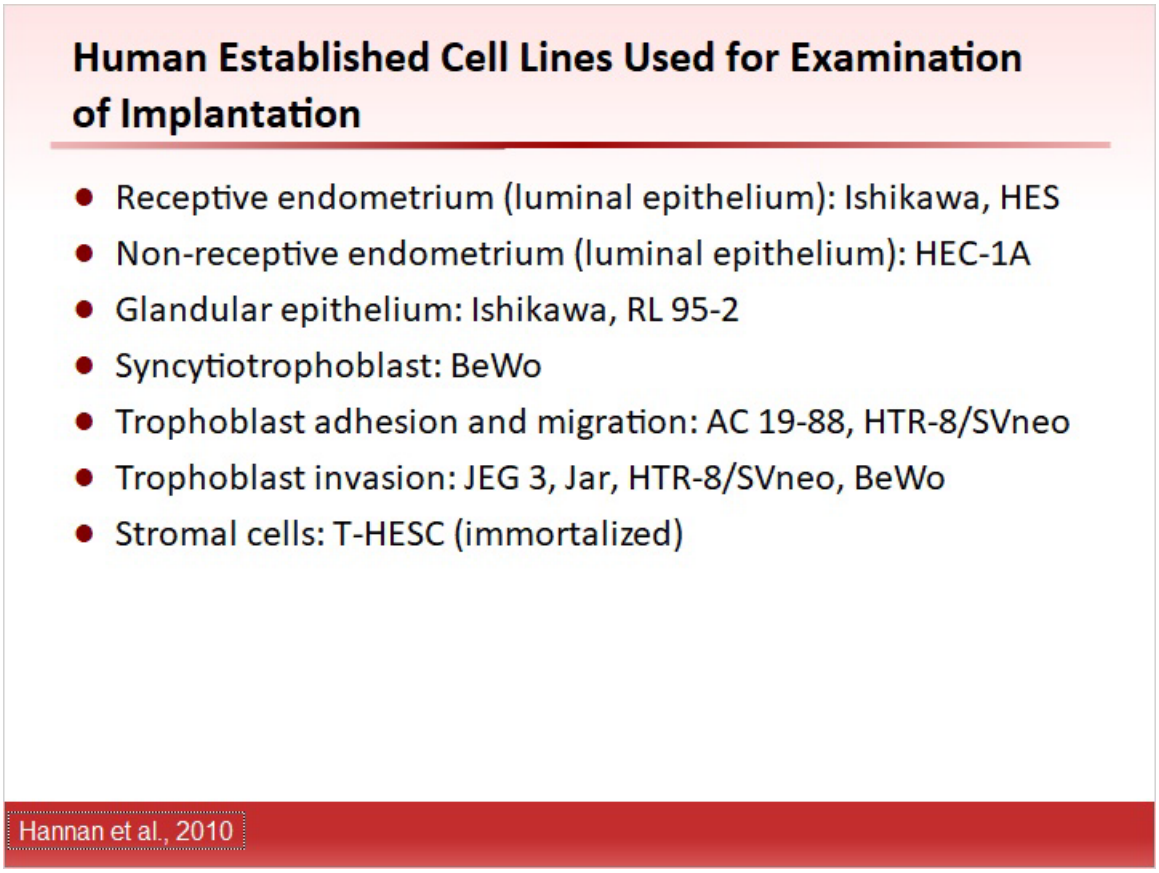
1.40 Models for Study of Human Embryo Implantation: Choice of Cell Lines



Notes:

Human implantation sites are not readily available for experimentation and animal models may or not represent the human physiology. Therefore *in vitro* studies continue to be important for elucidation of implantation mechanisms. Whole endometrial tissue, primary epithelial and stromal cells, and human established cell lines have been extensively used.

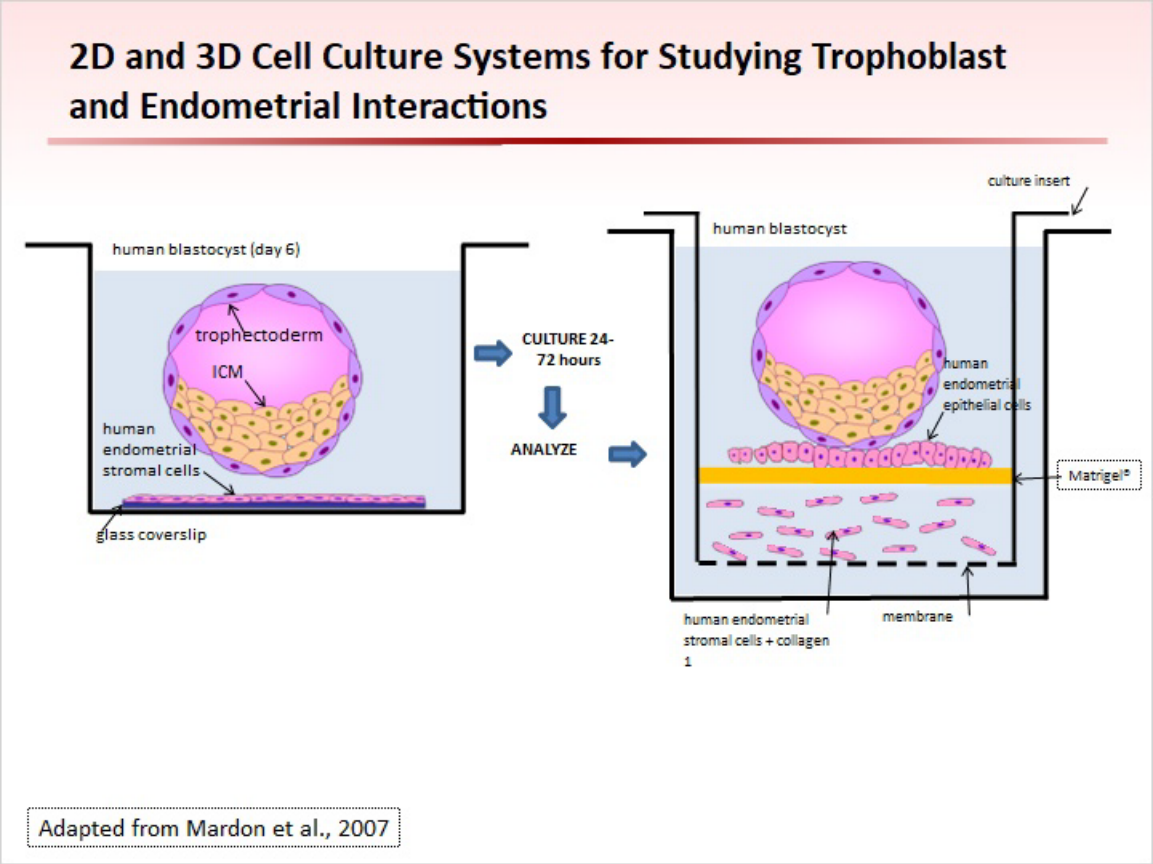
1.41 Human Established Cell Lines Used for Examination of Implantation



Notes:

Cellular interactions during implantation and potential model cell lines are used for analysis of adhesion, invasion and migration. Better understanding of these processes is hampered by the difficulty in obtaining human tissue from which primary cells can be prepared and by the very limited access worldwide to human blastocysts for experimentation. Therefore, the use of appropriate cell lines, particularly for functional studies of implantation and placentation, is imperative.

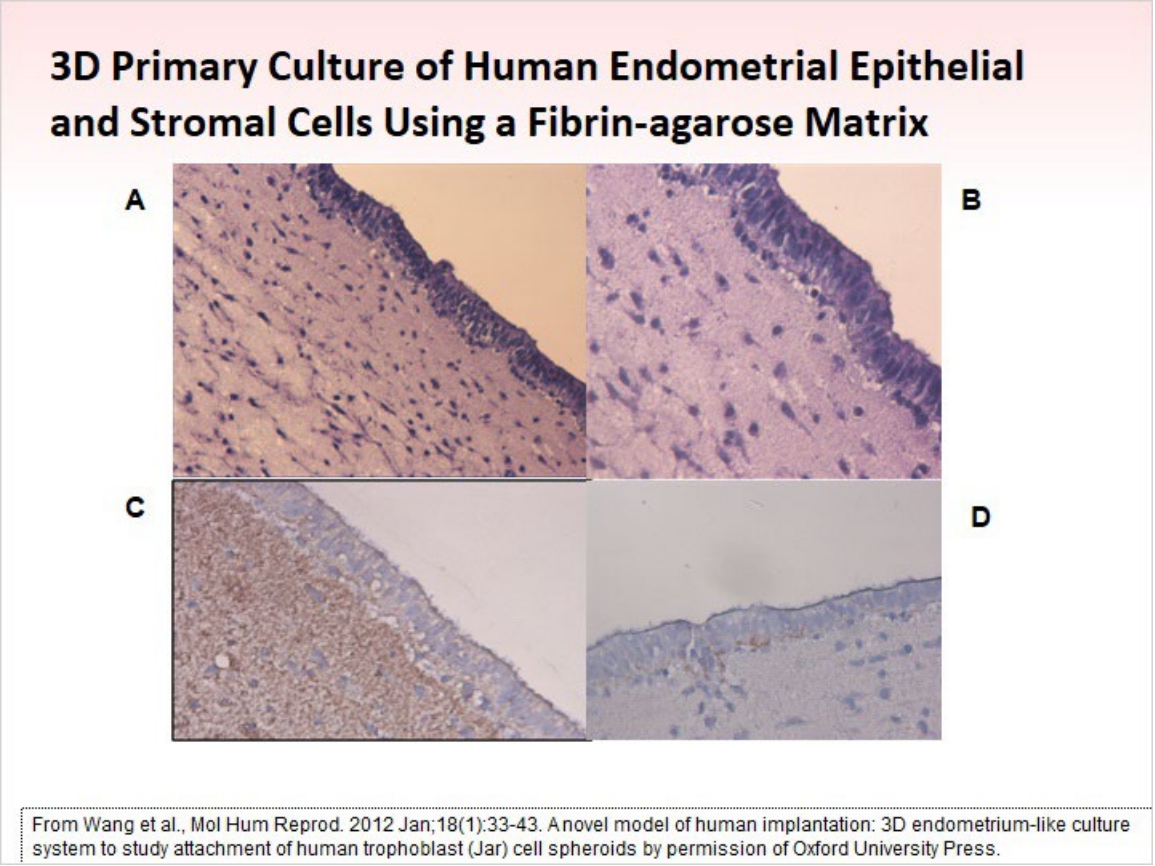
1.42 2D and 3D Cell Culture Systems for Studying Trophoblast and Endometrial Interactions



Notes:

Shown here is an *in vitro* model for stromal cell invasion during implantation of the human blastocyst. Human endometrial stromal cells were isolated from biopsies and cultured on coverslips to confluency. Human blastocysts (day 6) were then added to the stromal cell monolayers and were cocultured for 48 hours. A three-dimensional model of human endometrium was used: endometrial stromal cells are mixed with collagen type 1 layered into well inserts, followed by a thin layer of Matrigel®, with endometrial epithelial cells seeded onto the surface.

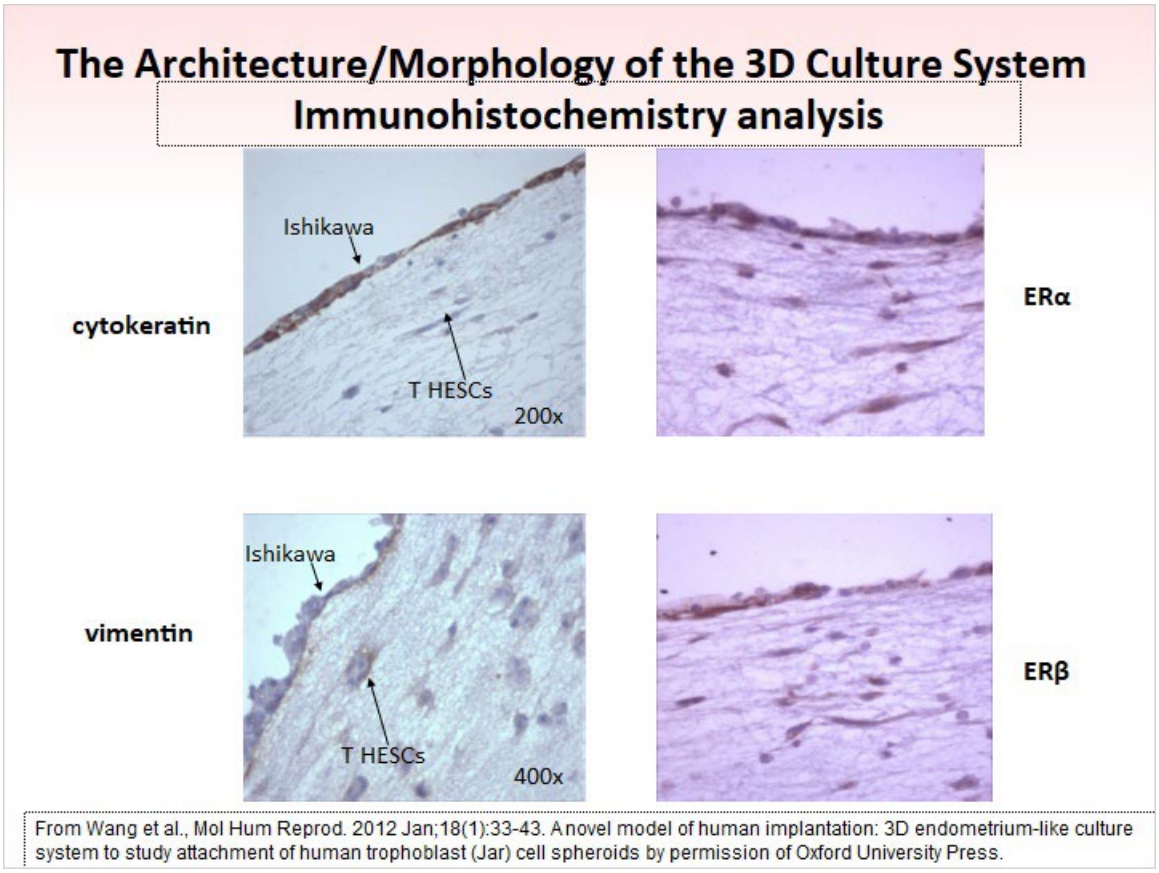
1.43 3D Primary Culture of Human Endometrial Epithelial and Stromal Cells Using a Fibrin-agarose Matrix



Notes:

A novel model of human implantation consisting of a 3D endometrium-like culture system has been developed to study attachment of human trophoblast cells (Jar spheroids). In this case primary endometrial cells were used. Formalin fixed sections (A) Hematoxylin/ eosin stained 20X (B) Hematoxylin/ eosin stained 40X. (C) Immunohistochemical analysis for vimentin 20 X (D) Immunohistochemical analysis for Cytokeratin 18 40X.

1.44 The Architecture/Morphology of the 3D Culture System

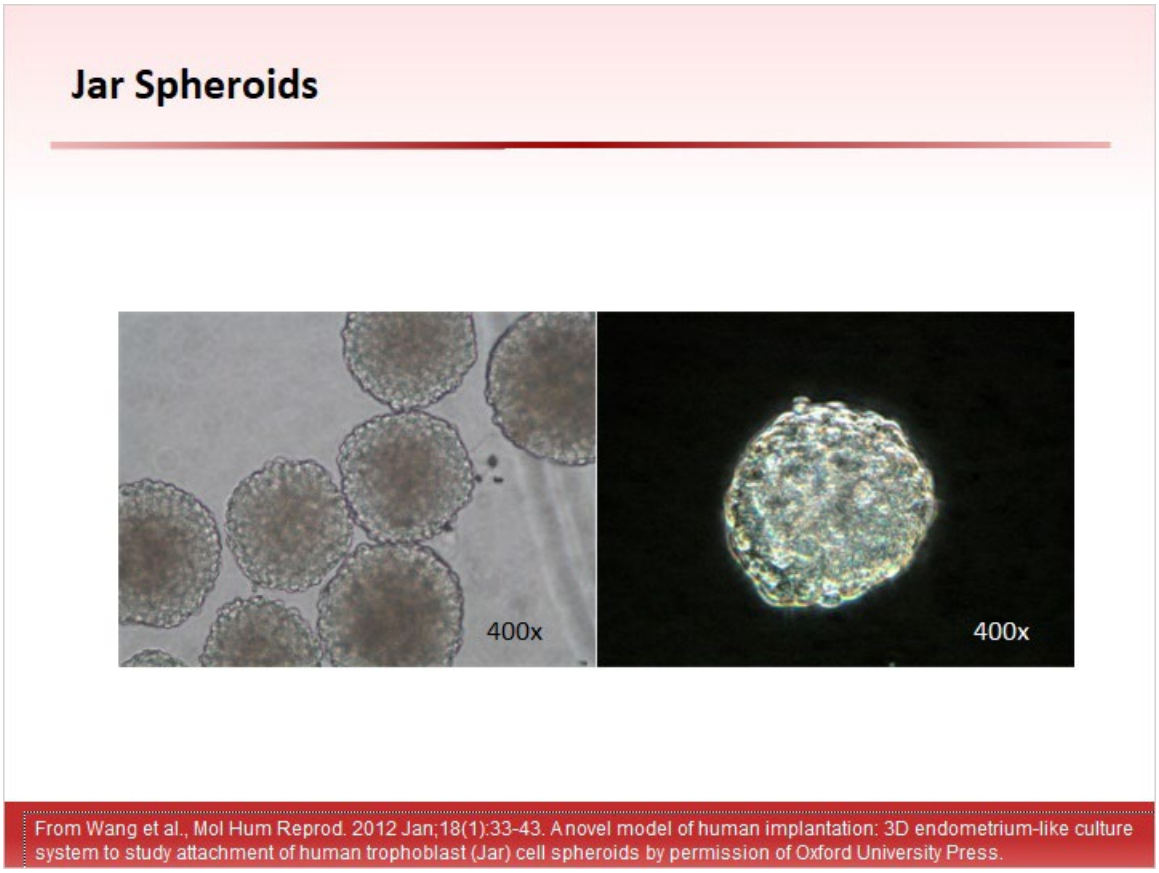


From Wang et al., Mol Hum Reprod. 2012 Jan;18(1):33-43. A novel model of human implantation: 3D endometrium-like culture system to study attachment of human trophoblast (Jar) cell spheroids by permission of Oxford University Press.

Notes:

Next, a 3D primary culture with epithelial Ishikawa cells and immortalized human stromal cells (T HESC) embedded in the fibrin-agarose matrix was constructed. Ishikawa cells were positive for the epithelial marker cytokeratin, whereas stromal cells were positive for the stromal marker vimentin. Both cell types were ER positive in the constructs (Wang et al., 2012).

1.45 Jar Spheroids



Notes:

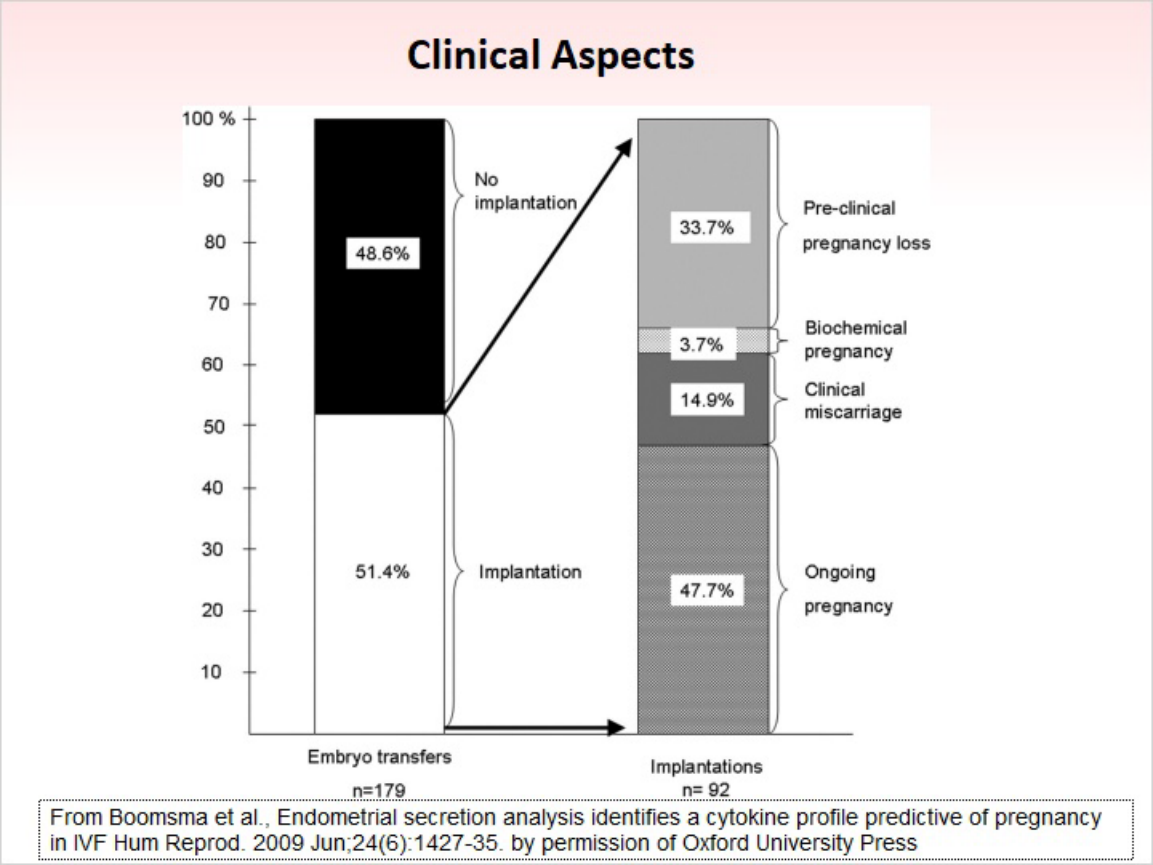
Cultured Jar cells form spheroids mimicking the trophoblast.

1.46 Attachment of Jar Spheroids to 3D Culture System

Notes:

In further experiments a 3D culture system was established with human established cell lines immersed in the fibrin-agarose matrix (Ishikawa epithelial cells and human immortalized endometrial stromal cells). Areas of attachment and initiation of invasion were observed for several trophoblast Jar spheroids.

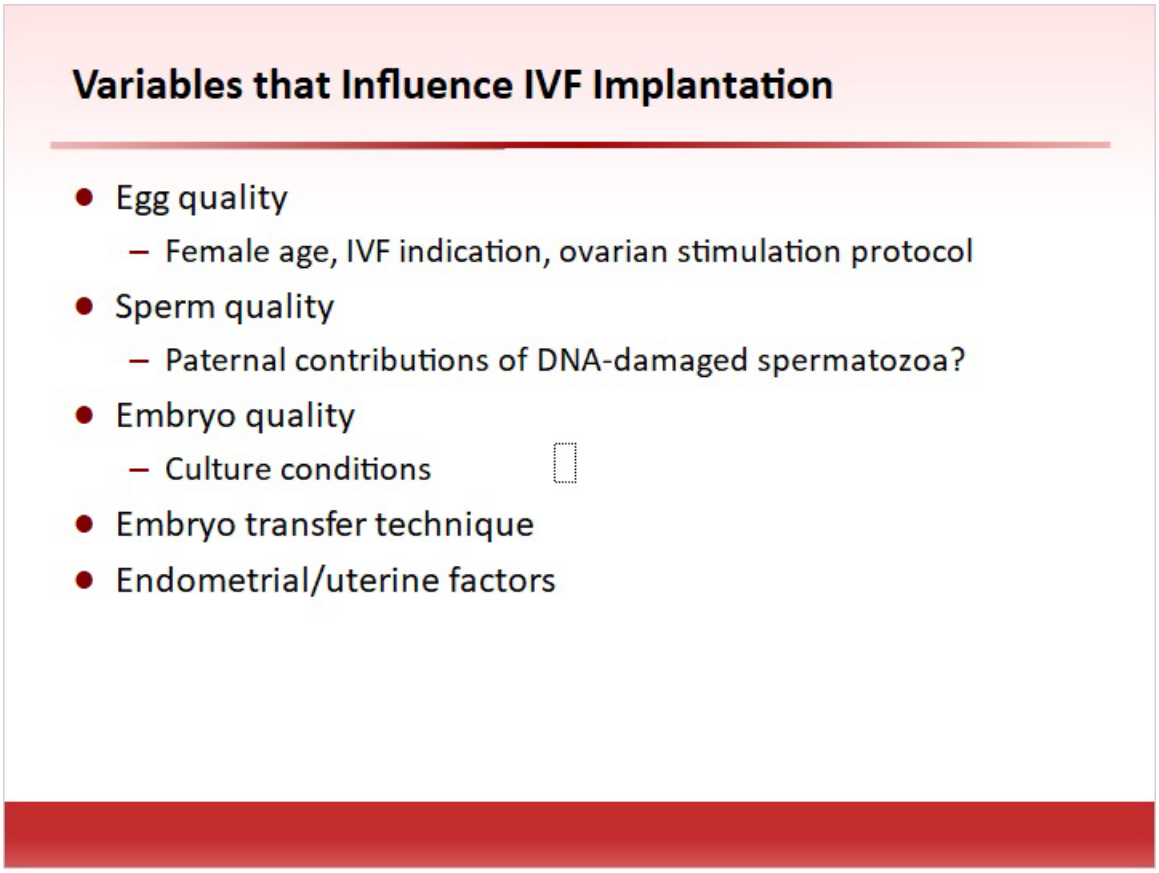
1.47 Clinical Aspects



Notes:

Estimates from natural cycles suggest implantation rates per embryo of only around 25% in human populations of normal fertility, and 75% of failed pregnancies are considered to be due to implantation failure (Norwitz et al., 2001, Koot et al., 2012). The window of receptivity is transient in humans, and implantation beyond this window results in spontaneous miscarriages (Wilcox et al., 1999), although a considerable number of losses result from embryonic abnormalities, including aneuploidy (Nagaoka et al., 2012). In IVF-ET, implantation rates can vary from 20% to 50%. Half of implantations are lost as biochemical, preclinical, or clinical miscarriages (Sharkey and Macklon, 2013). Embryonic and endometrial/uterine factors can be responsible for them.

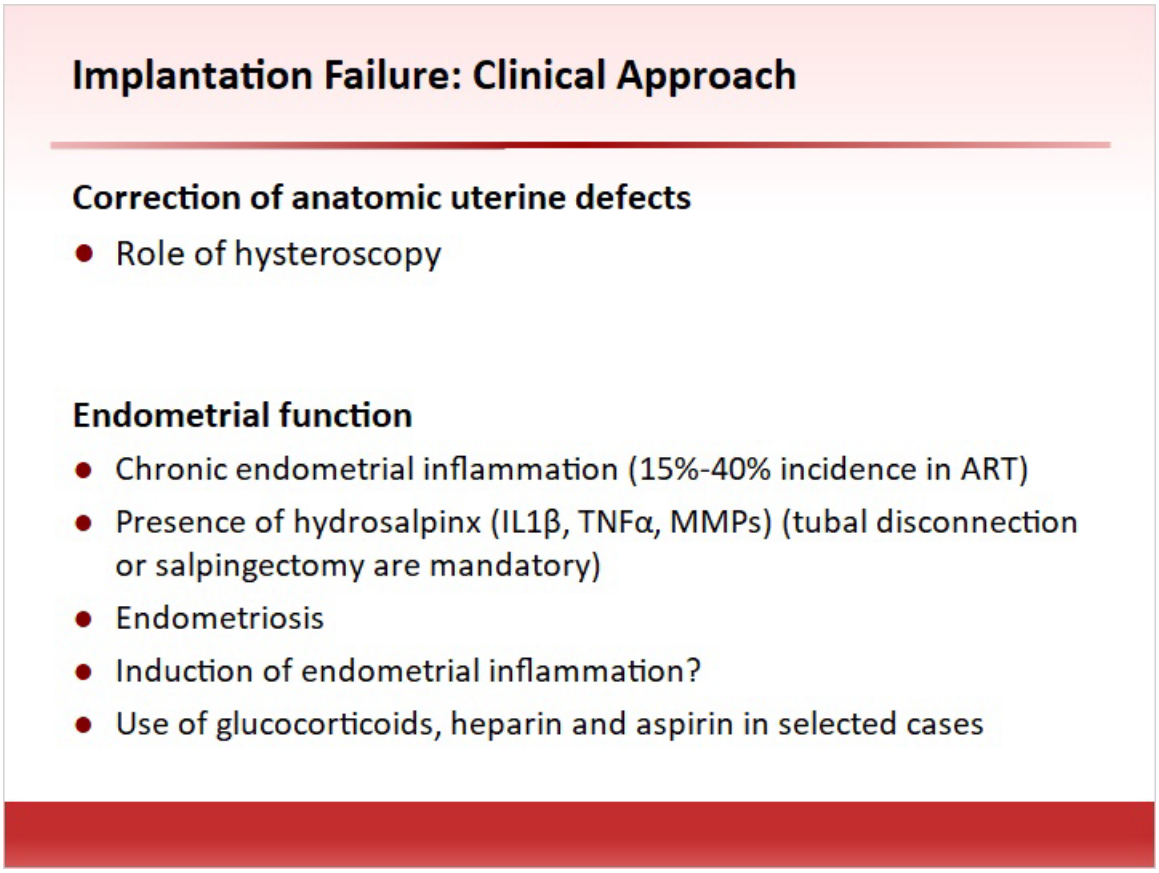
1.48 Variables that Influence IVF Implantation



Notes:

These are the main factors that influence implantation in the IVF scenario. Failed implantation can be the result of gamete (eggs and sperm) factors, embryonic factors (chromosomal anomalies and others), and inappropriate hormonally primed endometrium. The search continues for identification of key endometrial factors that may be absent or inappropriately expressed at the receptive stage. Other important factors to be considered include: uterine anatomical or structural anomalies, immune factors, thrombophilias, and thyroid disease (Simon and Laufer, 2012).

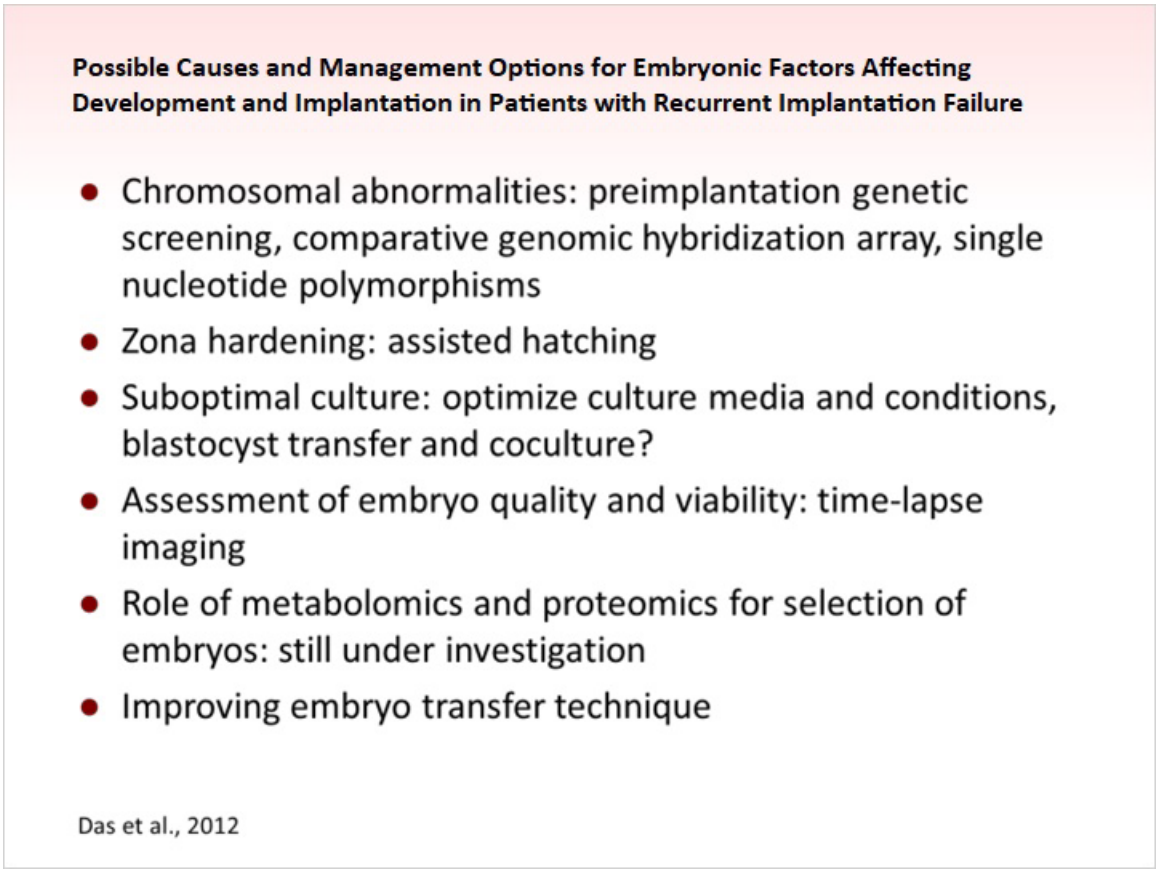
1.49 Implantation Failure: Clinical Approach



Notes:

Multiple factors may contribute to this failure, including genetic or metabolic abnormalities of the embryo. In addition, many of spontaneous early abortion cases are attributed to poor uterine receptivity. Although many fertility disorders have been overcome by a variety of assisted reproductive techniques, implantation remains the rate-limiting step for the success of IVF treatments. The clinical approach for cases with failed implantation in IVF includes correction of anatomic uterine defects and assessment and treatment of endometrial function (Barnhart et al, 2002; Simon and Laufer, 2012).

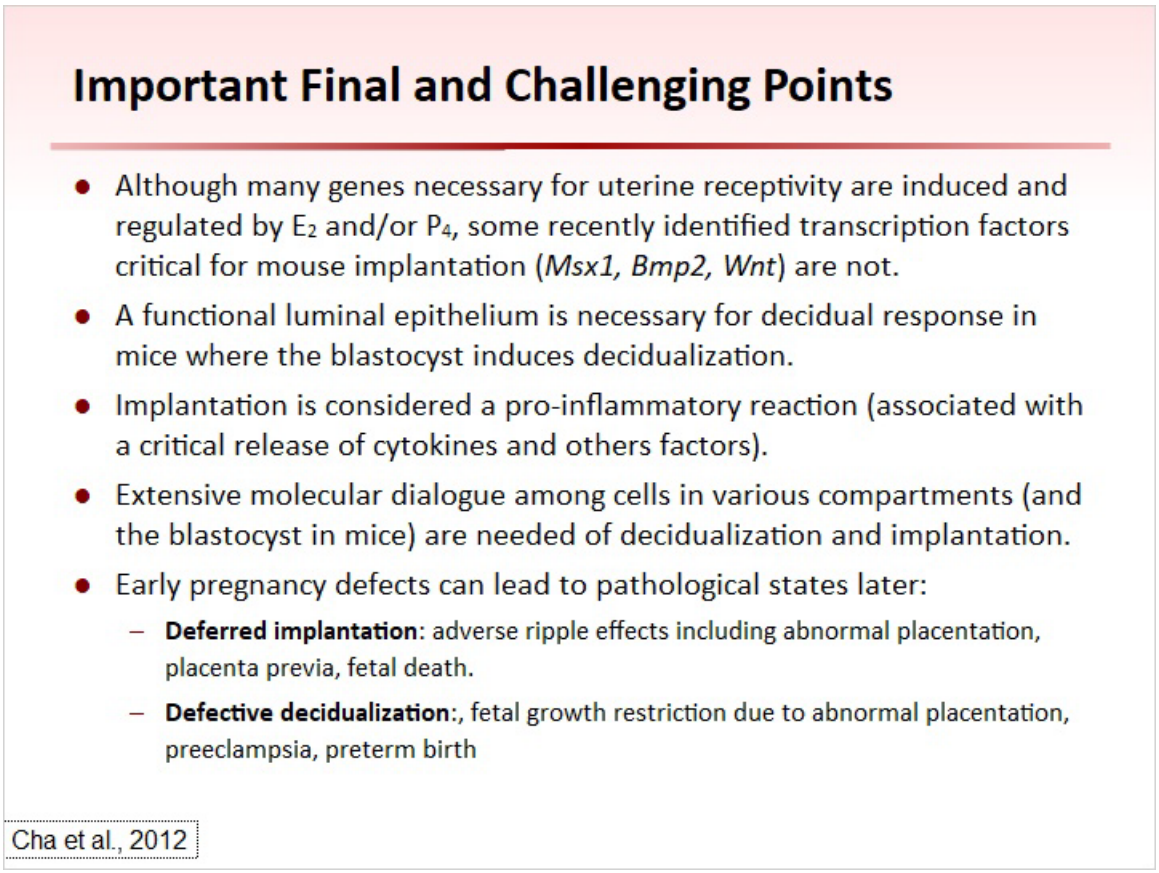
1.50 Possible Causes and Management Options for Embryonic Factors Affecting Development and Implantation in Patients with Recurrent Implantation Failure



Notes:

Chromosomal abnormalities, sperm DNA damage, zona hardening, inadequate culture conditions, and suboptimal embryo development **MAY ALL** play a significant role in the etiology of recurrent implantation failure. Comparative genomic hybridization array, analysis of single nucleotide polymorphisms, **AND NEXT GENERATION SEQUENCING** could enable a more comprehensive screening of chromosomes. Assisted hatching may help to overcome zona hardening in selected cases. Optimal culture conditions and blastocyst transfer could contribute toward improving implantation and pregnancy rates. Novel embryo assessment and selection procedures, such as time-lapse imaging and perhaps metabolomic analysis of embryo conditioned media, may help in better evaluation of embryo quality and viability and help in selecting embryos with the highest implantation potential. The safety and efficacy of emerging treatment modalities should be evaluated in prospective randomized clinical trials before being applied in routine clinical practice (from Das et al., 2012).

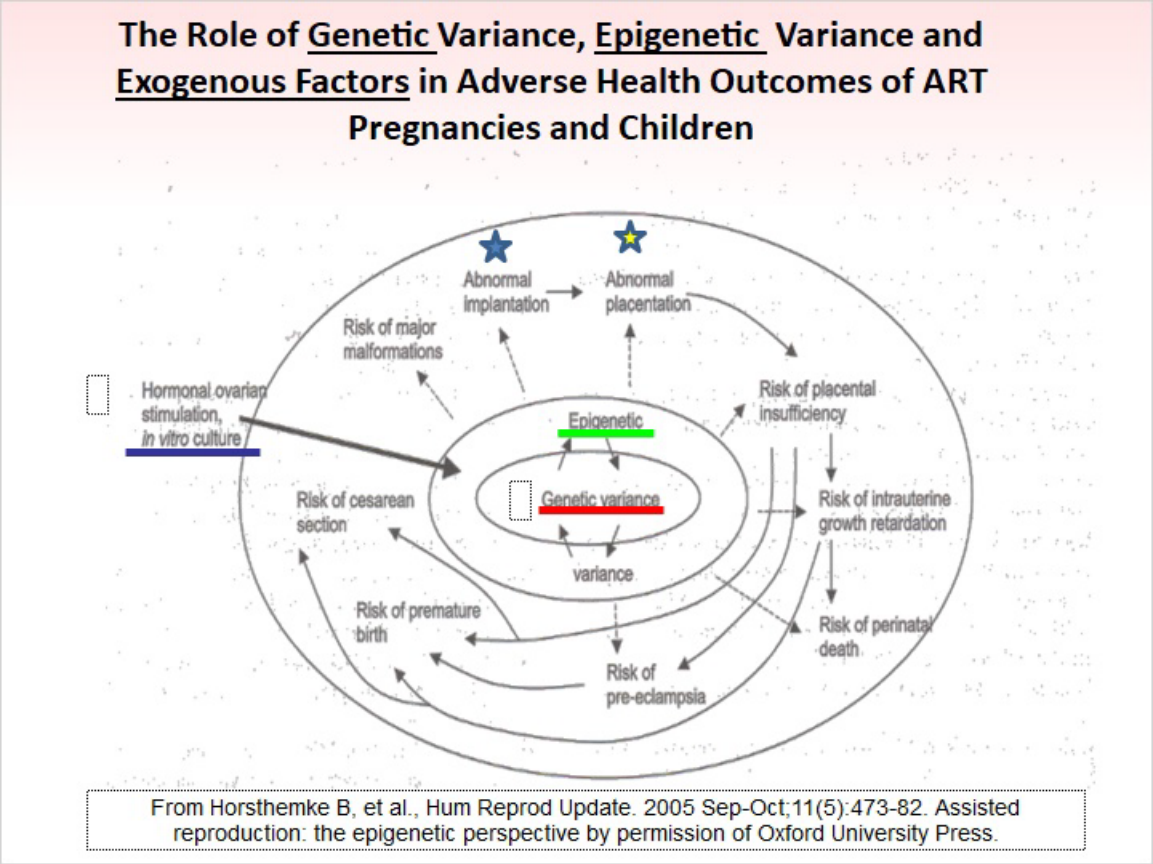
1.51 Important Final and Challenging Points



Notes:

Shown here are adverse pregnancy outcomes stemming from aberrant implantation and defective decidualization based on murine data (Cha et al., 2012).

1.52 The Role of Genetic Variance, Epigenetic Variance and Exogenous Factors in Adverse Health Outcomes of ART Pregnancies and Children



Notes:

The arrows indicate relationships between different events observed in pregnancies and children born after ART. The broken arrows indicate the possible role of genetic or epigenetic factors in increasing the risk for certain problems. The bold arrow indicates the effect of exogenous factors on epigenetic variance. The adverse health outcome in some A-R-T children may be due in part to the loss of epigenetic control during development. Although there are several studies on the influence of hormones and *in vitro* culture on methylation patterns and imprinted gene expression, next to nothing is known about possible genetic factors that predispose to the loss of epigenetic control. The loss of epigenetic control, triggered by environmental and/or genetic factors, may directly change developmental trajectories and/or expose hidden genetic variance (Horsthemke and Ludwig, 2005). The authors argue that both normal and abnormal development in children conceived by ART can be explained by epigenetic mechanisms, which control the establishment and maintenance of gene expression patterns in the placenta and fetus. Imprinted genes are of special importance in this respect. There is increasing evidence that genetic factors in infertile couples as well as environmental factors (hormones and culture media) may have adverse effects on epigenetic processes controlling implantation, placentation, organ formation and fetal growth. In addition,

loss of epigenetic control may expose hidden genetic variation.

1.53 Thank you!

Notes:

Thank you for participating in this educational activity.