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Prostaglandin E2 and prostaglandin F2 alpha as endometrial receptivity biomarkers in successful embryo implantation

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CERTIFICA:

Que el trabajo de investigación titulado: ***"Prostaglandin E2 and prostaglandin F2 alpha as endometrial receptivity biomarkers in successful embryo implantation"*** ha sido realizado íntegramente por Dña. Leslie B. Ramírez Lima bajo mi dirección. Dicha memoria está concluida y reúne todos los requisitos para su presentación y defensa como TESIS DOCTORAL ante un tribunal.

Y para que así conste a los efectos oportunos, firmo la presente certificación en Valencia a 10 de Septiembre de 2013.

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Que el trabajo de investigación titulado: ***"Prostaglandin E2 and prostaglandin F2 alpha as endometrial receptivity biomarkers in successful embryo implantation"*** ha sido realizado íntegramente por Dña. Leslie B. Ramírez Lima bajo mi dirección. Dicha memoria está concluida y reúne todos los requisitos para su presentación y defensa como TESIS DOCTORAL ante un tribunal.

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Fdo. Dr. Felipe Vilella Mitjana

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ABSTRACT

Failure in the adhesion of the human blastocyst to the endometrium has been described as an important cause of infertility. Establishing the period of the so-called window of implantation and understanding the molecular mechanisms associated with embryo implantation has clinical and scientific implications. While over the last decades histological evaluation has been used to determine the phase of the menstrual cycle of the endometrium, the poor information obtained has made the case of using new technologies to identify specific markers, understand and characterize the receptive stage. This doctoral thesis investigates the existence, function and clinical impact of two specific lipids, the prostaglandins E(2) and F(2α), which are abundant in human endometrial fluid (EF) during the window of implantation in natural, IVF, and ovum recipient cycles, which is abrogated with the insertion of an IUD. Developments in endometrial receptivity diagnosis using lipidomics demonstrate the correlation between those PGs and the receptive stage of the endometrium. The mechanisms that influence the production of these individual PGs in the endometrium were studied with a clinical approach that sheds light on the sequence of events that leads to the development of endometrial receptivity. Our results indicate that PG synthases required for the production of PGE_2 and $PGF_2α$ are located in the endometrial epithelium and EF for the regulation of PGs concentrations during the window of implantation. Most of the accumulated evidence, using an *in vitro* model of embryonic adhesion, indicates that inhibition of PGE_2 and $PGF_2α$ or the PG receptors EP2 and FP prevents embryo adhesion, which can be reversed by adding back these molecules or by using EP2 and FP agonists. Finally, our pilot study demonstrates that PGE_2 and $PGF_2α$ concentrations in EF aspirated 24 hours prior to embryo transfer showed to be predictive of a successful pregnancy outcome. In summary, our findings indicate that embryo implantation is associated with an active crosstalk of PGE_2 and $PGF_2α$ via EP2 and FP receptors, respectively, that might serve to nurse the blastocyst at the time of embryo implantation. Likewise the levels of these PGs in EF could potentially serve as non-invasive biomarkers to define the receptive phase of the endometrium and, therefore, have a significant impact in clinical traslation.

RESUMEN

El fallo en la adhesión embrionaria al endometrio es una de las principales causas de infertilidad humana. Establecer el periodo receptivo denominado ventana de implantación así como la comprensión de los mecanismos moleculares asociados a la implantación embrionaria tiene una gran implicación clínica y científica. En las últimas décadas, la evaluación histológica ha sido utilizada para determinar las etapas del ciclo menstrual en el endometrio humano, sin embargo la poca información clínica que ofrece y la limitación impuesta por la invasión de esta técnica han sido motivo para el uso de nuevas tecnologías para identificar marcadores específicos para comprender y caracterizar la fase receptiva. En esta tesis se analizan los resultados de la investigación lipidómica centrada en dos lípidos específicos, prostaglandinas E (2) y F (2α), que son abundantes en el fluido endometrial humano durante la ventana de implantación en pacientes en ciclos naturales, de fertilización in vitro y de receptoras de ovulos, que se suprime con la inserción de un DIU. Los avances en el diagnóstico de la receptividad endometrial mediante la lipidómica demuestran la correlación entre las prostaglandinas (PGs) y la fase receptiva del endometrio. Los mecanismos que influyen en la producción de estas PGs individuales en el endometrio se estudiaron con el fin de dar a conocer la secuencia de acontecimientos que conducen a una exitosa implantación del embrión y el impacto en área de la clínica reproductiva. Los resultados indican que las sintasas de las PGs tanto en el epitelio endometrial como en el fluido uterino son necesarias para la producción de PGE_2 y $PGF_2α$ para la regulación en las concentraciones de PG durante la ventana de implantación. La mayor parte de las evidencias se llevaron a cabo utilizando un modelo *in vitro* de la adhesión embrionaria, el cual indica que la inhibición de PGE_2 y $PGF_2α$ o sus receptores EP2 y FP, evitan la adhesión del embrión, que puede ser revertida por la adición de estas moléculas o mediante el uso de agonistas de EP2 y FP. Por último, las concentraciones de PGE_2 y $PGF_2α$ en líquido endometrial se aspiraron 24 horas antes de la transferencia de embriones mostrando que estos lípidos pueden ser predictores de un embarazo exitoso. En resumen, nuestros resultados indican que la implantación del embrión se asocia con una comunicación activa de la PGE_2 y la $PGF_2α$ a través de sus receptores específicos el EP2 y el FP, respectivamente, los cuales podrían servir para determinar el momento de la implantación del embrión. Del mismo modo las PGs, podrían servir potencialmente como biomarcadores no invasivos para definir la fase receptiva del endometrio con un impacto significativo en el ámbito clínico.

ACRONYMS, ABBREVIATIONS

AA	Arachidonic acid
AEA	N-Arachidonoylethanolamine
2-AG	2-arachidonoylglycerol
AKR1C3	11-ketoreductase
Ca^2	Calcium
CBR	9-ketoreductase
COX	Cyclooxigenase
cPGES	Cytosolic prostaglandin E_2 synthase
cPLA2	Cytosolic phospholipase A2
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
dNTPs	Deoxyribonucleotide triphosphate
E_2	Estradiol
EDTA	Ethylene-Diamine- Tetra- Acetic acid
EE	Endometrial epithelium
EF	Endometrial Fluid
EIA	Enzyme immunoassay
EGF	Epidermal growth factors
ESC	Endometrial stromal cells
ESI	Electrospray ionization
EGF	Epidermal growth factor
ERA	Endometrial Receptivity Array
ERS	Endometrial Receptivity Signature
FAAH	Fatty acid amide hydrolase
FSH	Follicle-stimulating hormone
FT-ICR	Fourier transform ion cyclotron resonance
GAPDH	Gliceraldehído 3-fosfato deshidrogenasa
GC	Gas chromatography
GE	Glandular epithelium
GnRH	Gonadotrophin-releasing hormone
hCG	Human chorionic gonadotropin
H-PGDS	Hematopoietic PGDS
IGFBP-1	Insulin-like growth factor binding protein 1
ICM	Inner cell mass
IL	Interleukin
LE	Luminal epithelium
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor

LPA	Lysophosphatidic acid
L-PGDS	Lipocalin-type PGD synthase
IVI	Instituto Valenciano de Infertilidad
MALDI	Matrix-assisted laser desorption/ionization
MAPEG	Membrane-Associated Proteins involved in Eicosanoid and Glutathione metabolism
M-CSF	Macrophage colony-stimulating factor
mPGES	Membrane prostaglandin E synthase
MRP	Multidrug resistance protein
MS	Mass spectrometry
MS1,2	Mass analyzer
MS-MS	Tandem mass spectrometry
NAEs	N-*a*cylethanolamines
NMR	Nuclear magnetic resonance
OEA	Oleoylethanolamide
P_4	Progesterone
PAF	Platelet-activating factor
PBS	Phosphate buffer saline
PEA	Palmitoylethanolamide
PGs	Prostaglandins
PGDS	PGD synthases
PGHs	Prostaglandin endoperoxide H synthase
PGSs	PG synthases
PGFS	Prostaglandin F(2α) synthase
PGI_2	Prostacyclin
PGIS	PGI synthase
PI3K	Phosphatidylinositol 3-kinase
PLA2	Phospholipase A2 enzyme
PPAR	Peroxisomal proliferator activated receptor
qPCR	Quantitative PCR
RT-PCR	Real Time Polymerase Chain Reaction
SEA	Stearoylethanolamide
sPLA2	Secretory phospholipase A2
TOF	Time-of-flight
TUN	Tophouteronectin
VEGF	Vascular endothelial growth factor
ZP	Zona pellucida
WOI	Window of implantation

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I.- INTRODUCTION

I.- INTRODUCTION

Embryonic implantation is one of the most important processes in human physiology. However, in spite of its importance, the complexity and the relative inefficiency of this process remain unexplained (Cha *et al.* 2013). It has been estimated that 12% of couples attempting to conceive suffer from infertility and, of the total pregnancies that are lost, 30% represent a failure in implantation (Macklon *et al.* 2002). For that, the implantation process has become an important topic of scientific investigation as the understanding of its process can potentially improve fertility in women. The onset of this process requires the communication between the endometrium and the embryo, and although both components are indispensable to complete the process, most studies have separated them in two fields, the endometrial receptivity and the embryo development.

1.Endometrium

Human endometrium, the mucous membrane that covers the uterine cavity, is a complex tissue that undergoes periodical, morphological, and functional changes. These changes are based on its proliferation, differentiation, and tissue breakdown that take place as a response to the fluctuating levels of circulating ovarian steroid hormones, estrogen and progesterone (Maruyama *et al.* 2010). These transitions are also needed in the endometrial preparation for its receptive state so that embryo implantation and its development for gestation can take place (Simon *et al.* 2009).

The endometrium consists of basal and functional layers that provide an optimum environment for the growth of the embryo and its implantation. The **basal layer** is the deepest part of the uterine lining that houses the blind ends of the tubular uterine glands. This layer is not released during any

phase of the menstrual cycle, and the functional layer develops from it (Hawkins & Matzuk 2008). The **functional layer** is composed of spongy and compact layers of the endometrium that are considered as a highly regenerative tissue undergoing monthly cycles of growth, differentiation, and shedding (Gargett *et al.* 2012). If conception does not take place, this layer is shed during menstruation and subsequently regenerated from the remaining endometrial basalis. Both the basal and functional layers are composed by a combination of epithelial, stromal and vascular compartment together with the existence of immune resident cells.

1.1 Endometrial epithelium (EE)

EE contains two types of epithelial cells, the luminal epithelium (LE) and the glandular epithelium (GE), which are regulated by ovarian steroid hormones that induce morphological and functional changes in the preparation for implantation. While the LE lines the lumen of the uterus that suffers plasma membrane and cytoskeleton changes along the menstrual cycle, the GE of the endometrial or uterine glands are responsible for secreting molecules required for the embryo implantation and development (Huang *et al.* 2012). EE is the first barrier in the interaction between embryo and endometrium in which the transformation of a non-adhesive endometrium to an adhesive phenotype occurs. It has the capacity to accept embryo adhesion at receptivity state or to refuse it during the pre-receptive or nonreceptive phase of the menstrual cycle (Nimbkar-Joshi *et al.* 2012).

1.2 Stromal compartment

This tissue is formed by an extracellular matrix and endometrial stromal cells (ESC) composed basically of fibroblasts that change along the menstrual cycle promoting a regulatory role in the epithelial development and its

differentiation (Simon *et al.* 2009). In the process of decidualization, the fibroblasts become decidualized by the presence of estrogens and progesterone 6-7 days after the appearance of progesterone generating biochemical and morphological changes that are essential to direct implantation and to the maintenance of pregnancy (Imai *et al.* 1992).

1.3 Vascular compartment

The architecture of the endometrial vascularization reaches the myometrium through the arcuate arteries. Those give rise to the radial arteries at the myometrium, leading to small branches known as basal arteries. The basal arteries are muscular, with little elastic and fibrous tissues, which supply the basal endometrial zone, and continue towards the lumen as spiral arteries. Each spiral arteriole passes towards the functional layer and supply blood to the upper endometrial zones (Knobil *et al.* 2006).

Angiogenesis is the process by which new microvessels develop from existing ones. It is induced regularly during the development of the ovarian follicle until the formation of the corpus luteum and the endometrium as part of the rapid growth and regression that occurs in these tissues during the menstrual cycle (Gargett *et al.* 2001).

2. Menstrual cycle

Menstrual ciclicity begins at puberty, and last until menopause. It occurs over 28 days as a series of physiological changes where the endometrium becomes receptive to the implanting blastocyst. If implantation does not take place, the functional layer of the endometrium is sloughed and then replaced as a preparation for the next cycle (Treloar *et al.* 1967).

The **menstrual phase** is considered the start of a new menstrual cycle, corresponding to the first 4 to 5 days. After hormonal drop-out due to the senescence of the corpus luteum the tunica media of the spiral arteries contract stopping the blood supply to the tissue, inducing necrosis. Then the functional layer of the endometrium is sloughed translated in menstrual bleeding that accounts for 35ml/day of menstrual blood (Schoenwolf *et al.* 2008).

The **proliferative phase.** In response to follicular growth, around day 5, the granulosa cells of the follicle secrete estrogens that promote the proliferation of the luminal and glandular epithelial cells associated with the thickening of the endometrial lining. The proliferation causes a daily growth of the endometrium of approximately 0.5mm up to a maximum thickness of 8-14 mm around the periovulatory moment, which undergoes remodelation until day 14, when ovulation takes place (Hawkins & Matzuk 2008).

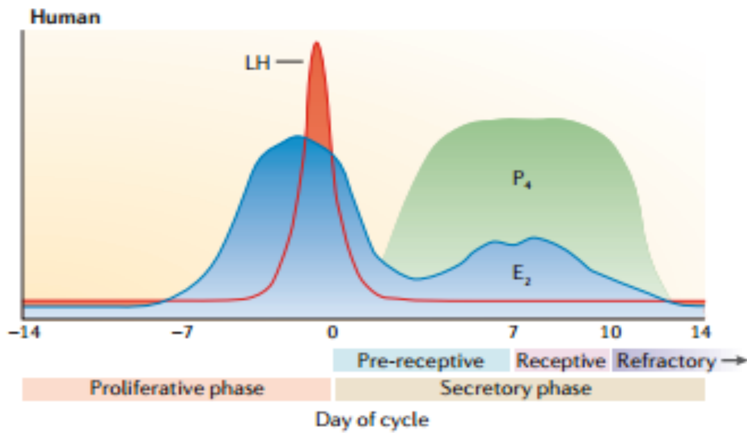
The **secretory phase** is the third phase of the menstrual cycle after ovulation and last about 13 days. In this phase, while the corpus luteum continue to secrete estrogens, the lutheal cells differentiate and secrete progesterone acting trough the endometrium in preparation to receive the embryo.

The action of hormones induces morphological changes from proliferative to secretory phase that have been identified histologically and functionally (Cakmak & Taylor 2011). In humans, while during the proliferative (follicular) phase the blastocyst is unable to implant because the endometrirum is less receptive for its survival, the secretory (lutheal) phase is divided into three stages in which one of them is receptive. The first one is the **prereceptive,** which covers the first 7 days after ovulation (day 0) when the endometrium is

unable to initiate implantation and the environment is hostile to blastocyst survival. The second one is the **receptive** state during the midsecretory phase, between days 20 to 24 of a regular 28 days menstrual cycle that is the optimal period for implantation (Norwitz *et al.* 2009). Finally, the third phase, known as **postreceptive**, is a refractory state that involves the last remaining days of the secretory phase, where the uterine environment is unfavorable to the blastocyst (Figure 1.1A).

In the case of mice, the endometrium is prereceptive on days 1-3 of pregnancy or pseudopregnancy, while on day 4 the endometrium is receptive, accompanied with localized increase in stromal vasculature permeability at the site of blastocyst attachment, and by the end of day 5 it becomes nonreceptive (refractory) to implantation (Figure 1.1B)(Wang *et al.* 2006).

A.



B.

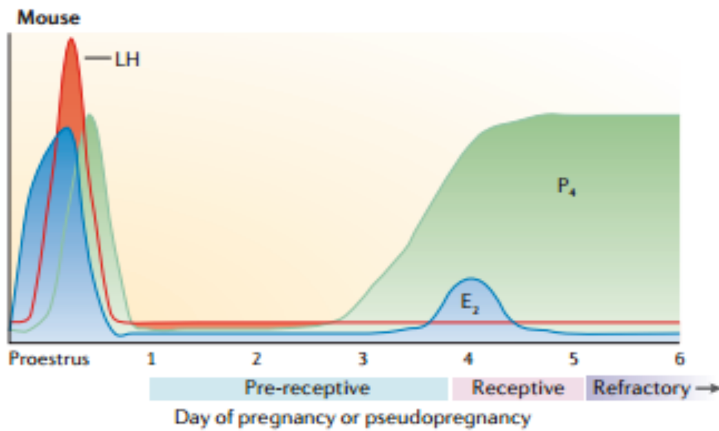


Figure1.1 Changes in human endometrium during the menstrual cycle and in mouse at estrous cycle. From (Wang & Dey 2006).

3. Implantation

Implantation is a crucial stage in human reproduction that involves a dialogue between the embryo and endometrium that leads to further post-implantatory embryonic development. The synchronization among the embryonic maturation until the blastocyst stage with the uterine differentiation to produce the receptive state is critical for successful implantation, and therefore for pregnancy outcome. Although implantation involves the interaction of many signalling molecules, the dialogue that coordinates the instructions of the embryo–endometrium are not well understood (Wang & Dey 2006).

Most studies of implantation focus primarily on rodent models, in which the first signal of this event is determined by the increase in vascular permeability. However, the timing for implantation and the anatomy of the reproductive system differ among species (He *et al.* 2010). While implantation for some species such as human, mouse, rabbit and all primates except lemurus and lorises are invasive, in other species like pig, sheep, cow, and horse it is not invasive. As shown in Fig 1.2, in mice and rats the attachment of the blastocyst to the luminal epithelium leads to a local apoptosis in the site of adhesion, allowing the penetration of trophoblastic cells through the stroma. In pigs, the trophoblasts make a specific projection through the zona pellucida and are placed between the epithelial cells, embedding the embryo in the uterine stroma. In rabbits, a group of trophoblastic cells fuse with the luminal epithelium, whereas in primates the trophoblastic syncytium is formed near the inner cell mass which is inserted between epithelial cells, penetrating in the basal lamina (Wang & Dey 2006).

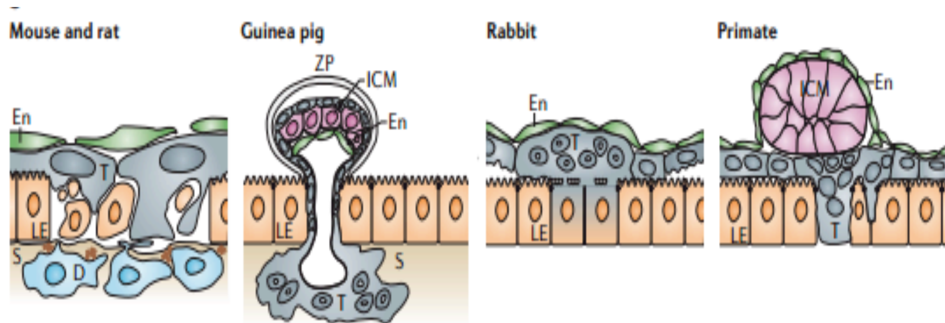


Figure 1.2 Implantation in different species. From (Wang & Dey 2006).

Similarly, studies based on microarrays showed that the genetic expression along the menstrual cycle differs in the mechanism that regulates the pre-implantational and implantational steps among species, including humans, as the global genetic expression patterns are different (He *et al.* 2010). These results support the idea that the mechanism regulating the steps through the menstrual cycle cannot be extrapolated among species (Clancy *et al.* 2009)

4. Embryo development

In humans after oocyte is fertilized in the fallopian tube, within 24 to 48 hours after ovulation, the zygote undergoes several mitotic cell divisions called cleavage, in which the zygote containing a single diploid nucleus divides to produce identical sets of daughter cells. About two to three days after fertilization, cellular division continues without an increase in size and the embryo remains enclosed to its zona pellucida (ZP). Around day 4 to 5, the embryo composed of about 16 to 32 cells leaves the oviduct and enters the uterine cavity under the influence of luteal progesterone that provides the

adequate nutrients for the development of the embryo (Lechniak, 2008). In this stage, cleavage changes the embryo morphology by undergoing the process of compaction, producing a morula: that involves maximum intercellular contact and transformation of the cell phenotype from radially symmetrical to highly polarized or epithelioid (Johnoson, 2007). The division of the morula continues to a minimum of 100 blastomeres suffering a transition to form a blastocyst (Lechniak *et al.* 2008).

The blastocyst, characterized by the reorganization of cells known as blastomeres, suffers a cellular differentiation with the appearance of a fluid in the inner cavity within the mass of cells. Blastomers that are localized in the periphery surrounding the blastocoelic cavity containing blastocoelic fluid are known as trophoblast cells, and they are responsible for the formation of the extraembryonic structures, including the placenta. Moreover, blastomers in the blastocoelic cavity, localized either in the trophoblast wall or embedded within it, compose the inner cell mass that generate the cell lineages of the embryo. In this phase, the blastocoele is stimulated by growth factors EGF and TGF-alpha, producing its expansion (Norwitz *et al.*2009; (Wu, 2012).

Within 1 to 2 days after the blastocyst enters the uterine cavity, it enzimatically hatches from the ZP, exposing the trophoblastic cells and revealing its outer covering to the endometrial epithelial cells (EECs) (Uchida *et al.* 2011). Six days after ovulation, endometrial secretions under the influence of luteal progesterone, provide the necessary nutrients for embryo development. This last event is one of the first synchronized processes that mark the endometrial relationship with the blastocyst where both components are in crosstalk to achieve implantation.

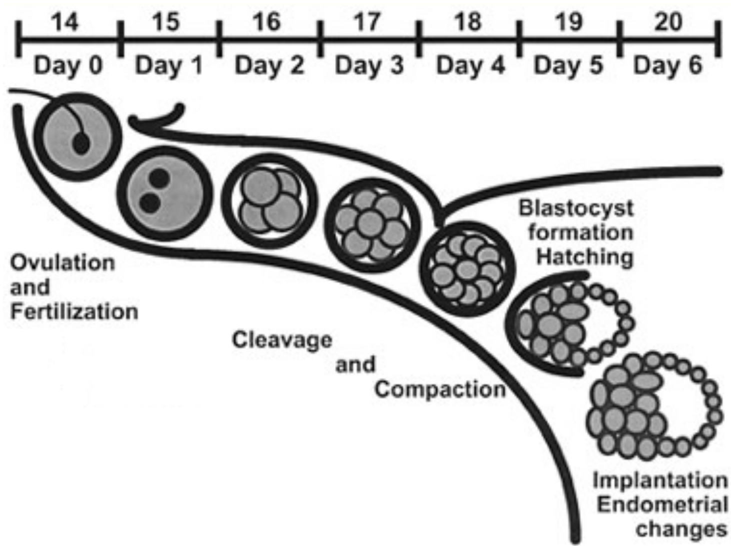


Figure 1.3. Development of the Preimplantation Embryo in Humans.

5. Phases of Embryo Implantation

After the human blastocyst enters the uterine cavity and hatches from the zona pellucida, implantation takes place, 6 or 7 days after fertilization. The process of blastocyst implantation involves complex synchronized interactions between embryonic and uterine cells that occur over a very narrow period of time, in a sequential manner, and that has been classified into three phases: apposition, adhesion and invasion. Each phase is connected to guarantee the establishment of pregnancy.

5.1 Apposition

The first phase takes place around day 5 and 6 post-fertilization, and is characterized by an unstable adhesion, where a blastocyst of approximately 300-400um is oriented to an area of the luminal epithelium and rotates so that the trophectoderm overlying the inner cell mass apposes to the endometrial surface (Wu 2012). During this phase, soluble mediators such as cytokines and chemokines are part of the communication between the blastocyst and endometrium. Those molecules act in a bidirectional manner to guide the blastocyst onto endometrial cells. Also hormones like progesterone, combined with estradiol, promote an up-regulation in the expression of MUC1 at the receptive endometrium that acts as an antiadhesive molecule, creating a barrier to the embryo attachment that must be locally removed at the time of the blastocyst adhesion. (Meseguer *et al.*1998). The blastocyst then comes into apposition with the endometrium where the contact of the microvilli on the apical surface of trophoblasts interdigitate with the pinopodes of the uterine epithelium, initiating the maternal component of the placenta. Contact with the uterine endometrium induces cell differentiation of the trophoblast at the embryonic pole.

5.2 Adhesion

Subsequently to apposition, the blastocyst acquires a stable adhesion onto the EEC layer by physical contact with the endometrium at the endometrial basal lamina and the stromal extracellular matrix (ECM), exposing its embryonic pole to the epithelium (Dey *et al.* 2004). This phase is mediated by steroid and embryonic influence of the adhesion molecules in the cellular surface of the luminal epithelium. Molecules responsible for the adhesion cell-cell and cell-ECM are essential for the adhesion of the blastocyst to the maternal endometrium, followed by the influence of molecules such as integrins, cadherins and selectins (Simon *et al.* 2009). The endometrium at this stage is integrin-dependent, which allows the blastocyst to firmly attach to the uterine wall where trophoblasts transmigrate across the luminal epithelium (Wu, 2012).

5.3 Invasion

Finally, implantation is completed when the embryo penetrates through the anatomic barriers that allowed the creation of the hemochorial placenta. The first barrier is endometrial epithelial cells where trophoblasts are attached. Below this layer a basement membrane, composed mainly of type IV collagen, functions as an anchor for surface epithelium and as a separating layer which sheaths blood vessels, muscle cells, and nervous tissue. Beyond the basement membrane lies the interstitial stroma, which contains vessels and lymphatic channels. Since the blastocyst is not able to go through the epithelial layer because of its size, trophoblasts that are attached induce an apoptotic reaction in endometrial epithelial cells by paracrine activity (Wu, 2012). The apoptotic reaction is mediated by the ligand Fas-Fas that allows the blastocyst to break through the epithelial barrier, allowing it to embed into

the stroma (Galan *et al.* 2000). This process is mediated by trophoblastic cells differentiation, which produces highly secretory synciotrophoblasts and anchoring cytotrophoblasts. While the first ones are formed by the fusion of trophoblasts forming a syncytium without cell membranes, the cytotrophoblasts are cells of the trophoblast that line the wall of the blastocyst and retain their cell membranes. These cells are able to break down the extracellular matrix between the endometrial cells with proteolitic enzymes such as metaloproteases and other proteases like serinprotease colagenasas (Simon *et al.* 2009). Cytotrophoblasts invade in a controlled manner the stroma and the myometrium as well as the uterine vasculature that establishes the uteroplacental circulation, which places the placental trophoblasts in direct contact with maternal blood. After 10 days of fertilization, the blastocyst is completely embedded in stromal tissue of the endometrium with the epithelium regrown over it to cover the site of implantation (Wu 2012). Moreover, the expression of molecules such as integrins, that are hormonally regulated with progesterone, are related to invasion. While integrins change the invasive phenotype of the trophoblast, cytokines are associated with invasive proteins, and interleukines IL-1, IL-11, IL-15, as well as other chemokines attract different groups of lymphocyte cells related to invasion. Galectins are also involved in adhesion cell-cell, migration, quimiotaxis, and inflammation (Simon *et al.* 2009).

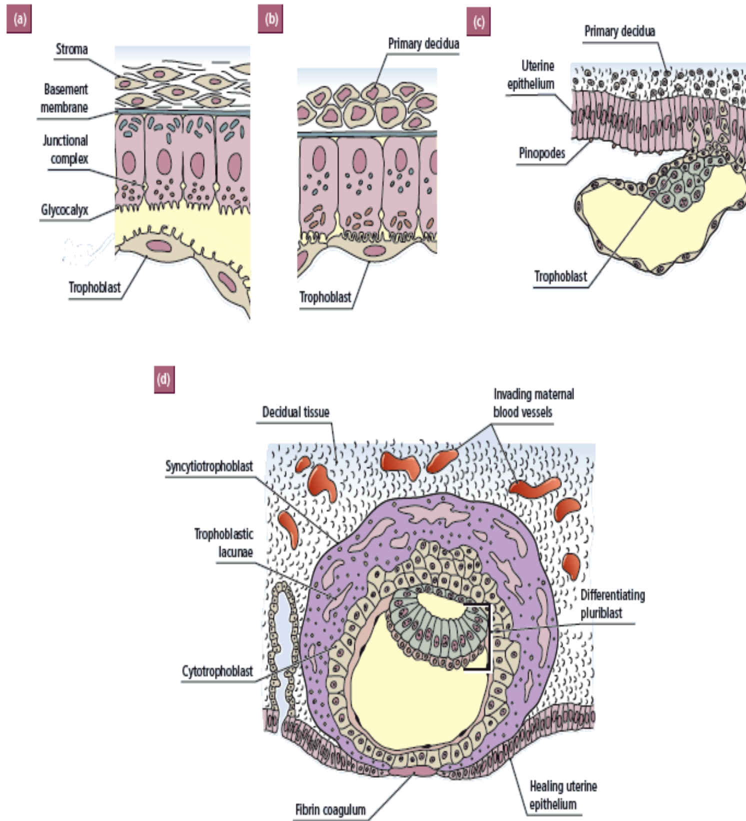


Fig. 1.4. Phases of embryo–emdometrium implantation relations in humans. From Johnson, 2007.

6. Endometrial Receptivity

Endometrial receptivity is one of the most important stages of reproductive process, in which a temporally unique sequence of factors, as molecular and physiological changes, makes the endometrium adopt a receptive phenotype where the blastocyst can intimately be associated to maternal endometrial surface (Achache *et al.* 2006).

This period of receptivity results from a programmed sequence of ovarian actions by estrogen and progesterone on the endometrium that induces endometrial cells to undergo cyclic developmental changes. In all mammals it has been determined that the hormone that directs uterine receptivity is P_4 which is essential for implantation and pregnancy maintenance, whereas the requirement for ovarian estrogen is species-specific. While in mice and rats ovarian P_4 and estrogen are essential to implantation, in humans it remains unknown whether ovarian or embryonic estrogen is necessary for implantation (Wang *et al.* 2006).

Phenotypic changes that the endometrium suffers from the pre-receptive to the receptive phase of the menstrual cycle involve the proliferation and differentiation of specific endometrial cell types in the luminal and glandular epithelium. The endometrium becomes more vascularized and edematous as the endometrial glands exhibit higher secretory activity with the appearance of protrusions known as pinopodes that develop in the luminal surface of the epithelium. Moreover, the basal membrane becomes more sinuous, and therefore the frequency of desmosomes decreases and the microvilli disappear. During this period, it has also been described a participation of molecules that are up-regulated like several endometrial growth factors, cytokines, and adhesion molecules (Aghajanova *et al.* 2008).

Although these changes are useful predictors for the outcomes of pregnancy, molecular mechanisms underlying them are largely unknown. (Norwitz *et al.* 2009).

7. Window of implantation (WOI)

The timing for successful implantation involves a short period when the uterus is receptive. Using assisted reproduction techniques (ART) in humans, it has been determined that the endometrium is remodeled to acquire a receptive state between days 19 to 21 (LH+7 to LH+9) of a regular 28 day menstrual cycle, the so called “window of implantation” (WOI) (Norwitz *et al.* 2009).

8. Markers of Endometrial Receptivity

Biological markers of endometrial receptivity are useful for the identification of a receptive endometrium. They must be present in the endometrium during the WOI, reflecting a change after this period. There are morphological and molecular markers used as predictors for the endometrial receptivity. While morphological markers focus on changes in the cells and structures of the endometrium, molecular markers are part of a pattern that aims distinguishing the stages of the endometrium.

8.1 Morphological markers

Histological morphology dating of the endometrium has been used for decades. As early as the 1950s the Noyes criteria was considered as the gold technique to determine the morphology of a normal and abnormal human endometrium, as well as to date the endometrium throughout the menstrual cycle (Lessey *et al.* 2000).

The Noyes criteria consist of eight histological characteristics of the endometrium: degree of glandular mitosis, stromal edema, pseudodecidual reaction, stromal mitosis, pseudostratification of nuclei, basal vacuoles, secretion, and leukocyte infiltration (Noyes *et al.* 1950,1975). According to these features, the phase of the endometrium can be determined: proliferative or secretory (pre-receptive, receptive, post-receptive). Using the traditional dating criteria of Noyes, Murray and col. 2004 demonstrated that histological dating of the endometrium is not a valid clinical diagnostic tool. They observed an abnormal delay in the endometrial maturation, as well as variations in the histological features of the secretory endometrium between cycles in fertile and infertile women. Moreover, they verified that the effectiveness of Noyes criteria using endometrial histological dating is subjective, with high variability between intra and interobserver, especially among infertile women during the window of implantation. Other studies support this conclusion, and demonstrate that histological dating of the endometrium should not be used in the routine evaluation of infertility. The technique adds little significant information that is not well elucidated and therefore is losing power in clinical evaluation. Nowadays, new and updated methods for the evaluation of the endometrium are replacing this technique (Lessey *et al.* 2000).

Pinopodes

The use of scanning electron microscopy has revealed the emergence of protrusions on the apical surface known as pinopodes or uterodomes, where their appearance depends on progesterone condition that coincides with the development of uterine receptivity for embryo implantation (Murphy *et al.* 2000). These epithelial protrusions might control the concentration of EFs absorbing uterine fluids, reducing uterine volume of the cavity and so

bringing into close apposition the opposing epithelia, helping attachment of the embryo (Wafaa *et al.* 2005). Pinopodes have been mentioned as a morphological parameter to determine the timing of the window of implantation on the uterine luminal epithelium. Although it has been proposed as a useful marker to determine the receptivity state of the endometrium, some limitations preclude its use as a marker (Norwitz *et al.* 2009). The necessity to use scanning electron microscopy in daily clinical work to detect the pinopodes makes it an expensive technique. Moreover, different studies compared that the appearance and duration of pinopodes during the menstrual cycle, and their results have not been reproducible. While in some investigations the duration of pinopodes persist for less than 48h during the mid-luteal phase of the menstrual cycle, in others studies pinopodes appear after ovulation and persist to the end of the luteal phase (Quinn *et al.* 2009).

Transvaginal ultrasonography

This technique is an alternative method to determine the time in which the endometrium is receptive especially because of its non-invasive property. Using this technique it has been suggested that endometrial thickness and pattern have diagnostic value to detect receptive endometrium (Gonen *et al.* 1990). However, although ultrasound is informative can not be considered as diagnostic (Aghajanova *et al.* 2008).

8.2 Molecular markers

The era of molecular and cellular biology and the development of new analytical techniques have aided the quest for more consistent predictors for human endometrial receptivity. Upon induction with steroid hormones, a large number of structural and molecular mediators have been identified as

potential markers of endometrial receptivity (Aghajanova *et al.* 2002; Lessey *et al.* 2003).

Molecular techniques together with specific molecules have been investigated to detect uterine receptivity. Biomarkers like cytokines, growth factors, homeobox transcription factors, lipid mediators, and morphogens have been described to function as autocrine, paracrine and juxtacrine factors. These biomarkers have been determined to participate in either a positive or a negative way in the process of implantation and the localization of each one determines its possible contribution for implantation (Wang *et al.* 2006). These findings on the surface of the luminal epithelium represent factors in the preembryo-endometrium, while the ones that have been described at the extracellular matrix of the endometrial stroma represent factors at the time of trophoblast invasion (Acosta *et al.* 2000).

Cell Adhesion Molecules

Integrins are part of adhesion molecules on the plasma membrane with two glycoprotein subunits: α and β. These cell surface receptors are present in the trophoblast and the epithelium, which play important roles during implantation (Wu, 2012). They have been suggested as the best of the immunohistochemical markers of endometrial receptivity during implantation window, because their pattern coincides during secretory phase (Lessey *et al.* 2000). While trophoblast integrins are involved in cell-cell and cell-matrix interactions in trophoblast attachment, migration, differentiation, and apoptosis, the integrins on endometrial surface mark the boundaries of the WOI. The expression of $\alpha_v\beta3$ in the luminal epithelium on day 19 to 20 gives an evidence of the opening of the window of implantation while the integrin α4β1 only appears on day 14, in the glandular epithelium and disappears on

day 24, closing the window of implantation (Acosta *et al.* 2000). Although these changes are useful predictors for pregnancy outcomes, their expression present a high variability in different cycles with a low reproducibility for the mechanisms underlying them are largely unknown (Norwitz *et al.* 2009).

Mucins (MUCs) are structural proteins, which are known to form mucus layers produced by epithelial cells of many tissues, including the endometrium. (Dekker *et al.* 2002). MUC1 is a cell-surface glycoprotein on the uterus that is abundant in the human endometrium, and plays a crucial role in embryo implantation, as it is thought to provide a barrier to trophoblast invasiveness by controlling the accessibility of integrin receptors to their ligands. It has been suggested that alterations in MUC1 expression levels of several epithelial cell surface proteins may affect the receptivity of the endometrium (Casado-Vela *et al.* 2009). Because MUC1 has anti-adhesive properties and in humans increases from proliferative to secretory phase of the endometrium and then decreases in the late secretory phase, it has been postulated that MUC1 may thereby hinder blastocyst attachment to the uterine epithelium until 3 days after entrance to the uterus.

Cytokines

Leukemia inhibitory factor (LIF), secreted from the endometrium, is considered as an important factor in embryo implantation. Among the cytokines, LIF is an interleukin (IL) 6-type that belongs to the family of epidermal growth factores (EGF), and that has been proved via LIF-null female mice to be essential in blastocyst implantation (Menhorst *et al.* 2011; Stewart *et al.* 1992). In humans, the production of LIF by uterine epithelium is maximal between days 19 and 25 of a normal menstrual cycle, around the

time of implantation (Menhorst *et al.* 2011). On the other hand, the oncofetal fibronectin trophouteronectin (TUN) mRNA, which is responsible for adhesion to endometrial integrins, increases its expression by LIF, suggesting that LIF is a regulator in human embryo implantation by modulating trophoblast differentiation and promoting luminal epithelial receptivity to attachment and the subsequent stromal decidualization (Acosta *et al.* 2000). In humans LIF levels in serum do not reveal fertility status, but its low concentrations in uterine flushing are predictive of unsuccessful implantation (Aghajanova *et al.* 2008).

Interleukin-1 (IL-1) has been reported to be one of the first possible signals between the blastocyst and the endometrium, since *in vitro* IL-1 increases endometrial secretion of prostaglandin E_2, LIF, and of integrin β_3 subunit expression IL-1 receptor antagonist prevents implantation in the mouse attachment, indicating its importance in embryo implantation. It has also been shown that it can induce trophoblast invasion (Simon et al.1994; Staun-Ram & Shalev 2005).

Homeobox transcription factors

There are two homeobox transcription factors upregulated during the secretory phase in the human uterus, Hoxa10 and Hoxa11. Even though Hoxa10 has been proved not to be essential for implantation in mice, the absence of LIF expression in Hoxa11-/- uterous points out to its importance in the uterine receptivity and in later events of implantation (Wang & Dey 2006).

9. Invasive methods to diagnose endometrial receptivity

9.1 Genomics

Genomics studies the functions and interactions between genes in the entire genome with a broader reach than genetics does. In health sciences, genomics directs experimental access to the genome for the identification of genes involved in common diseases by their high penetrance (Guttmacher *et al*. 2002). The aim is to screen the genetic information of entire populations or specific subgroups to make a predictive medicine for individual patients, trying not only to prevent diseases before the appearance of symptoms but also to decrease the frequency of diseases in subsequent generations and to diagnose individual susceptibility in common disorders (Khoury *et al.* 2003).

9.1.1 Endometrial Receptivity Array

Based on the large amount of information generated about the regulation and dysregulation of the genes implicated in (WOI), our group developed a molecular diagnostic tool that can identify a receptive endometrium using a specific transcriptomic signature present in both natural and in hormonal replacement therapy (HRT) cycles. The endometrial receptivity array (ERA) consists in a customised array containing 238 genes expressed at the different stages of the endometrial cycle and is coupled to a computational predictor which is able to identify the receptivity status of an endometrial sample and diagnose the personalised WOI (pWOI) of a given patient regardless of the sample's histological appearance (Diaz-Gimeno *et al.* 2011). The accuracy of the ERA test is superior to endometrial histology and results are completely reproducible 29 to 40 months after the first test (Diaz-

Gimeno *et al.* 2013). Compelling evidence indicates that there is an endometrial receptivity alteration in patients with RIF in term of WOI displacement (Ruiz *et al*. 2013).

9.2 Proteomics

Compared to an organism's genome that is constant, the proteome differs from cell to cell, making it more complex. For that, new developments using protein profiling and sequencing have been implemented to elucidate the underlying biological processes. The study and comparison of the proteome in different biological situations allows the identification of the presence, absence or alteration of the proteins correlated with specific physiologic and pathologic states that can be used as diagnostic biomarkers.

At the proteomic level, five proteins were differentially expressed in the endometrial transition from non-receptive to receptive stage, of which the glutamate NMDA receptor subunit zeta 1 precursor and FRAT1 were the most interesting proteins. Other studies support that seventy-eight proteins were differentially expressed in the receptive endometrium, of which Annexin A2 and Stathmin 1 were the most consistent expressed (Dominguez *et al.* 2009).

However, these approaches have not yet rendered applicable clinical results to the everyday-IVF practice, since no single specific factor has been identified to be crucial for implantation in humans (Altmäe *et al.* 2009; Domínguez *et al.* 2009; Strowitzki *et al.* 2006). Furthermore, major setbacks in the application of biopsy-dependent techniques lie on their invasive nature that disrupts the necessary endpoint of implantation, and the difficulty to extrapolate results obtained to the next cycle. However, the analysis of EFs

is a new, non-disruptive possibility for the investigation of endometrial receptivity.

10. Non-invasive methods to diagnose endometrial receptivity

10.1 Secretomics

Secretomics identifies factors that are secreted by cells or tissues at any given time under particular physiologic, pathologic, or experimental conditions (Berlanga *et al.* 2011). In the area of reproduction, the endometrium can be an interesting topic of research for secretomics as the EF is an important regulator of uterine biology.

The fluid in the uterine cavity is a complex biological fluid, generated by glandular secretion and transudation from stromal blood vessels in the endometrium. This fluid is composed by a multitude of proteins, lipids, aminoacids, electrolytes, glucose, urea, cytokines, growth factors, and metalloproteinases secreted from the endometrium that serve as a protection against pathogens, signals fertility, and sperm migration. However, its composition varies among the menstrual cycle, specifically during the transition from the proliferative phase to the secretory phase in response to changes in ovarian steroid production. This approach provides reliable read-outs of individual molecules correlating with the day of cycle (van der Gaast *et al.* 2009; Simón *et al.* 1993) and has proven to be effective in combined molecule readouts using a luminex system (Boomsma *et al.* 2009).

Endometrial secretions in acquisition of endometrial receptivity has been investigated using animals models in which absence of endometrial glands leads to an impaired uteri without endometrial secretions, causing infertility in

animals due to the inability to support blastocyst survival. (Berlanga *et al.* 2011). Since the human endometrium generates secretions during the WOI into the lumen that are in direct contact with the blastocyst, it has been suggested that human EFs have essential components to promote pregnancy (Casado-Vela *et al.* 2009).

One of the most important advantages of secretomics analyses in the context of the uterus is the recovery of the biological sample in real time without the need of biopsy, with a minimally invasive method that might be relevant for the assessment of the endometrium with reproductive and therapeutic purposes (Berlanga *et al.* 2011). More importantly, the aspiration of EF does not affect pregnancy rates, thus opening the field for a timely synchronized, non-invasive application for the investigation of endometrial receptivity (van der Gaast *et al.* 2003). On the other hand, one limitation of the E.F. is that the amount of material obtained by aspiration sometimes is not enough for its analysis. However, using the advantages of secretomics, a molecular profile of a receptive endometrium could be designed, as well as the determination of the specific molecules released by the endometrium that might be critical for the maturation of the blastocyst and its implantation (Berlanga *et al.* 2011). For that, depending on the type of molecules found in the E.F., the necessity to draw upon the other "omics" is necessary.

10.2 Proteomics

Proteomic studies have shown that defects in the expression of proteins in the E.F. may result in the failure of embryos to implant. Therefore, it can be used for the identification of biomarkers for endometrial diseases (Casado-Vela *et al.* 2009). The endometrial secretion contains proteins from the

transudation of serum, products of apoptotic epithelial cells, and proteins secreted from the glandular epithelium. These proteins are cytokines such as leukemia inhibitory factor (LIF), glycodelin (PP14), macrophage colony-stimulating factor (M-CSF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin-like growth factor binding protein 1 (IGFBP-1), and interleukins, as well as steroid and non-steroid hormones (estrogen, progesterone, prolactin, human chorionic gonadotrophin, and precursors). However, one of the limitations in applying proteomics in the study of protein patterns in E.F. is that the majority of identified proteins correspond to serum proteins, thus masking the identification of proteins present at low concentrations (Garrido *et al.* 2010).

10.3 Lipidomics

Lipidomics has emerged as a new field on the analysis of lipids and factors that interact with lipids, and it has become an interesting topic in EFs (Vilella *et al.* 2013). Lipidomics can be defined as the large-scale study of lipid species and their related networks to integrate the investigation of the role of genomics, transcriptomics, proteomics and metabolomics in the cell function, of cells or any other biological system (Quehenberger *et al.* 2010). The aim of this '–omics' is the full characterization, identification, and quantification of molecular lipid species and their biological roles with respect to the expression of proteins involved in lipid metabolism and function, including gene regulation (Lagarde *et al.* 2003). As an emerging '–omics' field, lipidomics provides a powerful approach for the understanding of lipid biology (Quehenberger *et al.* 2010).

Lipids are organic biomolecules essentially formed by carbon, hydrogen, and oxygen, although the latter forms have a much lower proportion of these

molecules. In addition, they may also contain phosphorus, nitrogen and sulfur. Lipids do not have a characteristic functional group that allows us to classify them according to specific functions. However, they share a common feature: they are water insoluble but are soluble in organic solvents like chloroform or ether. These molecules play an important role in diverse biological functions, constituting the main energy reserve of living beings (triglycerides), forming cell membranes and giving them structure (i.e. the phospholipids of lipid bilayers), and regulating the activity of cells and tissues (as steroids and other hormones). It is also important to note that most of the non-proteic components of membranes are lipids, which are generated and metabolized by enzymes, which are in turn influenced by the environment of a given biological system.

The lipidomic approach is possible today due to the newly developed instrumentation, protocols, and bioinformatics tools that are now available (Brown *et al.* 2007). The lipidome, characterized by global changes in lipid metabolites, is part of the metabolome; however, the widely differing physicochemical properties of many lipids compared to most water soluble metabolites means they deserve separate analysis (Griffiths *et al.* 2011).

10.3.1 Technical approaches

The primary technology used for lipid research is mass spectrometry (MS), a technique that can provide the structure, molecular mass, and concentration of analyzed molecules (Griffiths *et al.* 2011; Ho *et al.* 2003). However, to study complex lipid mixtures new technologies have been developed to improve the measurement of diverse components whilst using only small sample volumes. Methods using MS, such as electrospray mass spectrometry, have been employed to gently ionize biological molecules,

and are known as electrospray ionization (ESI) techniques. These allow the structure of molecular species, such as polar lipids and some non-polar lipids, to be determined (Fortier *et al.*, 2008). ESI has proven to be a useful technique for large molecular mass biological molecules, as they are not fragmented into particles; instead, they are ionized into small droplets using electrical energy to transfer ions from a solution into a gaseous phase. ESI is a sensitive and reliable technique which is able to determine sample compositions from micro-litre volumes, and identifies non-volatile and thermally labile bio-molecules that cannot be studied by other techniques (Ho *et al.* 2003).

Another ionization method is the matrix-assisted laser desorption/ionization (MALDI), which has the advantage of having a higher tolerance for salts and other contaminants. This method can analyze not only proteins but also lipids in small sample volumes, with high sensitivity, wide mass range, and with little fragmentation. The MALDI technique works by dissolving the sample in a matrix which is able to absorb laser radiation thus causing the volatilized ions to flow down a tube allowing their separation according to mass (Jackson *et al.* 2009).

Particularly for analysis of complex lipid mixtures, tandem mass spectrometry (MS-MS) has not only been designed for the identification of molecules based on their molecular weights but also to provide structural information of the molecule, which is achieved by combining two mass spectrometers. The first mass analyzer (MS1) is used to select a precursor ion that is specifically fragmented by collision with an inert gas, producing ion fragments that are then passed through a second mass analyzer (MS2) (Griffiths *et al.* 1974). These methods have been coupled with techniques like time-of-flight (TOF) to improve the resolution, as well as complementary

mass analyzers like ion traps and Fourier transform ion cyclotron resonance (FT-ICR) to determine the mass of ions. Those techniques are mainly focused on directly profiling lipids, increasing the sensitivity and the mass measurement accuracy (Fortier *et al.* 2008; Wenk 2005). However, MS is also limited by its poor ability to ionize, and therefore quantify, low abundance lipids within a complex mixture. Independent of the specific method used, MS gives information about the molecular weight of the analytes by measuring the mass to charge ratio, *m/z* (Griffiths *et al.* 1974).

The methods usually used for lipid analysis are gas chromatography (GC), thin layer chromatography (TLC), and high performance liquid chromatography (HPLC) (Willman *et al.* 2011). GC is an important technique generally used for the analysis of fatty acids. However, its restriction to volatile lipids requires the derivatization of non-volatile lipid components before the method can be applied, as well as requiring use of internal standards. On the other hand, HPLC is a simpler method in which derivatization is not required. It can be applied across diverse chromatographic conditions in which reverse phase and normal-phase chromatography would normally be used. HPLC is highly selective and efficient in detecting lipid classes, allowing it to determine and quantify analytes with high resolution. For lipid class separations TLC is usually used for lipid mixtures, and has the advantage of being cheaper and faster than other methods, including HPLC and GC; however, its low resolution and sensitivity restricts this technique (Petersson & Cummings, 2006).

The NMR technique, designed especially for lipid membranes, uses a principle based on the presence of active nuclei atoms that form lipids (Clogston & Patri 2011). Although this method studies the physical properties of membrane compounds, nowadays the focus of this approach

has been changed towards the study of complex lipid mixture properties to determine their function and structure. NMR has even contributed to the development of other '–omics' such as metabolomics and proteomics, although its sensitivity is a significant limiting factor. Because NMR is a powerful technique for structural analysis many new technologies have focused on improving it (Grélard *et al.* 2010).

The availability of instrumentation, protocols, and bioinformatics tools such as LIPID MAPS, have made the characterization of changes in lipid metabolites, and the comprehensive analysis of the mammalian lipidome possible (Quehenberger *et al.*2010).

11. Role of lipids in embryo implantation: animal models.

In recent years, several studies in animal models have shown the importance of lipids at the time of embryo implantation. Lipidomics studies have enabled the identification and characterization of these lipids at the time of endometrial receptivity. Furthermore, functional studies in mice have confirmed the important role played by these lipids in endometrial receptivity and implantation. Lipid molecules such as endocannabinoids, lysophosphatidic acid (LPA), and prostaglandins (PG) have been reported to be some of the most widely studied mediators of embryo implantation (Gawrish *et al.* 2002). Consistency between the results observed in different animal models suggests that this trend might also be found in humans, meaning that the receptive state of the endometrium may be characterized by a specific pattern of lipids.

11.1 Endocannabinoids

Endocannabinoids are lipids that have been detected in different human tissues and reproductive fluids. The anandamides (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) have been described as the two principal endocannabinoids involved in mouse implantation events; moreover, it has been described that aberrant levels of these lipids lead to deferred implantation and compromised pregnancy outcome (Wang & Dey 2006). These studies are supported by genetic evidence showing that fatty acid amide hydrolase (FAAH), the major degrading enzyme for endocannabinoids, is linked to successful continued pregnancy after *in vitro* fertilization and subsequent embryo transfer (Gawrisch *et al.* 2002). While low FAAH levels are associated with the up-regulation of AEA and are therefore related to non-receptivity of embryo implantation, down-regulation of AEA correlates with uterine receptivity (Maccarrone *et al.* 2009). These data support work that shows that cannabinoid AEA signaling is important in both mouse embryo and uterus during implantation period (Paria *et al.* 2002).

11.2 Lysophosphatidic acid (LPA)

This lipid is a water-soluble phospholipid that acts as a potent signaling molecule with wide-ranging effects on many different target tissues. LPA, acting through its receptor LPA3, is essential for normal embryo size and spacing in mice, which is linked to a positive effect on implantation (Mizugishi *et al.* 2007). It has also been shown that deficiency of LPA3 in uteri during preimplantation leads to down regulation of COX-2 and therefore reduces the levels of PGs, thus directly affecting the process of implantation and decidualization. Studies with ovine trophoectoderm, porcine and bovine

endometrium also demonstrated that LPA is an important mediator in the process of implantation. A recent study has suggested that LPA controls the levels of endocannabinoid and prostaglandin mediators that act via LPA3 to rearrange the endometrium for implantation during the receptive stage (Wang & Dey 2006; Downie *et al.* 1974).

11.3 Prostaglandins

Prostaglandins (PGs) are lipid mediators with important roles in reproductive processes, including ovulation, implantation, and menstruation (Jabbour *et al.*2004). PGs have been classified as members of the eicosanoids family produced by all nucleated cells of the body that act locally in a paracrine and autoctrine manner to hold homeostasis (Kobayashi *et al.* 2002). Its production from lipid precursors involve different enzymes that are in charge of its biosynthesis, such as phospholipase A2 (PLA2), cyclooxigenase (COX), and prostaglandin synthetases, in which each one regulates different phases of PG pathway (Hara *et al.* 2010). In different animal models the importance of the correct production of the PGs and their synthases are essential to achieve implantation. This topic will be described below in more detail.

12. Prostaglandins biosynthesis

PG biosynthesis begins with the sequential metabolism of arachidonic acid (AA) from the plasma membrane phospholipids by phospholipase A2 enzyme (PLA2). Chronological oxidation of AA by cyclooxygenases (COX-1 and COX-2) and the action of terminal PG synthases lead to the generation of individual PGs, namely PGD_2, PGE_2, $PGF_2\alpha$, and PGI_2. The enzymes cPLA2, COX-1 and 2 act as rate-limiting factors in the production of PGs,

and their role in the endometrium is well established in mouse and human models (Stavreus-Evers *et al.* 2005; Marions *et al.* 1999; Achache *et al.* 2009; Wang & Dey, 2006). These proteins represent upstream common factors in the signaling pathway that leads to PG production. Manipulating their levels or activity has an effect on the synthesis of all PGs, and thus this approach is not informative of the production mechanisms of individual PGs. PG synthases (PGSs), on the other hand, represent the downstream terminal mediators in that pathway, and mediate the catalysis from inactive PGHs to the terminal active PGs.

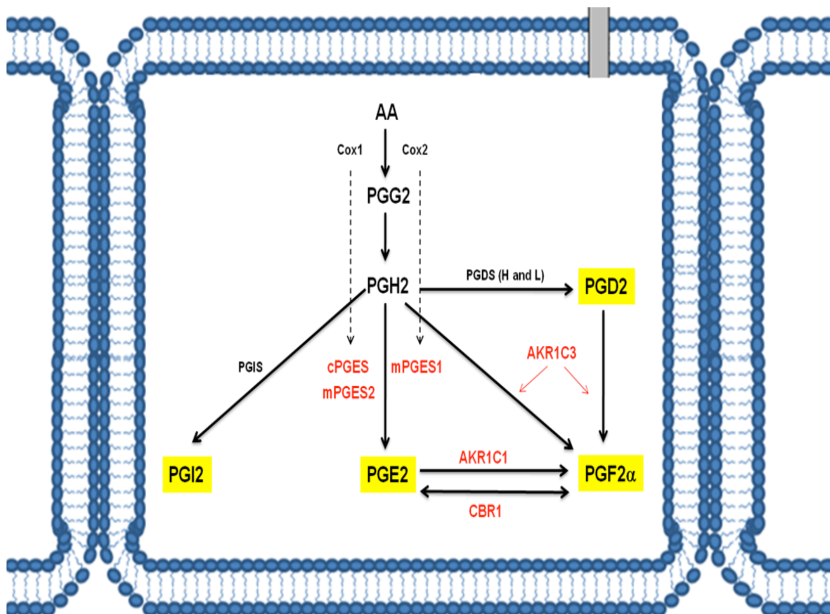


Figure 1.5. Prostaglandins production.

12.1 Upstream signalling pathway

Phospholipase A2, especially cPLA2 and the two cyclooxygenases (COX-1 and COX-2), represent the enzymes of the upstream signalling pathway that are in charge of PG production.

12.1.1 Phospholipases A2 (PLA2)

Arachidonic acid is a 20-carbon unsaturated fatty acid that is released by PLA2. PLA2 is an enzyme that hydrolyzes the ester bonds of the fatty acids at the sn-2 portion of phospholipids to generate bioactive lipid mediators that participate in several cellular functions, as well as in phospholipid metabolism, immune functions, and signal transduction. In mammalians four PLA2 subtypes have been described: cytosolic (cPLA2), secretory (sPLA2), Ca2+-independent (iPLA2), and platelet-activating factor (PAF) acetylhydrolase. The main isoforms differences are attributed in their substrate specificity and requirement for Ca2+. While PLA_2 and $sPLA_2$ generates free fatty acids, $iPLA_2$ and PAF acetylhydrolase participate in membrane remodelation and attenuation of PAF bioactivity. (Lister & Van Der Kraak 2008).

PLA2: Among the four PLAs subtypes, cPLA2 is the most important enzyme for PGs biosynthesis. It is responsible for releasing bioactive lipid mediators that are indispensable substrates for PGs formation. In humans two isoforms, $cPLA_{2\rho}$ and $cPLA_{2\gamma,}$ have been described; unlike cPLA2α, they do not show substrate preference for arachidonic acid (Wang & Dey 2005). By in situ hybridization techniques performed in mice uterus during the first days of pre-implantation and implantation period it was observed that the expression of cPLA2α presents a parallel pattern with COX-1 (Tranguch *et al.* 2005). However, the expression throughout the stage of adhesion is more

similar to COX-2 in the zones of the epithelial cells that surround the blastocyst expression in the sites of implantation (Wang & Dey 2005).

12.1.2 Cyclooxygenase

Once arachidonic acid undergoes oxidation producing PGG2, it suffers a reduction to an unstable prostaglandin endoperoxide H synthase (PGH2) by cyclooxygenasein the two isoforms, COX-1 and COX-2. A COX-3 enzyme derived from alternative splicing of the COX-1 gene has been also described; however, its function is still unclear (Simmons *et al.*, 2004). Although COX-1 and COX-2 have similar structural and kinetic properties, they are encoded by different genes with distinct cell-specific expression, regulation, and subcellular localization (Wang & Dey 2005). While COX-1 is constitutive and mediates normal physiological functions as a “house-keeping”, COX-2 is inducible by growth factors, cytokines, oncogenes, and inflammatory stimuli (Achache *et al.* 2006). The absence of COX-2 produces multiple failures in female reproductive processes, including ovulation, fertilization, implantation, and decidualization (Zhao *et al.*2012).

In mice the functional role of COX-1 and COX-2 expression has been described. **COX-1** is expressed in uterine luminal and glandular epithelial cells before the embryo gets attached, but its expression disappears in the luminal epithelial cells by the time of attachment. Three days after adhesion it is expressed again in the mesometrial and anti-mesometrial secondary decidual. Because COX-1 has been observed during postimplantation period, it has been associated with the production of PGs in decidualization and/or continued localized endometrial vascular permeability (Tranguch *et al.* 2005; Wang & Dey 2005). **COX-2** gene, on the other hand, is expressed in the luminal epithelium and subepithelial stromal cells surrounding the

blastocyst at the time of attachment. PGs produced at this site by COX-2 are involved not only in the phase of adhesion but also in angiogenesis and in the establishment of the placenta (Wang & Dey 2005).

12.2 Downstream signalling pathway

12.2.1 PG synthases

Once COX enzymes form the intermediary PGH2, it is used as a substrate for the generation of the four PGs (PGD, PGE, PGF, and PGI) by specific terminal synthases, such as PGE synthases (cPGES, mPGES-1, mPGES-2), PGF synthases (AKR1C3, AKRB1), PGD synthase (PGDS), and PGI synthase (PGIS) (Fortier *et al.* 2008). Interconversion between PGE_2 and $PGF_2\alpha$ is mediated by CBR1 and AKR1C1 in response to physiological stimulus (Colombe *et al.* 2007).

PGE_2 is synthesized by 3 types of PGE synthases (PGES): one cytosolic (cPGES) and two membrane associated PGE synthases (mPGES)-1 and -2. cPGES belongs to the glutathione transferase family that is ubiquitously expressed and preferentially coupled to COX-1, and therefore implicated in maintenance production of PGE_2. On the other hand, mPGES-1, which belongs to the Membrane-Associated Proteins involved in Eicosanoid and Glutathione metabolism (MAPEG) superfamily is coupled with COX-2 and its induction is usually synchronized with COX-2 (Samuelsson *et al.* 2007). Moreover, mPGES-1 is up-regulated by proinflammatory stimuli and down-regulated by anti-inflammatory glucocorticoids, often in coordination with COX-2, and its expression *in vivo* has been associated with various physiological and pathological events, such as inflammation, cancer, and reproduction. Finally, mPGES-2 is able to couple with both COX isoenzymes, and it is expressed relatively constitutively, rather than

inducibley, although its functional significance is still unclear (Murakami, 2003).

PGD2 is synthesized by two isoforms of PGD synthases (PGDS), lipocalin-type PGD synthase (L-PGDS) and hematopoietic PGDS (H-PGDS). L-PGDS belongs to the lipocalin superfamily that is mostly expressed in the brain, especially abundant in the cerebrospinal fluid, and it is also found in the seminal fluid. H-PGDS is a cytosolic enzyme that isomerizes PGH_2 to PGD_2 in a glutathione-dependent manner. It is also responsible for the biosynthesis of PGD_2 in immune and inflammatory cells such as mast cells, antigen-presenting cells, and Th2 cells (Kanaoka *et al.* 2003). However, PGD2 can be also metabolized by dehydration to PGJ2, delta12-PGJ2, and 15-deoxy-delta(12,14)-PGJ2, which is a ligand for the nuclear transcription factor peroxisomal proliferator activated receptor (PPAR)-gamma with an anti-inflammatory activity (Díaz-Muñoz *et al.* 2008).

Prostacyclin PGI_2 is synthesized from PGH_2 by PGI synthase (PGIS), a membrane-bound heme protein of the P450 family. In reproduction PGIS is produced by luminal epithelia and tubal smooth muscle of human fallopian tubes. As well it is localized in the nucleus and cytoplasm of stromal cells around the implanting mouse blastocyst, with distinct expression in the stromal vasculature on day 5 of mice implantation. COX-2 protein exhibits similar differential subcellular pattern with PGIS in the endometrium, suggesting generation of COX-2-derived PGI_2 (Huang *et al.* 2002; Lim *et al.* 1999).

In particular, $PGF_{2\alpha}$ is the result of not only prostaglandin F(2α) synthase, (PGFS) but can also be produced from PGD in a reaction catalysed by 11-ketoreductase (AKR1C3), or from PGE_2 through the action of 9-

ketoreductase (CBR1) and AKR1C1, illustrating how these pathways can converge in the production of PGs. AKR1C members regulate androgen, estrogen, and progesterone receptors and PPARγ (Bauman *et al.* 2005).

12.2.2 Importance of PGs synthases using animal models

Prostaglandins are lipids derived from membrane phospholipids by the action of phospholipase A2 (PLA2) and cyclooxygenase (COX-2). These enzymes are responsible for the production of PGs that become elevated in both the lumen and stroma over the receptive period, although only at the site of blastocyst implantation in murine models. In reproduction, $cPLA_2$ knockout mice demonstrate a delay in the window of implantation with an impact in diverse reproductive aspects. Some of them have been described as defective postimplantational development in embryos with small litters due to a delayed growth with hemorrhagic placentas and preponderance of big trophoblastic cells. Abnormal uterine spacing of embryos and slow fetoplacental development has also been observed, in which the main contributor for the time of implantation is of maternal cPLA2*α* origin and not embrionary (Tranguch *et al.* 2005). However, administration of PGs on day 4 can rescue the normal time of implantation in *cPLA2α−/−*, but the abnormal embryo spacing cannot be recovered (Zhao *et al.* 2012; Wang & Dey 2005). This pattern also shows up in other studies, suggesting that the PGs have an essential role during a specific time, in which any delay leads to an irreversible phenotype (Hama *et al.* 2007).

Because $cPLA_2$ is responsible for the production of arachidonic acid as a substrate for COX, deficiencies in COX-2 corroborate that the lack of either PLA2 or COX-2 leads to an absence of PG synthesis, which in mice results in several implantation defects, thus confirming the importance of PGs in

murine reproductive efficiency (Fortier et al., 2008; Lousse J-C *et al.* 2010). It has also been demonstrated that any alteration in the PG production pathway has a dramatic effect on the process of implantation, decreasing the probability of achieving pregnancy at the end of the process. For that reason it has been established that PGs significantly contribute to establish the microenvironment required during implantation and decidualization, and are therefore strong molecular candidates for implantation regulation in humans (Achache *et al.* 2010).

13. Prostaglandin studies

13.1 Animal models

It has been demonstrated that COX-2 is indispensable for blastocyst implantation and decidualization. However, the candidate PG(s) that is or are responsible in these processes and the mechanism of its action in humans remain unknown. Using different animal models to understand this riddle it was shown that the type of PG implicated in implantation varies among species, hindering the search for candidate PGs responsible for implantation in humans.

PGE_2 and $PGF_2\alpha$ have been revealed as the most important PGs during the implantation stage in mice (Pakrasi *et al.* 1997). This work reported that PGE_2 was increased at implantation sites, whereas $PGF_2\alpha$ was higher at inter-implantation areas. However, a more recent investigation differed from previous studies, showing that PGI_2 levels were the highest, followed by PGE_2, and no differences in the $PGF_2\alpha$ levels during implantation were found when compared to other phases of the menstrual cycle (Stavreus-Evers *et al.* 2005). Due to the properties of PGI_2 as a vasoactive agent that

participates in vascular permeability changes, this PG has been associated with localized vascular permeability in the uterus during implantation, indicating that PGE_2 and PGI_2 are important for ovulation but also for the initiation of implantation in mouse uteri (Marions *et al.* 1999). In contrast, studies performed with rats as a model showed that PGE_2, but not PGI_2, is an important mediator of increased vascular permeability at the implantation site (Catalano *et al*. 2011).

In hamsters, blastocyst implantation is related with an increase of PGE_2 at the implantation site through the co-expression of the activity enzymes: mPGEs-2 and COX-2 (Catalano *et al.* 2011). In rabbits, it was reported that PGE_2 concentrations were higher at implantation sites (Durn *et al.* 2010). However, other studies showed that both PGE_2 and $PGF_2α$ were elevated. In pigs, PGE_2 was able to act locally through endometrial PGE_2 receptors, especially PTGER2, and may be involved in a positive feedback loop during increased PGE_2 synthesis in porcine uterus in the peri-implantation window (Waclawik 2009).

13.2 Human model

Despite the limited number of reports on the role of PGs in human implantation, it is known that in human blastocysts PGI_2 stimulates embryo development and hatching through its cognate nuclear receptor PPARγ, whereas PGE_2 interacts with its receptors EP1-4 stimulating the migration of human trophoblast by activating Rac1 and CDC42 and inducing changes in the cytoskeleton (Huang *et al.* 2007; Nicola et al., 2008; Fournier et al., 2007). However, most of the endometrial studies have been focused on the role of PGs in the increase of vascular permeability and decidualization, leaving behind the expression and function of individual PGSs in

endometrium, as well as the dynamics of their production in receptive versus non-receptive human endometrium. A recent study has shown that defective endometrial PG synthesis is linked with repeated implantation failure in patients undergoing IVF (Achache *et al.* 2009). Using non-human systems, it has been proposed in the issue of reproduction that the ratio of PGE_2:$PGF_2\alpha$ could be an important parameter to control the embryo implantation, thus placing PGs at the forefront of implantation events in humans (Zieck *et al*. 2008; Ghosh & Sengupta, 1998).

14. PG Transporters

Once PGs are synthethized they are rapidly transported outside of the cells. PGs predominate as charged anions that are characterized by its low intrinsic permeability across the plasma membrane (Schuster *et al*. 1998). Due to their lipophilic nature, their transport through plasma membranes is poorly understood. It has been suggested that PGs could be transported through different mechanisms like simple diffusion, passive transport, active transport, counter-current, and carrier-mediated transport (Banu *et al*. 2008).

Although it is known that PGs can cross cell membranes by simple diffusion, it is very difficult to maintain a biological function by this mechanism because their flow rate is too low: PGs diffuse poorly across biological membranes and the presence of a carrier is necessary. PGT, a broadly expressed 12-membrane-spanning domain integral membrane protein, could mediate the transport of PGs through the membrane in both ways of efflux and influx. It has also been shown that PGT has a high-affinity for PGs by uptaking and transporting them ((Banu *et al*. 2008). During the human menstrual cycle, PGT expression is elevated in proliferative and early-secretory phase and

low in the mid- to late-secretory phase. Moreover, it is immunolocalized in luminal, glandular epithelium and stromal cells. PGT modulation in epithelial cells during the menstrual cycle suggests an important role in regulation of PG action in the human endometrium. However, their specific role in human implantation is still not well defined (Achache *et al.* 2006).

PGH_2, a precursor of PGs inside cells, is also released into the extracellular compartment mainly by diffusion, whereas its influx is mediated by both passive diffusion and by PGT carrier. PGH_2 translocation from the intracellular to the extracellular compartments or vice-versa is critical for the cellular levels. Based on the structural similarity between PGH_2 and PGE_2, PGT also transports PGH_2; however, its transport has a lower affinity, but a higher rate, compared to PGE_2. Moreover, PGT slightly prefers PGE_2 over PGH_2 in the sense that PGT balances the uptake of PGE_2 into the cell against a concentration gradient (Chi & Schuster 2010).

The members of the multidrug resistance protein (MRP)/ABCC subfamily of ATP-binding cassette transporters, MRP4 (ABCC4) are organic anion transporters. They have, however, the outstanding ability to mediate the effux of prostaglandins (Ritter *et al*. 2005).

Recently, a new mechanism of PGs transportation has been proposed. Exosomes appear to be involved in additional intercellular signaling by interacting with cell peripheral receptors such as G protein coupled receptor (GPCR). Although the participation of exosomes in lipid metabolism is not yet known, the presence of arachidonic acid, as well as either COX-1 or COX-2 and terminal prostaglandin synthases in exosomes has been found. However, no specific peripheral receptors or mechanisms of entry have been identified for this prostaglandin. It has been suggested that exosomes may

function as a vehicle allowing the plasma membrane with PGs to be bypassed where they would be released into the cytosol after fusion between exosome and endosome membranes (Subra *et al.* 2010).

15. PG Receptors

Once PGs are biosynthesized, they are quickly transported outside the cell, acting in an autocrine or paracrine manner that might bind to specific plasma membrane receptors to produce a diverse range of biological responses. These receptors are surface G-protein coupled receptors (GPCRs) with seven trasmembrane domains that activate an associated G-protein (Gα , Gi, Gq) (Narumiya *et al.* 1999).

Different prostaglandins (PGE_2, $PGF_{2\alpha}$, PGI, PGD) exert their biological function through interaction with their corresponding receptor EP, FP, IP and DP (Blesson *et al.* 2012), which have been classified according to their response to agonists/antagonists (Myatt *et al.* 2004). Among PGs, the only one that possesses four receptors subtypes is EP receptor, termed EP1, EP2, EP3, EP4 (EP1-4) (Lim & Dey 1997). Because EP subtypes have different structures, ligand bindings, and signal properties between them, PGE has a multiplicity of biological responses (Tsuboi *et al.* 2002). In addition to that, the EP_3 receptor also possesses isoforms in bovine, mouse, rabbit, and human, that are produced by the alternative RNA splicing with different carboxyl terminal sequences associated to different G protein, and those subsequently activate different second messenger systems (Okuda-Ashitaka *et al.* 1996).

Moreover, pharmacologic studies have identified two contractile receptors EP1and EP3 and two relaxing EP2 and EP4 (Myatt & Lye 2004). Despite the

conserved sequences, homology between PGs receptors is relatively limited to 20-30% even among the four subtypes of PGE_2 receptors. However, the homology of a type or subtype of receptors among various species is considerably higher: between human and mouse, for instance, the homology of IP, EP1, EP3, EP4, and FP receptors goes to around 85% (Narumiya *et al.* 1999).

Signal intracellular pathways of these receptors have been determined by changes of the second messengers such as cAMP, intracellular calcium release, and inositol phosphates via their G protein. EP1 receptor is coupled to Gq protein and activates phospholipase C, which results in the generation of two second messengers: inositol triphosphate, that liberates intracellular calcium (Ca^{2+}), and diacylglycerol, which activates protein kinase C (Banu *et al.* 2009). While EP2 and EP4 are coupled to $G\alpha_s$ with the activation of adenylate cyclase, EP3 is coupled to Gi via the inhibition of adenylate cyclase. On the other hand, FP is coupled to the stimulation of phospholipase C–inositol (IP3) pathway and Ca2+ mobilization by $G\alpha_q$ (Wanggren *et al.* 2006)

PGs, PGE_2 and $PGF_2\alpha$ have been determined to be implicated in many reproductive functions in which their receptors are expressed. However, their distribution in the human endometrium and/or embryo during the menstrual cycle state remains unknown. Using animal models in mice it has been shown that deficiencies in EP1, EP3, EP4, and IP do not alter female reproduction, the same being true for FP. However, although $PGF_2\alpha$ seems not to be part of the indispensables PGs for implantation in mice, its action varies among species, raising the question of what role each PG receptor plays in human implantation (Narumiya *et al.* 1999).

There are different agonists for the EP-receptors, but only a few are selective for each EPs subtypes. Among the existing EP1 receptor agonists the most commonly used is 17-phenyl-omega-trinor PGE_2 (17-Ph-PGE_2) principally in studies of the nervous system (Oka *et al.* 1997). However, although this agonist specially activates EP1, it also has low affinity for EP3.

Butaprost is the most potent agonist for EP2-receptor as it shows high selectivity for this receptor subtype, with little or no activity for the other receptors (Senior *et al.* 1991). It has demonstrated that the activation of EP2 receptor leads to intracellular accumulation of cAMP as it also increases CXCR4 expression with a rapid phosphorylation of ERK1/2. CXCR4 is regulated in the endometrium by embryonic signals and its expression is synchronized with EP2 expression in endometrial tissues during the mid-secretory phase of the cycle, suggesting a potential crosstalk between these two receptors (Sales *et al.* 2011; Abera *et al.* 2010).

Sulprostone, a stable acyl sulphonamide analogue of PGE_2, shows selectivity for both EP1 and EP3 receptors, although it has the highest affinity with EP3. Sulprostone produces potent contractile responses, supporting the presence of contractile EP3-receptors in the non-pregnant human myometrium *in vitro*, and it confirms PGE signaling pathway leading to ERK activation (Senior *et al.* 1991; Chuang *et al.* 2006). The last PGE receptor, EP4 agonist 16S-9-Deoxy-9β-chloro-15-deoxy-16-hydroxy-17,17-propano-19,20-didehydro PGE-2 (ONO-AE1-329) has been widely used in studies of inflammation, possibly via induction of VEGF-C and VEGF-D (Hosono *et al.* 2011). On the other hand, fluprostenol is an analog of $PGF_2\alpha$ with potent FP receptor agonist activity, which can only bind to this receptor. It activates phospholipase C and Ca2+ mobilization in human

myomentrial cell and induces contractions in rabbit uterus (Chen *et al.* 1998; Carrasco *et al.* 1996).

On the other hand, there are limited antagonists available for EP receptors. Selective EP1 antagonists such as SC51089, or SC53122 have been described as nonsteroidal anti-inflammatory drugs (Breyer 2001). Also recently, a selective EP2 receptor antagonist, PF-04418948, has been discovered as an important tool for investigating the biological activity of PGE_2 and the role of EP2 receptors in health and disease (Birrell *et al.* 2013).

The EP3 antagonist *N*-[(5-Bromo-2-methoxyphenyl)sulfonyl]-3-[2-(2-naphthalenylmethyl)phenyl]-2-propenamide (L-798,106) has been used in different species for being a potent and highly selective antagonist by its inhibitory action on sulprostone (Bassil *et al.* 2008). As well as *N*-[[4'-[[3-Butyl-1,5-dihydro-5-oxo-1-[2-(trifluoromethyl)phenyl]-4*H*-1,2,4-triazol-4-yl-]methyl][1,1'-biphenyl]-2-yl]sulfonyl]-3-methyl-2-thiophenecarboxamide (L-161,982), EP4 receptor antagonist has a selective antagonism over all other prostanoid receptors.

From the variety of FP receptor antagonists that have been reported in the past AL-3138 and AL-8810, both $PGF_2\alpha$ analogues, have received acceptance as FP antagonists (Jones *et al*. 2009).

II.- HYPOTHESIS

II.- HYPOTHESIS

Endometrial receptivity, a crucial stage in the human endometrium is governed by endocrine, paracrine and autocrine regulators. Different approaches have been used to identify biomarkers to diagnose the endometrial receptive period but none of them are non-invasive.

Based on our preliminary data, our hypothesis relies in lipidomics and specifically on the quantification of PGE_2 and $PGF_2\alpha$ lipids in endometrial fluid as biomarkers that can diagnose the endometrial receptivity status during the window of implantation.

III.- OBJECTIVES

III.- OBJECTIVES

General Objectives

- To identify the lipidomic profile in human endometrial secretions throught the menstrual cycle, with special attention to the WOI.

- To understand the production mechanism/s of these lipids by human endometrial epithelial cells and their putative effects on the blastocysts.

- To prove the diagnostic sensitivity and specificity of PGE_2, $PGF_2\alpha$ levels in endometrial fluid obtained 24 hours before embryo transfer, by correlating with cycle outcome.

Specific Objectives

- To compare lipidomic profiles in endometrial fluid among optimal, suboptimal and refractory endometrial cycles.
- To determine the expression and localization of PGE_2 and $PGF_2\alpha$ synthases in the human endometrium
- To describe specific enzymes involved in the production pathway of PGE_2 and $PGF_2\alpha$ in the endometrium and endometrial fluid
- To demonstrate the functionality of PG synthases
- To determine the expression and localization of PGE_2 and $PGF_2\alpha$ receptors in the human endometrium and mouse embryos
- To proof the relevance of these molecules in embryo adhesion using an *in vitro* model
- To determine the sensitivity and specificity of PGE_2 and $PGF_2\alpha$ concentrations in endometrial fluid predicting IVF cycle outcome.

IV.- MATERIAL AND METHODS

IV. MATERIAL AND METHODS

1. Lipidomic Profile

1.1 Chemicals. HPLC grade water, methanol, and acetonitrile used for mass spectrometric studies were purchased from VWR International (Plainview, NY, USA). Mass spectrometry/HPLC grade acetic acid, formic acid, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

1.2 Patient recruitment and endometrial fluid collection. This study was approved by our IRB 1002-C-069-OB (27/07/13) and all patients involved signed informed consent. EF was extracted with the woman lying in lithotomy position; the cervix was cleansed after inserting the speculum and an empty flexible catheter (Wallace, Smith Medical International) was gently introduced 6 cm transcervically into the uterine cavity guided by abdominal ultrasound, and suction gradually applied with a 10 ml syringe. To prevent contamination by cervical mucus during catheter removal, suction was dropped at the entrance of internal cervical oss (ICO), and cervical mucus was also aspirated prior to EF aspiration. Approximately 20-40 µl of EF was obtained per patient and was snap-frozen and stored at -80 °C until analysis. This study was approved by our IRB 1002-C-069-OB (27/07/13) and all patients involved signed informed consent. In studies I and II were ovum donors aged between 18 and 35, were healthy with regular menstrual cycles, normal karyotype, and a BMI of 19-29 Kg/m^2.

1.3 Lipidomics profiles across the natural cycle. Endometrial fluids were obtained throughout the natural cycle from 51 patients with regular cycles

and classified as: group I (days 0-8; n=10), group II (days 9-14; n=15), group III (days 15-18; n=9), group IV (19-23; n=9), and group V (days 24-28; n=8).

1.4 Lipidomics profiles in HRT, COS and HRT+IUD. This study aimed to investigate endometrial lipidomic profile in different assisted reproductive technologies (ARTs) such as ovum donation in which hormonal replacement therapy (HRT) is used, and IVF in which controlled ovarian stimulation (COS) is performed, as well as to understand the lipidomic profile in the refractory endometrium induced by the insertion of an IUD. We analyzed the lipidomic profile in the EF of 30 ovum donors undergoing first an HRT cycle, then a COS cycle, and finally an IUD was inserted and an HRT cycle was performed again (HRT/IUD) to induce a refractory endometrium in the same patients. EF was obtained and analyzed at the following days in HRT: P+0, P+1, P+3, P+5, P+7, and P+9 (WOI corresponded to days P+5 and P+7), COS: hGC+0, hCG+3, hCG+5, hCG+7, hCG+9, and hCG+11 (WOI corresponded to days hCG+7 and hCG+9) and HRT with the insertion of an IUD: P+0/ IUD, P+1/IUD, P+3/IUD, P+5/ IUD, and P+7/IUD (no WOI is present in the refractory endometrium induced by an IUD even during HRT).

1.4.1 HRT protocol. A baseline transvaginal scan was carried out prior to downregulation to ensure that the endometrium and ovaries were in basal conditions. For all patients who were still cycling, down-regulation was performed using an IM dose of 3.75 mg of triptorelin (decapeptyl; Ipsen Pharma; Barcelona, Spain) in the mid-luteal phase of the previous cycle. HRT was initiated on day 1–3 of the following cycle, and doses of estradiol valerate (progynova; Schering Spain, Madrid, Spain) were increased as follows: 2 mg/day for the first 8 days of treatment, 4 mg/day for the following 3 days and then 6 mg/day. On day 15, ultrasound was performed to evaluate endometrial growth and when endometrial lining reached 7 mm with a typical

triple layer pattern, 800 mg/day of micronized intravaginal progesterone (Progeffikw; Effik Laboratories, Madrid, Spain) was administered. The day of initial progesterone administration was considered as P0.

1.4.2 COS protocol This protocol was carried out by following a GnRH agonist long protocol, with a combination of 200 IU recombinant FSH (Gonal F; Merck-Serono). When six or more follicles were more than 17 mm in diameter, recombinant Chorionic Gonadotrophin (rCG; Ovitrelle; Merck-Serono) was administered to trigger ovulation. Doses were adjusted according to ovarian response as judged by serum estradiol concentrations and ultrasound scans every 3 days.

An inert IUD (Lippes Loop Intrauterine Double-S; Ortho Pharmaceutical Corp., Raritan, NJ) was used in this study. It was inserted at the time of menstruation and HRT was initiated in the next cycle as indicated above. After EF was obtained IUD was removed.

1.5 Lipidomics profiles in EF collected 24-hours prior to embryo transfer in IVF patients and ovum recipients. For the pilot study, EF was obtained 24 hours prior to the day of elective embryo transfer in IVF patients undergoing day-3 embryo transfer (n=20), and in ovum recipients undergoing day-5 embryo transfer (n=17). IVF patients eligible for this trial were women between 20-38 years of age with regular menstrual cycles,a BMI 19-29 Kg/m^2 undergoing COS for their first IVF attempt and who gave informed consent. Oocytes were fertilized either by ICSI or IVF, and an elective single or double embryo transfer to rule out poor embryo quality was performed at day-3 of embryo development corresponding to hCG+5. Inclusion criteria for ovum recipients were women between 20-50 years of age (both inclusive) undergoing routine HRT treatment, with a BMI 19-29

Kg/m^2 who were having blastocyst(s) transferred from ovum donation, and who gave informed consent. Oocytes were fertilized either by ICSI or IVF, and an elective single or double embryo transfer to rule out poor embryo quality was performed at day-5 of embryo development, corresponding to P+5.

1.6 Sample analysis. Lipids from endometrial-fluid extracts were identified by liquid chromatography combined with tandem mass-spectrometry. Tandem mass-spectrometers include triple quadrupole, ion trap, and quadrupole/time-of-flight instruments, among others. These instruments typically use quadrupole technology to isolate a compound based upon its molecular weight prior to collision activation (fragmentation) and mass analysis of fragmented components. This means that the mixture must be purified only to the point that the sample applied to the mass spectrometer is free of other compounds with the same mass. This can often be accomplished with a liquid-liquid extraction from the tissue followed by solid phase extraction methods. Quadrupole technology provides approximately 1 amu resolution; improved isolation within the mass spectrometer is accomplished using TOF/TOF instruments, which permit much finer resolution.

1.7 Quantification and analysis of prostaglandins using LC/MS/MS. Samples of EF were recovered in 5 ml total volume of HPLC-grade methanol in the presence of 1 µM N-arachidonoyl glycine-d8 (d8NAGly) that served as an internal standard to determine the recovery of the compounds of interest. Samples were then centrifuged at 19,000xG at 24ºC for 20 minutes. The supernatants were then collected with HPLC-grade water to make a final solution of 25% of supernatant in water. To isolate the compounds of interest, partial purification was achieved through extraction on C18 solid-

phase extraction columns. The columns were then washed with 2.5 mL HPLC grade water and 1.5 mL of 40% methanol. Elutions of 1.5 mL of 60%, 75%, 85%, and 100% methanol were collected in individual auto sampler vials. The vials were stored at -80ºC until mass spectrometry analysis. LC/MS/MS analysis was performed following the protocol described by Walker and co-workers (Walker et al. 1999). The amount of analyte in each sample was calculated by using a combination of calibration curves of the synthetic standards obtained from the Analyst Software and isotope dilution methods. The standards provided a reference for retention times by which the analytes could be compared. They also helped identify the specific precursor ion and fragment ion for each analyte, which enabled their isolation. These processes guaranteed that the compounds measures were, in fact, the compound of interest. The amount of each compound in each tissue was then converted to moles per gram of fluid.

2. Biological recovery and processing

2.1 Endometrial biopsies. Endometrial biopsies from natural cycle or progesterone-treated ovum donors were taken using pipelle catheters (Genetics, Belgium) under sterile conditions. Samples were rinsed with PBS and processed immediately or frozen at –80°C. A portion of each specimen was histologically examined for dating using the Noyes criteria (Noyes *et al.* 1950). Patients diagnosed with endometriosis and/or endometritis were excluded. All patients gave informed consent prior to entering the study.

Endometrial biopsies were processed to separate epithelial and stromal fractions, and epithelial cells. For some experiments whole endometrial tissue was used instead. For in vitro experiments, endometrial biopsies from ovum donors were regularly obtained and the epithelial fraction purified and

cultured for up to one week in appropriate tissue culture media (Dominguez *et al.* 2010).

2.2 Separation of epithelial and stromal fractions The process was made in sterile conditions to avoid contamination using laminar flow in aseptic cell culture areas. Processing of human endometrial biopsies took place within a day of their extraction, when they were aseptically removed, immediately placed in individual Eppendor tubes of 15ml, and stored at 4°C until its processing. Endometrial biopsies were cleansed of blood and mucous in a 60 mm Petri dishes containing 1.0ml of DMSO. By mechanical disaggregation the tissue was rapidly minced with blades to fragments less than $1mm^3$, and were then transferred to eppendor tubes of 15ml. The tissue minced was digested enzymatically using type IA-collagenase (Sigma-Aldrich Madrid, Spain) diluted in DMEM at 10mg/ml and then stored at 4°C for 24h. Collagenase is an endopeptidase that digests native collagen in the triple helix region releasing small fragments of tissue and cells. After digestion, the tissue was allowed to settle and the supernatant was removed. The tissue was washed three times with 10 ml DMEM for 10 min each one, and the supernatant removed. They were collected, pooled together, and filtered with a pore of 30 um to obtain the estromal cells. The sample was then centrifugated at 2000 rpm for 5min and resuspended in DMEM/F12 (Dulbecoco's Modified Eagle Medium: Nutrient mixture F12).

Cell suspension was diluted with 5ml of DMEM and added to a flask of $25cm^2$ for 20 min at 37°C. Supernatant was then collected, with a subsequent repetition of the process. Incubations eliminated the total stromal cells in the epithelial fraction, and the last fraction of the suspension was centrifugated at 2000rpm for 5 minutes. Finally, the pellet containing the

epithelial cells was resuspended in EEC medium (75% DMEM, 25%MCDB-105, 10%FBS, 220ul Insulin, 0,1% fungizone and gentamizine).

2.3 Primary Endometrial Epithelial Cell Cultures. Primary culture of human epithelial cells were prepared from endometrial biopsies. Endometrial epithelial cells were isolated from stromal cells and seeded on 24-well plaques with EECs. The culture medium was changed 24h after seeding and every other day thereafter. Cells were cultured in a sterile incubator at 37C, 95% relative humidity and 5% CO2 until they reached confluent EEC monolayer

3. Cell Lines

RL95-2 derived from a moderately differentiated adenosquamous carcinoma of the endometrium were used as a model for receptive endometrium. Cell line HEC-1A derived from human endometrial carcinoma served as a model for non-receptive state, and JAR cells derived from human endometrial choriocarcinoma used as an in vitro model for trophoblast cells.

3.1 Cell Lines Culture HEC-1A cells were cultured in Meckoy 5A medium (Ha'Emek, Israel) containing 10% fetal calf serum (FCS), penicillin and streptomycin. RL95-2 cells were cultured in DMEM F:12 medium containing FCS, penicillin, streptomycin, and 2.5 mM glutamine. Cell cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. RL95-2 cells (1–2 × 10^6) and HEC-1-A cells (1–2 × 10^6) were seeded in 24-well culture plates for 10 days, and the growth medium was renewed every 2–3 days.

4. Gene expression analysis

4.1 PCR primers design Appropriate primers for PCR were designed to span exon-exon boundaries to the PCR template sequence. Initially, the primer melting temperature (T_m), where the double strand become single stranded, was calculated. Primers were constructed based on the length between 18 and 25 bases (optimal 20-22 bases) and on the melting temperature in the range of 60 ^oC. The T_m was calculated according to the formula: T_m = 4(G+C) + 2(A+T). To avoid regions of homology, primers sequences were tested using NCBI Primer Blast software to improve specificity by not amplifying other sequences. Before the use of primers a primer efficiency test was made. Three concentrations of primers (200μM, 400μM, 800μM) were tested with 2μl of a cDNA control containing the expression of the sequence that we are interested in. The following primers were designed for further gene expression experiments:

Table 4.1:

β-actina	(F): GCCATGTACGTAGCCATCC (R): CTCTCAGCTGTGGTGGTGAA
cPGES	(F): TTTCCGCGCGGTGCATTCT (R): AGGGGGACGGGCGAACTG
mPGEs-1	(F): CATCATCACGGGCCAAGTGA (R): GTAGATGGTCTCCATGTCGT
mPGEs-2	(F): TCGGCAATAAGTACTGGCTCA (R): AGTCGCTTGCTGATGAGGTA
AKR1C1	(F): CCATCGACCAGAGTTGGTC (R): TTGGGATCACTTCCTCACCT
AKR1C3	(F): ATGATGGCCACTTCATGCCT (R): TGCAATCTTGCTTCGGATGG
AKR1B1	(F): AAGTCTGTGACACCAGAACG (R): GTAATCCTTGTGGGAGGTAC
CBR1	(F): GCTGGACATCGACGATCTG (R): TGAATATGAAAGGGTGTGGGA
COX-1	(F): AGGAACATGGACCACCACAT (R): GGTAGAACTCCAACGCATCA
COX-2	(F): TACGGTGAAACTCTGGCTAG (R): CAGCAAACCGTAGATGCTCA
EP1	(F): GGTATCATGGTGGTGTCGT (R): GGCCTCTGGTTGTGCTTAG
EP2	(F): ATTTCGGTCCCTCCCCTTT (R): GCGTCTCGCAGTCCTCAGA
EP3	(F): CGTGTCGCCCAGCTACCGGCG (R): CGGGCCACTGGACGGTGTACT
EP4	(F): ACCATCGCCACGTACATGAA (R): CCAATCGCTTGTCCACGTAGT
FR	(F): GCAGCTGCGCTTCTTTCAA (R): CGATGCCTTGGACTTCTGT
GAPDH	(F): GAAGGTGAAGGTCGGAGTC; (R): GAAGATGGTGATGGGATTTC

4.2 RNA extraction and quantification. The total RNA was extracted from hEEC and whole endometrial tissue using TRIzol reagent according to the manufacturer's protocol (Life Technologies, U.K.). Cells grown in monolayer were lysed directly in the culture dish. The amount of TRIzol was added based on the area of the culture dish (1ml per 10 cm2) and not on the number of cells present. Three steps were followed to extract the total RNA from EEC.

4.2.1 Phase separation. The samples were incubated for 5 min at 15 to 30ºC to allow complete dissociation of nucleoproteins complexes. 0.2ml of chloroform was added per 1ml of TRIzol reagent. Then tubes were shaken vigorously for 15 seconds and incubated at 15-30ºC for 2 to 3 minutes. This was followed by centrifugation at no more than 12,000 g for 15 min at 2 to 8ºC, and the mixture was separated into a lower red, phenol-chloroform phase, interphase, and a colorless upper aqueous phase. The RNA remained exclusively in the aqueous phase.

4.2.2 RNA precipitation. Following the separation of phases, the aqueous phase was transfered to a fresh tube. Cold isopropanol was then added in a volume of 1/2 per ml of Trizol. The samples were incubated at 15 to 30ºC for 10 min or 4ºC overnight. Total RNA was condensed by centrifugation at 12000g for 15 min -4ºC.

4.2.3 RNA wash. Supernatant was removed and rinsed with 1ml of 70% ethanol, then centrifugated at no more than 7500g for 5min-4ºC. Supernatant was eliminated and the dry pellet was reconstituted in RNasefree water (Invitrogen) according to the size of the pellet. RNA was diluted 50-fold in RNase-free water and quantified at an absorbance of 260

nm using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA). All samples had an A260/280 ratio between 1.8 and 2.2.

4.3. Retrotranscription. Reverse transcription RNA strand was reverse transcribed into its DNA complement (complementary DNA, or cDNA). RNA was diluted in RNase-free water to a concentration of 1µg/µl, and 1µg was used in the reaction. Each sample was treated with 1 µl of oligoT with a concentration of 20 µM. The samples were incubated in a thermoblock at 70ºC for 2 minutes. Then a cocktail consisting of 4ul per sample of 5X RT buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM MgCl2, Invitrogen), dNTPs (0.5 mM, Roche Molecular Biochemicals, Laval, QC, Canada), Recombinant RNase inhibitor (10 mM, Invitrogen), and M-MLV reverse transcriptase (200 U, Invitrogen) was added to each sample to obtain a final volume of 25 µl. The reverse transcription reactions were completed in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) for 1h at 37ºC followed by 5 min at 90ºC, then diluted 5-fold and stored at -20ºC until qPCR amplification.

4.4 Semiquantitative PCR. Semiquantitative PCR studies were used to assess the transcriptional expression. PCR was composed by a mixture containing < 250ng cDNA template derived from total RNA, PCR Master Mix 1X (Taq DNA polymerase, dNTPs, MgCl), upstream and downstream primers, (0.1–1.0µM) in a total volume of 25 µl. PCR was performed using a Biometra Thermocycle T3000 with the following conditions: 95 °C for 1 min and then 40 cycles at 94 °C for 45 s, 60 °C for 1 min, and 72 °C for 1min followed by 72 °C for 8 min. Samples were applied on 2% agarose gel prestained with 0.5 µg/ml ethidium bromide.

4.5 Quantitative Real Time RT-PCR. Real time RT-PCR is a variant of the polymerase chain reaction (PCR) that enables reliable detection and absolute quantification of the products generated during each cycle of the PCR process. For qPCR analysis, RNA was extracted from human epithelial cells and the whole endometrium tissue and reverse transcribed to cDNA using Superscript III (Stratagene). PCR reactions were carried out with a LightCycler 480 Real-Time PCR System (Roche Diagnostics, GmbH Mannheim, Germany) using SYBR Green PCR reagents.

PCR consisted of a series of 40 repeated temperature changes, called cycles, each one with 3 temperature steps. The first step is denaturation at 95 °C for 20–30 seconds that causes the disruption of the hydrogen bonds between complementary bases yielding single-stranded DNA molecules. This is followed by the annealing step at 59 °C for 20–seconds that allows the primers to anneal to the single-stranded DNA template. The last step is the extension/elongation at 72 °C, where the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs (deoxynucleotide triphosphate) that are complementary to the template. The amount of DNA target is exponentially amplified. Quantification data was analyzed with the Lightcycler analysis software version 3.5. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as the housekeeping gene control. This gene is involved in basic functions in cell survival with a constitutive expression. Primers are described in Table 4.1.

5. Protein quantification and localization techniques

5.1 Protein Extraction. Whole-cell proteins, usually between1-5x10^6 cells, were extracted with 1ml RIPA buffer (25mM Tris-HCl (pH 7.6), 150mM NaCl,

1% NP-40, 1% sodium deoxycholate, 0.1% SDS) that lyses and extracts membrane, nuclear and cytoplasmic proteins. Adherent cells, for instance hEEC monolayers, were washed with cold PBS that was added straight into the plate and collected mechanically with a cell scratcher; however, for cells in suspension, the medium was removed. After samples were centrifuged at 2000 rpm for 10 min at RT, RIPA buffer was added and incubated for 30 min at 4°C. The samples were then centrifuged at 12000 rpm 4°C for 15 min and supernatants were collected for protein measurement using Bradford.

5.2 Protein Quantification. Bradford method, a colorimetric protein assay, is based on the deprotonation of Coomassie Brilliant Blue G-250 dye. The protonated form of the dye is stable in solution under acidic conditions and absorbs at 470 nm. Formation of the complex between dye and protein generates the deprotonization form of the dye and the amount of protein concentration is detected as an increase of absorbance at 595 nm.

Standards were prepared based on BSA stock solution (see Table 4.2). Bradford reagent (Bio-Rad) was diluted 2.5-fold in de-ionized water. A total volume of 200 µl of the diluted Bradford reagent was added to each well. Color reagent was mixed with 5ul of standards, incubated for 5 min for the reaction, and read on a Victor Machine with an absorbance at 595 nm. At the same time, samples of interest were prepared as above and their absorbance was measured after measuring the standards. The calibration graph was prepared by dividing the net absorbance values at 590 nm and at 450 nm. Then the concentration of the unknown sample was calculated based on the linear equation of the calibration curve.

Table 4.2: Values upon which the standard curve was prepared.

mg/mL	H2O in µl	BSA 2mg/ml in µl	Load in every well in µl	BioRad Protein assay (µL)
2	---	200	5	200
1.75	14.28	100	5	200
1.5	16.6	100	5	200
1.25	20	100	5	200
1	25	100	5	200
0.75	33.3	100	5	200
0.5	50	100	5	200
0.25	100	100	5	200
0	100	----	5	200

5.3 Western blot. An appropriate volume of 5x non-reducing Laemmli buffer was added to samples and proteins denatured for 5 min at 95°C. Equal amounts of approximate 20-30 µg of total protein (depending of the quantity of protein in the sample) were loaded into the wells of the SDS-PAGE in 12% polyacrylamide gels, along with molecular weight markers. The gel ran for 1 to 2 hours at 120 V and transferred onto polyvinylidene fluoride (PVDF) membranes by wet electroblotting using Tris/Glycine Transfer Buffer (Biorad, Munich, Germany). Membranes were blocked with 5% milk in TBST (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween-20) at RT for 1 hour, and incubated with appropriate primary antibodies overnight at 4°C. Primary antibodies were rabbit polyclonal for AKR1C1, AKR1C3, and mPGES-1 (Abcam Inc, Cambridge, Ma, USA); mPGES-2, cPGES, and PGDS (hematopoietic- and lipocalin-type) (Cayman Chemical, Ann Arbor, MI); and PGIS (Sta. Cruz Biotechnology, CA, USA). Rat polyclonal for PGDS (lipocalin-type) was from Cayman Chemical (Ann Arbor, MI); COX-2 and goat polyclonal for CBR1, as well as Mouse monoclonal for COX-1, were from Abcam Inc (Cambridge, MA, USA).

The membrane was washed three times with TBST for 5 min, followed by incubation with the recommended dilution of labeled secondary antibody in 5% blocking buffer in TBST at RT for 1 hour. Then it was washed again three times with TBST for 5 min, and protein bands were visualised with ECL SuperSignal Kit (Thermo Scientific, Rockford, USA) and photographed using Fujifilm LAS-300. Membranes were stripped and re-blotted with a mouse monoclonal antihuman GAPDH or Actin (Abcam Inc, Cambridge, MA, USA) for loading control. Densitometric analyses were carried out using Fujifilm Multi Gauge V3.0 software.

5.4 Immunohistochemistry. This technique was used to localize and verify the presence of COX-(1,2) enzymes, PGs synthases, and EP1-4 and FP receptors in human endometrial samples. Formalin-fixed and paraffin-embedded endometrial biopsies were sectioned with a thickness of 5 microns and mounted on glass slides coated with Vectabond TM (Vector Lab, Burlingame, CA, USA). After deparaffinization with three passes of xylene (5min), samples were dehydrated by triplicate with ethanol 100% (5min). Samples were limited with PAP PEN and then were rehydrated in decreasing concentrations of alcohols 95% (5min), 85% (5min), and 70% (5min), followed by a washing in distilled water (1min) and 1X phosphate-buffered saline (PBS) (1min). Immunohistochemistry was performed on endometrial sections using the LSAB Peroxidase Kit (DAKO, CA, USA). Primary antibodies were the same as above, except for mPGEs-1 (PGE synthase (A3), Sta. Cruz Biotechnology, CA, USA). For PGs receptors studies were conducted using polyclonal antibodies that recognize specific EP 1-4 and FP receptors. Rabbit IgGs recognizing EP2 and EP4 (Cayman Chemical, Ann Arbor, MI, USA) and EP3 (Abcam Inc, Cambridge, MA, USA), and goat IgGs for EP1 (Abcam Inc, Cambridge, MA, USA) and FP (Sigma-

Aldrich, Irvine, UK). Antibodies were diluted at the appropriate concentration in 1% BSA in PBS. Slides were placed in a wet chamber and 20ul of the antibody-containing solution were added to the sample. The chamber was covered and placed in an incubator at 37ºC for 60 minutes. Slides were then washed twice with PBS for 10 minutes at room temperature (RT) with gentle movement. Secondary antibodies were included in LSAB Peroxidase Kit (DAKO, CA, USA), valid for rabbit, mouse, and goat origin primary antibodies. Immunostaining was then visualized with 200ul of 3,30-diaminobenzidine (DAB) chromogen. After counterstaining with hematoxylin and washing with distilled water, slides were mounted with entellan (Merck, Darmstadt, Germany) and analyzed with a Nikon Eclipse 80i microscope. For negative controls, primary antibodies were omitted and samples were incubated in DAKO Antibody Diluent.

5.5 Immunofluorescence. For immunofluorescence microscopy, blastocysts and hEEC monolayers were fixed at room temperature for 25 min in 4% paraformaldehyde buffer in PBS. Following fixation, they were washed through 2 drops of BSA, and permeabilized with 1% TritonX-100 (Sigma) for 1 hour at room temperature. Thereafter, they were incubated overnight at 4°C with the same primary antibodies of EP 1-4 and FP receptors that were mentioned in the immunohistochemistry. Blastocysts and hEEC monolayers were washed through 3 drops of BSA to remove any unbound primary antibodies. Secondary antibodies Alexa 568 conjugated goat anti-rabbit (A11079, Invitrogen Corporation, Stockholm, Sweden) was diluted in blocking buffer and added to the embryos and hEEC monolayers, and subsequently incubated at 37°C for 1h. This was followed by incubation with DAPI [40,6-diamidino-2-phenylindole]), and the sample was mounted on a slide and stored at 4°C until its use. Stained embryos and hEEC

monolayers were analyzed in a confocal laser microscope (Zeiss, Germany) equipped with fluorescence optics and appropriate filters.

6. Enzyme immunoassay (EIA)

6.1 Prostaglandin-synthase activity in EECs. EECs were grown to confluence in 96-well plates. PG synthase inhibitors were added and cells grown for up to 48 h, with a fresh-medium+inhibitors change after the first 24h. The following inhibitors were used: DRB (5,6-dichloro-1-b-D-ribofuranosylbenzimidazole) (Cayman Chemical), a CK-II inhibitor that affects cPGES activity; MK886 (3-[3-tert-Butylthio-1-(4-chlorobenzyl)-5-isopropyl-1H-indol-2-yl]-2,2 dimethylpropionic acid) (Cayman Chemical) for mPGEs-1; bimatoprost (Cayman Chemical) for AKR1C3; 1-phenyl-1-cyclopentanecarboxylic acid (Sigma) for AKR1C1; and CAY10607 (4-chloro-6-[5-[2-(4-morpholinyl)ethyl]amino]-1,2-benzisoxazol-3-yl]-1,3-benzenediol) (Cayman Chemical) for CBR1. All inhibitors were prepared in DMSO, and control cells were treated with the same volume of DMSO (vehicle). Conditioned media was collected after 24h and 48h and stored at -80°C. PGE_2, $PGF_2\alpha$ and 11β-$PGF_2\alpha$ concentration was measured in conditioned-media from inhibitor-treated EECs using appropriate ELISA kits and following the manufacturer´s instructions (Cayman Chemical). All samples were measured in duplicates.

6.2 Kinetics of prostaglandin-release recovery after PGS inhibition in EECs. EECs were grown to confluence in 96-well plates as described above, adding 50 μM of indomethacin during 48h. Then the medium was collected after 1h, 2h and 4h. Using ELISA technique (Cayman Chemical) the concentrations of PGE_2 and $PGF_2\alpha$ were measured after they were released into the environment at the different times mentioned above.

7. Analysis of PGs synthases in EF

7.1 Albumin/IgG purification of PGs synthases in EF. Endometrial fluid was resuspended in 200 ul RIPA buffer and purified using the Vivapure Anti-HAS/IgG Kit (Vivascience AG, Hannover, Germany) to specifically immunodeplete serum albumin and class G immunoglobulins (Ig). Proteins were precipitated with 15% w/v trichloroacetic acid for 1h at 48C followed by centrifugation (10 min, 16,000g, 48C), and pellets were washed with 1 ml of prechilled acetone and centrifuged again. Supernatants were discarded, and pellets air dried at room temperature and resuspended with RIPA buffer. Protein content was determined with Bradford colorimetric assay and the sample was subjected to western blot analysis to detect the relative protein amounts of CBR1 and AKR1C, which mediate the inter-conversion between PGE_2 and $PGF_2α$.

7.2 Immunoprecipitation

7.2.1 Washing Beads. Before purifying the enzymes of EF, beads must be washed (50µl beads/20µl EF). They were centrifuged 1 min at 12000 rpm and supernatant was removed. Then 250 ul of PBS was added and centrifuged at 12000 rpm for 1 min and the supernatant was removed. The process was repeated 3 to 4 times. After the last step, 500 ul of PBS was added to the beads and stored at 4ºC overnight. Before the beads were used, they were centrifuged and the supernatant was removed and washed with 100 ul IP buffer.

7.2.2 Pre-washing cell lysate. This step will reduce non-specific binding of proteins to the agarose when it is used later on in the assay. For each 20µl of EF, 100µl of washed beads that were prepared in the previous step was

added. The sample was incubated at 4ºC for 2 hours in a cold room under constant agitation. Then it was centrifuged for 15 min at 4ºC at maximum speed to remove supernatant. The supernatant was transferred to a clean tube and for each 1 ml of supernatant, 10 ul of antibody was added. The sample was incubated at 4ºC in the cold room under constant stirring overnight**.**

7.2.3. Antibody incubation with beads. New beads were used in this step (100 µl). Beads were centrifuged 1 min at 12000 rpm and the supernatant was removed. Then 100µl IP buffer was added and again centrifugated (repeated 3-4 times). After the supernantant was removed 100µl IP buffer with protease inhibitors was added. For each 1ml of supernatant incubated with the antibody (previous step) 100 ul of beads was used and incubated at 4ºC in the cold room under constant agitation for 2 to 3 h. After the incubation, the sample was centrifuged at maximum speed for 2 min and the supernatant was removed. Then 1 ml of IP buffer was added and incubated them for 20 minutes on ice. The sample was centriguated and the supernatant was removed carefully adding back IP buffer (protease inhibitors).

7.3 Enzymatic Activity. CBR1 which directly converts PGE2 into PGF2α was used for this experiment. The enzyme was purified by immunoprecipitation and incubated with different concentrations of a synthetic PGE_2 substrate (5nM, 10nM and 50nM). The production of its $PGF_2α$ product was quantified at 15, 30, 60, 90, 120, 150 and 190 min with an EIA kit.

8. Adhesion Assay

8.1 Embryo Recovery. B6C3F1 mice of 6–8 weeks of age were housed under controlled temperature and lighting 16:8-hour light:dark schedule at the University of Valencia School of Medicine and they were provided food and water *ad libitum*. Mice were primed to ovulate by intraperitoneal treatment with 10 IU equine chorionic (eCG; Sigma-Aldrich, Irvine, UK) to stimulate follicular development. Forty-eight hours later (designated hour 0), mice were injected with human chorionic gonadotropin (hCG, 10 IU; Sigma-Aldrich) to stimulate ovulation and luteinization. Females were housed with a stud male immediately after hCG administration, paired (1:1) for overnight, and checked the next morning for the presence of a vaginal plug on day 1 of pregnancy. Pregnant females were killed by cervical dislocation at day 3 of pregnancy (48h post hCG), and the embryos in the oviducts were collected using a 30-gauge needle attached to a 2-ml syringe. Embryos were cultured for 3 days in CCM-30 medium (Vitrolife, Lübeck, Germany) to achieve blastocyst stage. Embryo development and morphological change were checked and only blastocysts with normal morphology at the stage of hatching were used.

8.2 *In vitro* adhesion assay. Mouse embryos were cultured with CCM until blastocyst stage. Only blastocysts without *zona pellucida* and multicellular spheroids were used in the study. Simultaneously, confluent monolayers of human endometrial cells in 24-well plates were treated with 50 μM of indomethacin PGs inhibitor for 48h. After indomethacin treatment the medium was restored with fresh medium containing mouse blastocysts or (5-10 per well), and a specific treatment in the case of the experiments discussed in the following subsections. Control cells were treated with DMSO as vehicle instead of indomethacin. The number of mouse embryos

adhered to EEC were recorded at 1h, 2h, 4h, 24h and 32h by movement along a 3-cm diameter circular path at a speed of one rotation per second for about 10 sec under the microscope. Embryos floating in the medium were considered as non-attached, while embryos not floating were assumed to be attached. Embryos were examined under an inverted microscope (Nikon Diaphot 300, Nikon Corporation, Tokyo, Japan). Every condition was made by triplicate.

8.3 Role of PGs on adhesion assay. Mouse blastocysts were used in our adhesion assay to study the functional relevance of PGs on embryo adhesion. After inhibition of PGs using indomethacin, no specific treatment was supplemented; only blastocyst were added to prove the importance of the presence (Control group) and absence of PGs (Indomethacin). Adhesion of blastocysts was counted as mentioned above.

8.4 Effect of PGE_2 and PGF2α on embryo adhesion. To determine the effect of PGE_2 and $PGF_2\alpha$ in our *in vitro* adhesion model using mouse embryos, the restored medium was administered in the presence of increasing concentrations of PGE_2 (10nM, 100nM, 1μM, 10μM) or $PGF_2\alpha$ (1nM, 10nM, 100nM, 1μM) (Cayman Chemical). To determine the effect of both PGs a combination of the lowest concentration of PGE2 (10nM) with PGF2α (1nM) and the highest PGE2 (1μM) with PGF2α (10μM) were used. The same steps of the adhesion assay were followed.

9. PGE_2 and $PGF_2\alpha$ receptors experiments

9.1 PGE_2 and $PGF_2\alpha$ receptor agonists. Agonist for EP2: Butaprost (Sigma) at doses 20 μM, 15 μM, 10 μM, 8 μM, 5 μM (n = 6), and AS701931 (Merk-Serono) at doses 20 μM, 15 μM, 10 μM, 8 μM, 5 μM (n = 6);

Sulprostone agonist (Sigma) for EP3 with 40µM, 20 µM, 15 µM, 10 µM, 5 µM (n = 6); Fluprostenol (Sigma) for FP receptor agonist at doses 4 µM, 8 µM , 10 µM ,18 µM and antagonist AS604872 (MERK-Serono) for FP at doses

9.2 PGE_2 and $PGF_{2}\alpha$ receptor agonist and antagonists on embryo adhesion. To determine the effect of each PGE_2 and $PGF_{2}\alpha$ receptors on embryo adhesion, specific receptor agonists were added to the medium after inhibitory treatment of PGs. Moreover, we used Butaprost (Sigma) and AS701931 (Merk-Serono) as EP2 receptor agonist, Sulprostone (Sigma) as EP3 receptor agonist, and Fluprostenol (Sigma) for FP receptor agonist. Finally, antagonist AS604872 (MERK-Serono) for FP receptor was added after pretreatment with $PGF_{2}\alpha$. Control cells were treated with DMSO as vehicle instead of indomethacin.

9.3 Effect of PGE_2 and $PGF_{2}\alpha$ receptor agonists on the embryo development. Mouse blastocysts development were observed after agonists were added to the culture .

V.- RESULTS

V. RESULTS

1. Lipidomic profile of the human EF throughout the menstrual cycle

In our initial experiments, we investigated the lipidomic profile of human EFs throughout the menstrual cycle. This study was carried out in healthy female donors. All samples were collected during a natural cycle with no pharmacological interventions and the lipid extracts of EF were identified by a non-invasive technique with LC/MS/MS analysis. A total of 51 EF samples were obtained throughout the menstrual cycle and classified as: group I (days 0-8) (n = 10), group II (days 9-14) (n = 15), group III (days 15 - 18) (n = 9), group IV (19-23) (n = 9), and group V (days 24-30) (n = 8). The samples were analyzed blinded in two independent experiments.

Results obtained in the first experiment with 13 EFs showed the identification of 9 lipids in the EF including: 2-arachidonoyl glycerol, N-arachidonoyl ethanolamine, N-linoleoyl ethanolamine, N-oleoyl ethanolamine, N-palmitoyl ethanolamine, N-stearoyl ethanolamine, prostaglandin E2 (PGE_2), prostaglandin F2α ($PGF_2α$), and PGF1α (Figure 5.1).

Of the 9 lipids found in EF, only two of them PGE_2 and $PGF_2α$, were significantly increased and regulated during the WOI, specifically between days 19 to 21 (Figure 5.2). Because of those results, PGE_2 and $PGF_2α$ lipids were analyzed in a second experiment in 38 new samples of EF, and PGE_2 and $PGF_2α$ were analyzed in the five different stages of the menstrual cycle.

Results demonstrated that PGE_2 values ranged from 0.09±0.03 nmol/g (group II) to 2.12±1.2 nmol/g (group III), except during the WOI (group IV), when the values increased to 6.10±1.88 nmol/g. However, the maximum increase was observed for $PGF_2α$, as levels in the groups I, II, III and V

varied from 0.10±0.04 nmol/g (group II) to 3.83±1.61 nmol/g in group III, and in group IV it reached 21.84±6.41 nmol/g. Therefore, PGE_2 and $PGF_2\alpha$ levels in the WOI were significantly increased compared to any other phase of the menstrual cycle ($p<0.01$, Kruskal-Wallis test) (Figure 5.2).

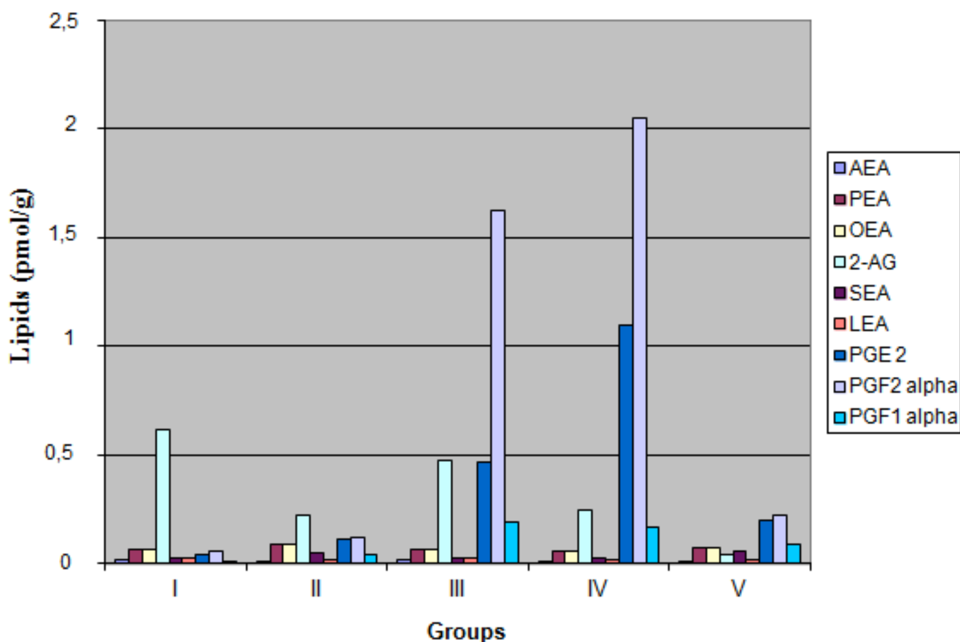


Figure 5.1. Lipids from endometrial-fluid extracts of women, during their natural menstrual cycle, identified by liquid chromatography combined with tandem mass-spectrometry [A: N-arachidonoyl ethanolamine (AEA); B: N-palmitoyl ethanolamine (PEA); C: N-oleoyl ethanolamine (OEA); D: 2-arachidonoyl glycerol (2- AG); E: N-stearoyl ethanolamine (SEA); F: N- linoleoyl ethanolamine (LEA); G: prostaglandin E2 (PGE2); H: prostaglandin F2 alpha ($PGF_2\alpha$); I: prostaglandin Fl alpha (PGFlα)]

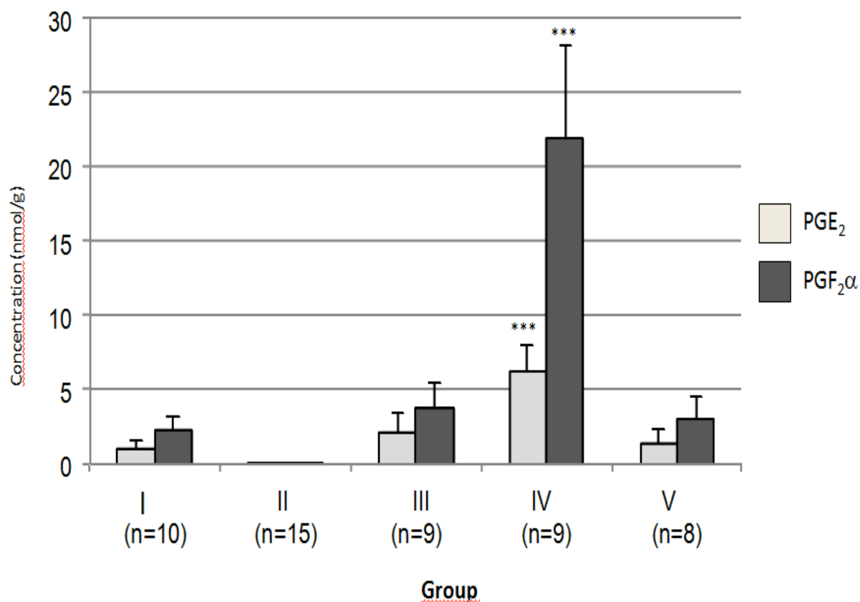


Figure 5.2 Levels of prostaglandin E2 (PGE2) and prostaglandin F2 alpha (PGF2α) in endometrial-fluid samples from women obtained throughout the menstrual cycle [Group I (days 0-8); Group II (days 9-14); Group III (days 15-18); Group IV (days 19-23) and Group V (days 24-30)].

1.1 Lipidomic profile of human endometrial fluid in HRT, COS and HRT + IUD

Because PGE_2 and $PGF_2\alpha$ were observed in the lipidomic profile studies in human endometrial fluid, we designed a series of studies to assess the reproducibility of this biomarker profile for the acquisition of endometrial receptivity under different hormonal conditions used in ART as well as in the induction of a refractory endometrium by inserting an intrauterine device (IUD). We analyzed the lipidomic secretomic profile in 30 ovum donors undergoing first a hormonal replacement therapy (HRT) cycle, then a controlled ovarian stimulated (COS) cycle, and finally a HRT cycle with the insertion of an IUD (HRT/IUD) inducing a refractory endometrium. Endometrial fluid was then analyzed during the following days, in five different patients, in HRT (P+0, P+1, P+3, P+5, P+7, P+9), COS (hGC+0, hCG+3, hCG+5, hCG+7, hCG+9, hCG+11), and HRT with the insertion of an IUD (P+0/ IUD, P+1/IUD, P+3/IUD, P+5/ IUD, P+7/IUD), as it is schematically shown in Figure 5.3.

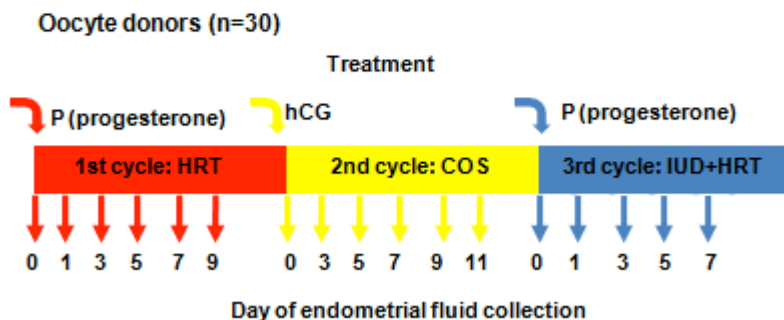


Figure 5.3. A schematic depiction of the treatments to obtain the EF samples used for the analysis of the lipidomic profile. First, a hormonal replacement therapy (HRT) cycle was induced, followed by a controlled ovarian stimulated (COS) cycle, and lastly an HRT cycle with the insertion of an IUD (HRT/IUD) inducing a refractory endometrium.

The analysis of their lipidomic profile further demonstrates that PGE_2 values ranged from 0.38±0.13 to 5.94±2.06 nmol/g and $PGF_2\alpha$ from 0.62±0.33 to 14.21±12.27 nmol/g, among the 6 different times investigated (n=30).

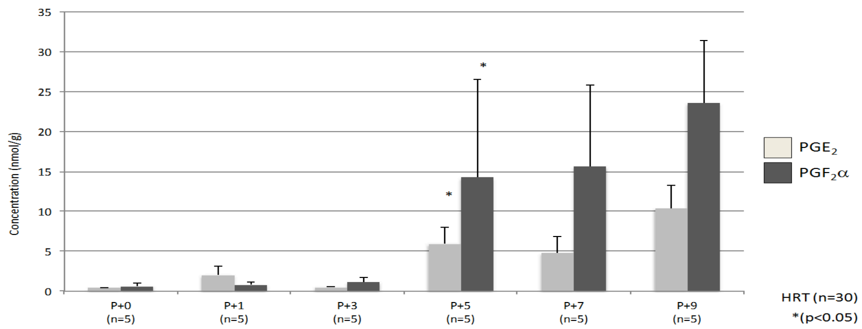
Significant differences were found between P+0 and P+5 (PGE_2 $p<0.01$ and $PGF_2\alpha$ $p<0.05$) (U de Mann-Whitney test) (Figure 5.4A).

In COS cycles, we observed that PGE_2 oscillated from 0.28±0.09 to 2.09±0.99 nmol/g, and $PGF_2\alpha$ values from 0.22±0.12 to 5.60±4.42 nmol/g among the 6 different days investigated (n=30). PGE_2 and $PGF_2\alpha$ were significantly different between hCG0 and hCG7 ($p<0.05$) (Figure 5.4B).

Interestingly enough, after the insertion of an IUD in HRT cycle, the opposite trend was registered with a significant decrease in the secretion of both PGs throughout the 5 different time points investigated (n=25), ranging from 2.69±1.07 nmol/g at P+0/ IUD to 1.94±0.46 nmol/g at P+5/ IUD for PGE_2 and 1.24±0.48 nmol/g to 0.41±0.12 nmol/g for $PGF_2\alpha$ (n=25) (Figure 5.4C).

In conclusion, PGE_2 and $PGF_2\alpha$ levels in endometrial fluid are compatible with putative biomarkers for the acquisition of endometrial receptivity in natural, HRT and COS cycles.

A.



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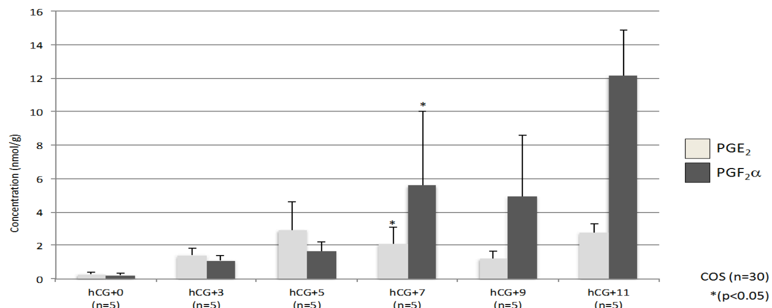

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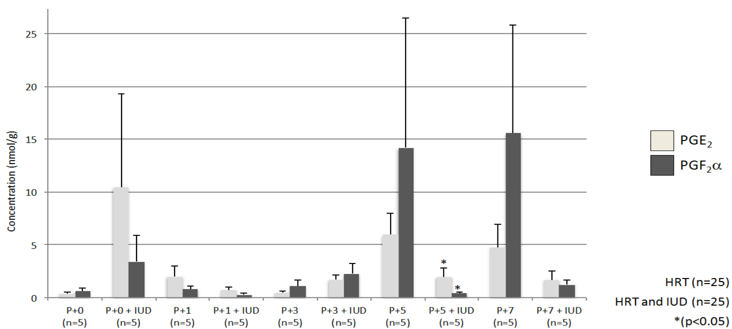


Figure 5.4. Concentration of PGE_2 and $PGF_2\alpha$ in EF samples of ovum donors in (A) HRT and (B) COS cycle and (C) HRT/IUD. Values are expressed as mean (± SEM). The increase (A,B) and the decrease (C) were statistically significant ($p<0.05$).

2. Investigation of PGs biosynthesis in human endometrium

2.1 Localization of COX enzymes

Since PGE_2 and $PGF_2\alpha$ are differentially secreted in the EF during the WOI, we proceed to investigate the origin and regulation of these PGs, studying the enzymes responsible for their production.

Cyclooxygenase is the rate-limiting enzyme in prostaglandin (PG) biosynthesis. However, studies in mice have described that COX-2 isoform is principally involved in normal blastocyst implantation due to its role in COX-2 derived PGs. Therefore, to document COX-1 and COX-2 in human endometrium, immunohistochemistry analyses were performed using human biopsies in pre-receptive (LH+2) and receptive (LH+7) stages.

Our results indicate that COX-1 expression were present in luminal and glandular endometrial epithelial cells, as well as stroma, in LH+2 and LH+7 stages with no significant intensity variations between both stages (Figure 5.5).

Expression of COX-2 showed a different pattern, while at LH+2 it was mostly localized in luminal epithelium and with lesser extent in the stroma compartment, at LH+7 expression increase specifically in luminal epithelium (Figure 5.5).

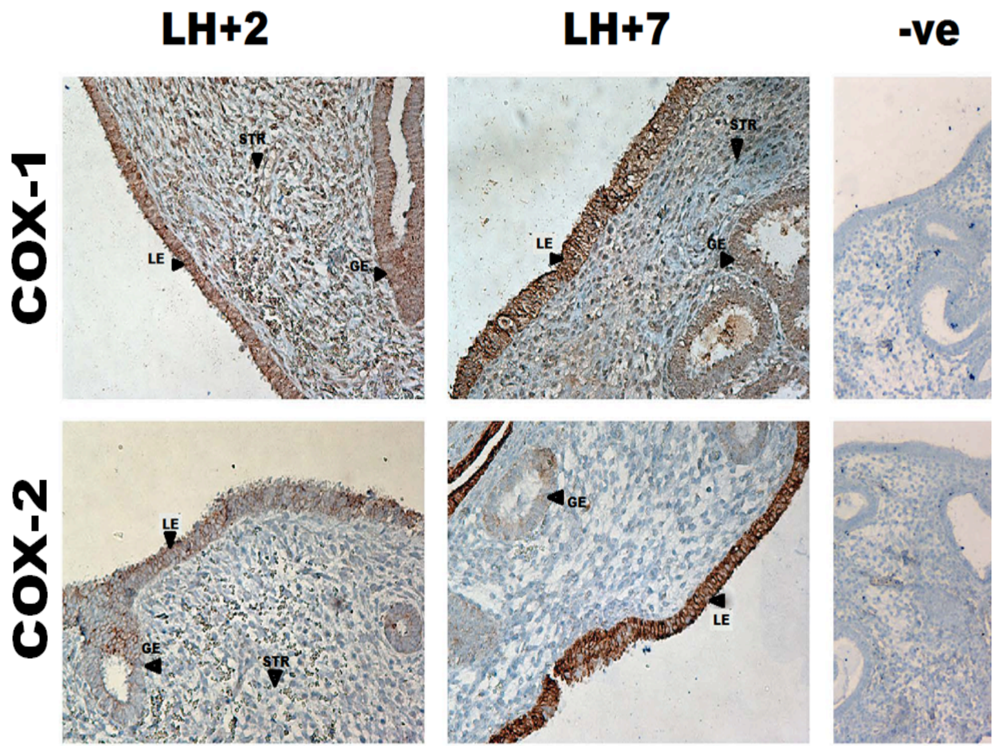


Figure 5.5 Immunohistochemical localization of COX-1 and COX-2 in human endometrium of patients in LH+2 (pre-receptive stage) and LH+7 (receptive stage). Positive staining is shown in brown. –ve: negative control; le: luminal epithelium; ge: glandular epithelium; str: stroma.

2.2 Expression and localization of endometrial PG synthases

We also investigated the presence of terminal prostaglandin synthases that act downstream of COX enzyme to catalyze the conversion of PGH2 into PGS (a scheme of the prostaglandin synthases is presented in Figure 5.6). We investigated at the mRNA and protein levels the most relevant PGE_2 synthases (cPGES, mPGES-1 and mPGES-2) and $PGF_2α$ synthases (AKR1C3 and CBR1), as well as AKR1C1 (that catalyzes a reversible reaction to interconvert PGE2 to $PGF_2α$), in human endometrial epithelium (hEEC) compared to the whole endometrium across the menstrual cycle.

Moreover, to obtain a quantitative RNA transcriptional profile, we performed qPCRs. In the complete endometrium, we found no major variations in the expression levels of PGE_2 synthases throughout the menstrual cycle (Figure 5.7). It must be pointed out as well, that the level of expression of mPGES-1 was always within the lower threshold of detection, indicating that this gene is expressed at very low levels in human endometrium. However, as expected, mRNA levels were higher when the epithelial compartment was analyzed separately, as it is primarily responsible for PGs production. When transcriptomic data was analyzed in detail, we found that enzymes leading to $PGF_2α$ synthesis (AKR1C3, AKR1C1 and CBR1) presented higher levels of expression compared to those synthesizing PGE_2 (mPGES2, mPGES1 and cPGES). These results are consistent with the higher concentrations of $PGF_2α$ detected by lipidomics in EF between days 19 and 23 of the menstrual cycle (Figure 5.7).

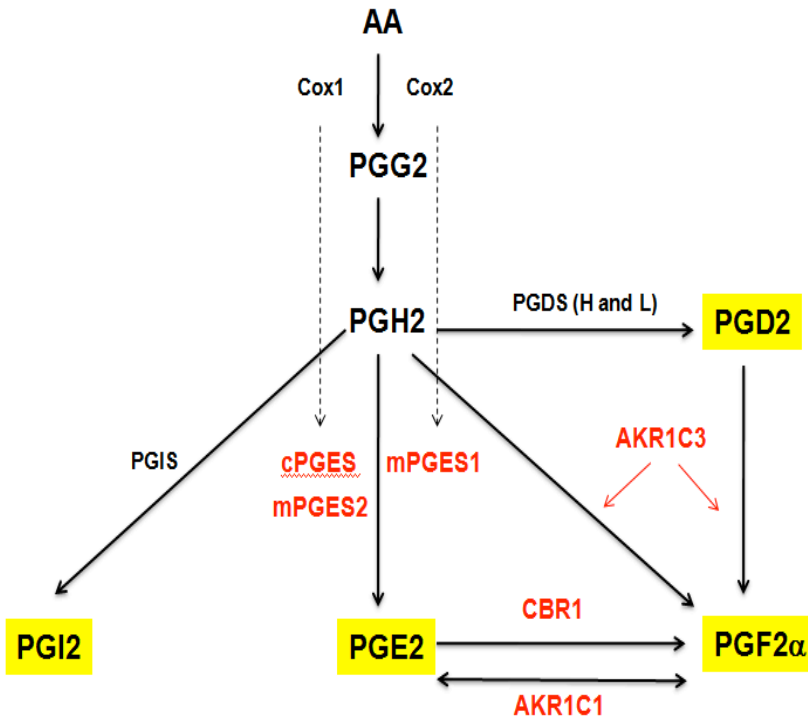


Figure 5.6. Overview of PGE_2 and $PGF_2α$ biosynthesis by their specific synthases. Arachidonic acid is metabolized by the cyclooxygenase (COX-1 and COX-2) and subsequently by specific synthases. PGE_2 is produced directly from PGH_2 by three different prostaglandin E synthases (PGES): microsomal PGES-1 (mPGES-1); cytosolic PGES (cPGES); and microsomal PGES-2 (mPGES-2). $PGF_2α$ can be synthesized by three different enzymes: aldo-keto reductase family 1, member C3 (AKR1C3); carbonyl reductase 1 (CBR1); and aldo-keto reductase family 1, member C3 (AKR1C1) that catalyzes a reversible reaction that interconverts PGE2 to $PGF_2α$.

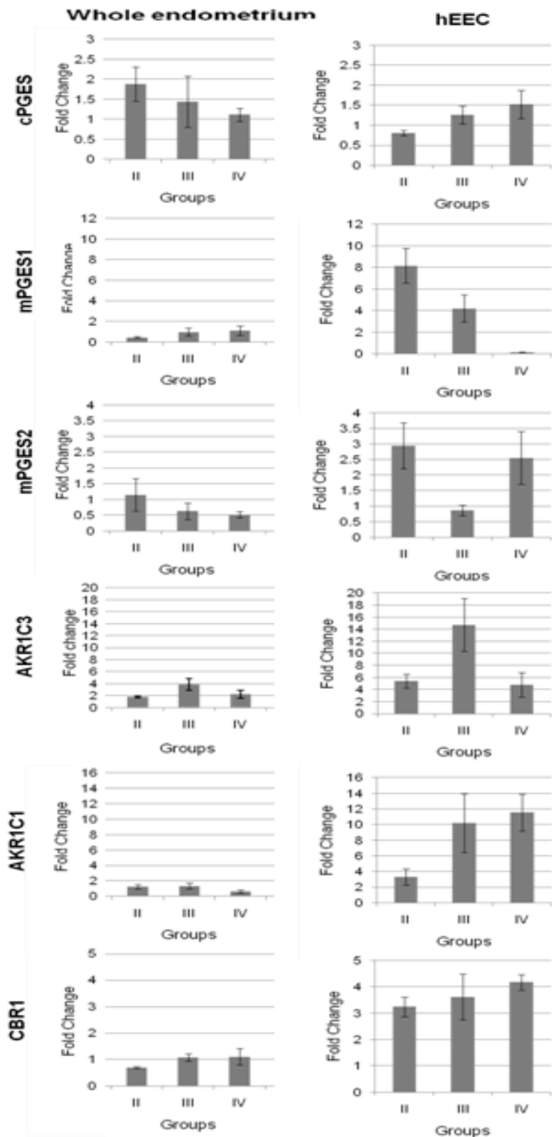


Figure 5.7. Comparative fold change of gene expression of prostaglandin E synthases (mPGES-1, cPGES and mPGES-2) and prostaglandin $F_2\alpha$ synthases (AKR1C3,CBR1, AKR1C1) in group II (days 9-14); group III (days 15-18); and group IV (days 19-23) between the whole endometrium and human endometrial epithelial cells (hEEC) obtained by using quantitative PCR.

To learn more about the regulation and location of these synthases in the endometrium, especially during the WOI, immunohistochemistry and western blot techniques were used. Immunohistochemical localization studies of the PG synthases in LH+2 versus LH+7 endometrium revealed that all enzymes tested were present at both stages. Nonetheless, cPGES staining decreased at LH+7 in luminal and glandular epithelium, the same was observed for mPGES-1, but only in luminal compartment. In contrast, both CBR1 and AKR1C1 enzymes were specifically increased at LH+7. However, CBR1 increased its location in the luminal and also in the glandular epithelium. Interestingly, AKR1C3 location shifted from luminal and glandular epithelium to exclusively luminal epithelium in receptive stage. On the other hand, mPGES-2 was expressed in luminal, glandular and stromal cells where the intensity of the signal appeared to be similar at LH+2 and LH+7 (Figure 5.8).

To determine the PG synthases protein levels in pre-receptive and receptive endometrium, western blot were employed using endometrial epithelium (EEC) from HRT cycles (P+0, the pre-receptive stage, and P+5, the receptive one). Results demostrated that while the enzymes responsible for PGE_2 production (cPGES, mPGES-1, and mPGES-2) did not change significantly between P+0 and P+5, AKR1C1, AKR1C3 and CBR1 increased in the receptive endometrium (Figure 5.9A & 5.9B). Increase of AKR1C3 and CBR1 in receptive endometrium parallels the sharp rise of $PGF_2\alpha$ in the E.F. during the implantation window. AKR1C1, as a reversible enzyme, can work as $PGF_2\alpha$ and/or PGE_2 synthase and is responsible for the production of both.

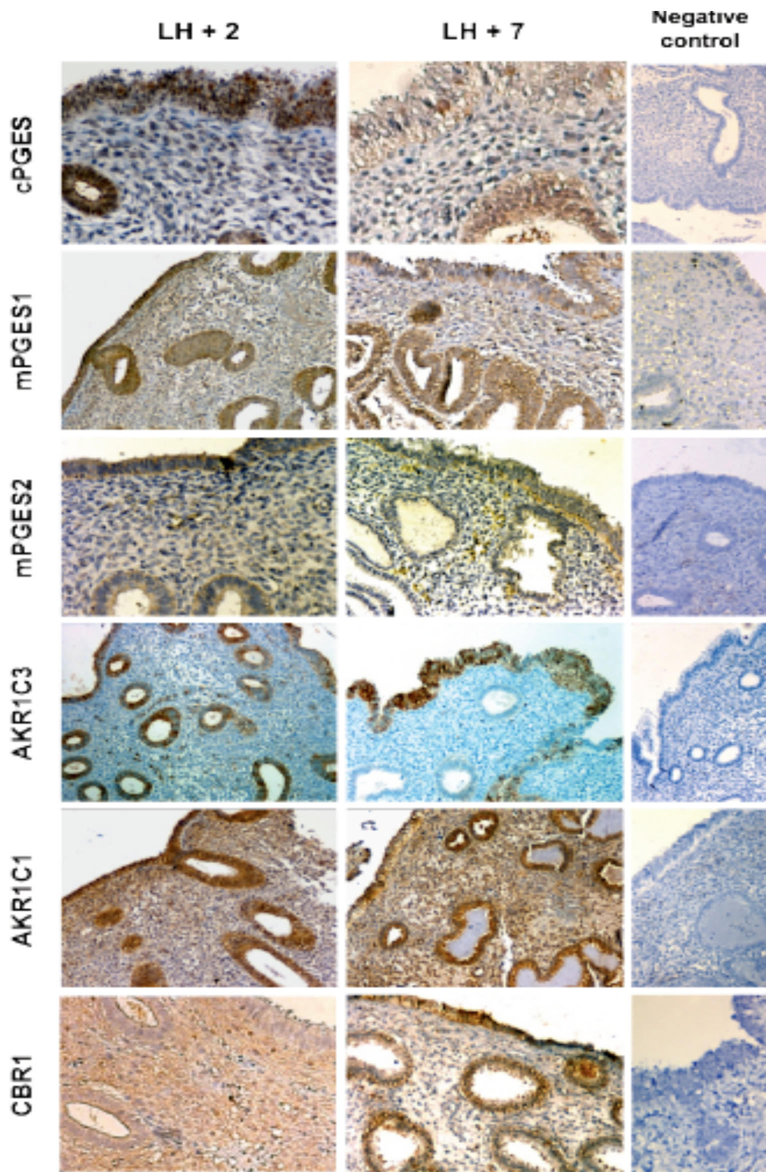
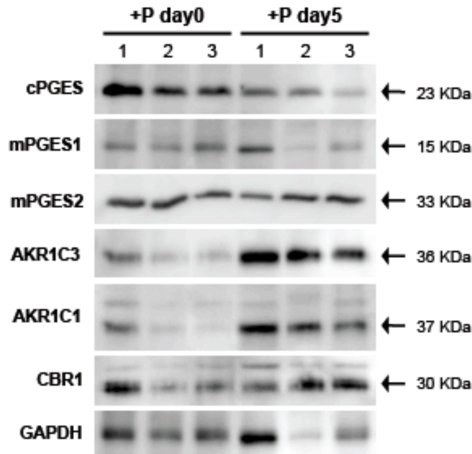


Figure 5.8. Immunohistochemical localization of cPGES, mPGES-1, mPGES-2, AKR1C3, CBR1, and AKR1C1 in pre-receptive (LH+2) versus receptive endometrium (LH+7).

A.



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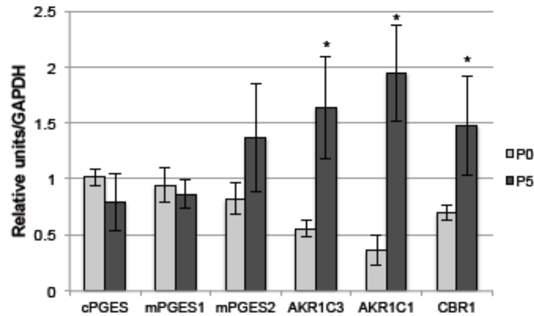


Figure 5.9. (A) Western Blotting analyses of PG synthases: cPGES, mPGES-1, mPGES-2, AKR1C1, AKR1C3, and CBR1 in the endometrial epithelium in HRT cycles (n=3) of non-receptive (P+0) versus receptive (P+5) endometrium. (B) Vertical bars represent the relative amounts of protein levels of the WB analysis, quantified by densitometry. Statistically significant increase in P+5 is expressed as *p<0.05 (Student's t-test).

2.3 Functionality of PG synthases in the endometrium

To determine the enzymes displaying prostaglandin synthase activity in endometrium, we measured their ability to mediate the synthesis of PGE_2 and $PGF_2\alpha$ in EECs *in vitro* using specific inhibitors. Semiquantitative PCR was used to confirm the expression of all enzymes in primary cultures of human EEC (Figure 5.10).

Then, to verify their functionality hEECs were first treated with different concentrations of the following enzyme-specific inhibitors. Indomethacin, a COX-1 and COX-2 inhibitor that blocks the synthesis of the whole PGs synthesis pathway, was tested at 5μM and 50μM. To evaluate terminal prostaglandin synthases downstream COX enzyme for the production of PGE_2, cPGES inhibitor DRB (1-β-D-ribofuranosylbenzimidazole) was tested to avoid the conversion from PGH_2 to PGE_2, with concentrations 1mM and 10mM, and the same test was performed for mPGEs-1 inhibitor MK886 (3-[3-tert-Butylthio-1-(4-chlorobenzyl)-5-isopropyl-1H-indol-2-yl]-2,2 dimethylpropionic acid 2) with concentrations of 10μM and 100μM. Moreover, because PGE can also be generated from $PGF_2\alpha$ through the action of AKR1C1, cyclopentane was used at 1μM and 10μm as AKR1C1 inhibitor. On the other hand, $PGF_2\alpha$ can also be synthesized from different precursors, including PGH_2 and PGD_2 through the action of AKR1C3, or from PGE_2 in a reaction mediated by CBR1 and AKR1C1.

Also, CBR1 inhibitor 4-chloro-6-[5-[2-(4-morpholinyl)ethyl]amino]-1,2-benzisoxazol-3-yl]-1,3-benzenediol (CAY10607) was tested at 30nM and 300nM, and AKR1C3, which mediates synthesis of 11β- $PGF_2\alpha$, was used as a bimatoprost inhibitor at 50μM and 500μM. Subsequently, after 24h and 48h

of treatment, conditioned media was collected and assayed for the inhibitory effect of PGE_2 and $PGF_2\alpha$ production by ELISA technique.

Results indicate that PGE_2, $PGF_2\alpha$ and 11β- $PGF_2\alpha$ (a $PGF_2\alpha$ isoform) concentrations in vehicle-treated cells media were 153±0.8, 76±8.23, 37.8±0 pg/ml respectively at 24h-treatment, and 113±2, 51.5±1.7 and 30.7±2.8 pg/ml at 48h-treatment, demonstrating that EEC are capable of producing PGs under basal conditions (Figure 5.11A, 5.11B & 5.11C). Treatment with 50μM indomethacin blocked almost completely the production of PGE_2, $PGF_2\alpha$, and 11β-$PGF_2\alpha$, which served as proof-of-principle for the functionality of the assay. In addition, the results showed no differences in levels of PGE_2 and $PGF_2\alpha$ after adding inhibitors at 24h-treatment in most of them.

Meanwhile, a different pattern was observed after 48h with a reduction in PGs production, especially in cPGES and mPGES-1 (Figure 5.11A). Likewise, AKR1C3 displayed PGF synthase activity, measured here by its ability to produce 11β-$PGF_2\alpha$ (Figure 5.11C). Conversely, inhibition of AKR1C1 and CBR1, which interconvert PGE_2 and $PGF_2\alpha$, had minor impact on $PGF_2\alpha$ production (Figure 5.11B). Moreover, CBR1 inhibition with 30nM of CAY10607 led to an increase in PGF2a. However, several concentrations tested, reveal toxic effect on hEEC proliferation.

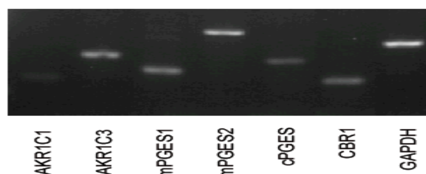
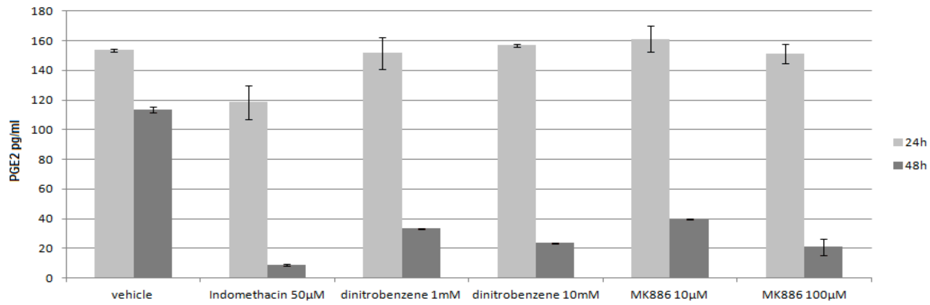


Figure 5.10. Semiquantitative RT-PCR analysis of prostaglandin E synthases and prostaglandin $F_2\alpha$ synthases in primary human EEC cultures.

A.



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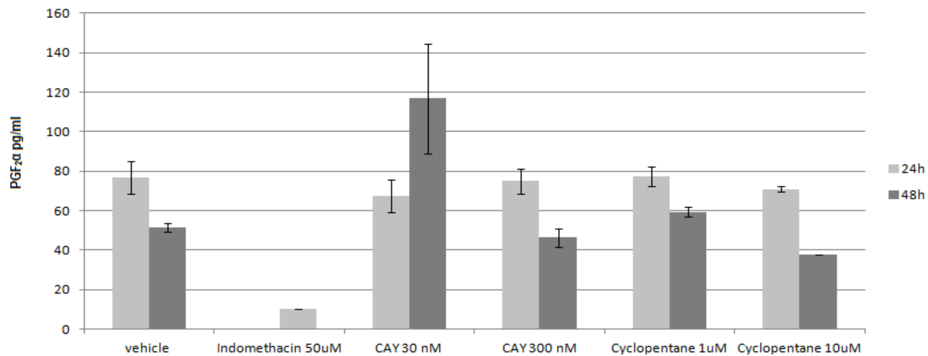

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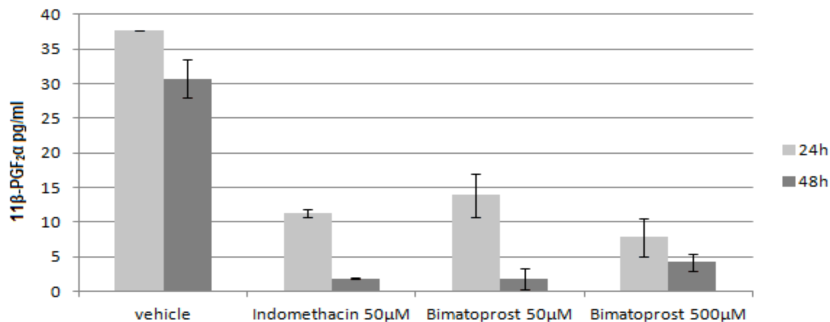


Figure 5.11. Enzymatic activity of individual PG synthases in EECs tested using specific inhibitors and measuring PG concentration in conditioned media by ELISA. (A) Graphs represent the concentration of PGE_2 measured 24h & 48h after hEEC were pre-treated with DRB and MK886 for the inhibiton of cPGES and mPGES-1 respectively. (B) Concentration of $PGF_2\alpha$ measured after CAY and cyclopentane were used for the inhibiton of CBR1 and AKR1C1, respectively and (C) Concentration of 11β-$PGF_2\alpha$ measured after bimatorprost was used for the inhibiton of AKR1C3.

The experiment was repeated using concentrations with a maximal inhibition on PGs in the 48h-treatment that did not cause toxicity to hEEC. A pool of six PG-inhibitors was employed, and results analyzed again for both PGE_2 and PGF_2.

Inhibition of mPGES-1, CBR1, and AKR1C1 using MK886, CAY10607, and cyclopentane, respectively, decreased the concentration of PGE_2. Differences were not significant when compared to the basal conditions similarly to increased $PGF_2\alpha$ production induced by the DRB (Figure 5.12B). These results suggest that since PGE_2 synthesis takes place by the action of different enzymes, others might compensate a deficiency in any of them.

However, different results were obtained in terms of $PGF_2\alpha$ production. Only a specific CBR1 inhibitor (CAY10607) was capable to nearly block $PGF_2\alpha$ synthesis, indicating that this enzyme has an important contribution in PGF_2 production from PGE_2 (Figure 5.12B). Mediation of the synthesis of 11β-$PGF_2\alpha$ and $PGF_2\alpha$ by AKR1C3 was demonstrated by the blockade of its secretion with bimatoprost, which inhibited the production of 11β-$PGF_2\alpha$ as much as indomethacin (Figure 5.12C). This points to AKR1C3 being one of the main responsible enzymes for $PGF_2\alpha$ production.

For the remaining enzymes inhibitors an opposite effect was observed. MK886 and DBR inhibitors that were used to avoid the conversion from PGH_2 to PGE_2 showed a significant increase in $PGF_2\alpha$ production, similarly to what happens with cyclopentane, which inhibits the interconversion between the two PGs (Figure 5.12B), indicating that in response to acute inhibition of these enzymes, the influence of PGE_2 and $PGF_2\alpha$ derived enzymes is exaggerated, compensating the absence specific enzymes to maintain basal conditions.

To completely avoid the production of both PGs, different combination of PGE_2 and $PGF_2\alpha$ enzymes inhibitors were used. The results showed a negative effect in the survival and proliferation of EECs, indicating that both PGs could be implicated in pathways that regulate growth of the EECs.

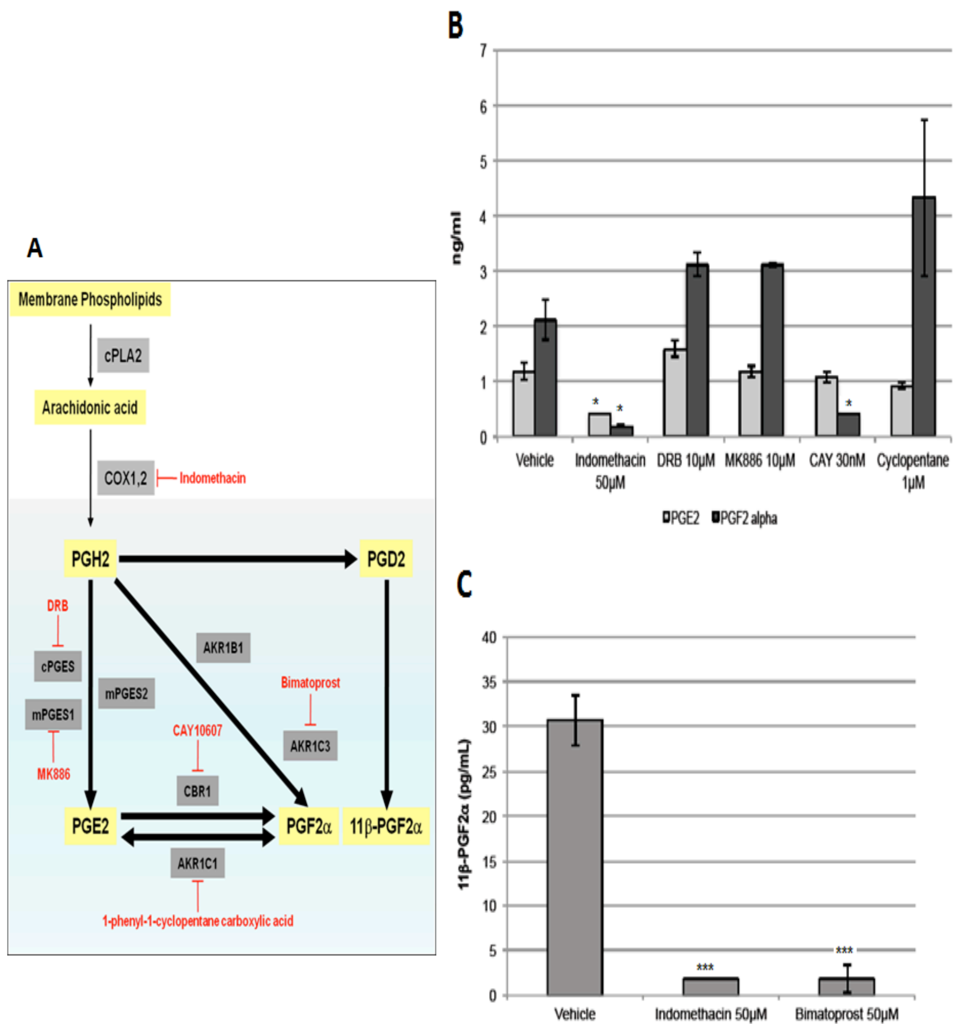


Figure 5.12. (A) Schematic diagram of prostaglandin E synthases and prostaglandin $F_2\alpha$ synthases and their specific pharmacological inhibitors. (B) Specific inhibition of individual PG synthases (mPGES-1, cPGES, CBR1, and AKR1C1) in EEC cultures and PGE_2 and $PGF_2\alpha$ concentration measured in the conditioned media. (C) Specific inhibition of AKR1C3 in EEC cultures using bimatoprost, and 11β-$PGF_2\alpha$ concentrations measured in the conditioned media. The significant decrease is statistically expressed as *$p<0.05$ and ***$p<0.001$ (Student's t-test).

3. Analysis of PGE_2 and $PGF_2α$ receptors in the endometrial receptivity

3.1 Localization of PGE_2 and PGF_2 receptors in human endometrium

PGE_2 and $PGF_2α$ exert their biological function through interaction with their corresponding receptors, EP and FP, that have been determined to be implicated in many reproductive functions (Blesson *et al*. 2012). Among the PGs, the only one that possesses four receptors subtypes is EP receptor: EP1, EP2, EP3, EP4 (EP1-4), that have been classified according to their response to specific agonists and antagonists of PGE_2 (Figure 5.13) (Lim *et al.* 1999). However, their distribution in human endometrium and/or embryo during the menstrual cycle state remains unknown.

Localization of EP1-4 and FP at the protein level was investigated using inmunohistochemistry analysis of endometrial epithelium in HRT cycles (at P+0 and P+5), and in natural cycles (at LH+2 and LH+7), representing the pre-recetive and receptive stages. In the endometrial epithelium, at P+0 EP1 and EP3 receptor subtypes were moderately expressed in glandular and luminal epithelium, although EP1 presented major intensity in the glandular compartment (Figure 5.14). At P+5 expression of EP2 and FP increased. In comparison to other EP subtypes, they were prominently expressed in stroma, luminal and glandular compartment during pre-receptive as well as in receptive stage. However, there was no expression of EP2 in the stroma during the receptive stage and FP staining was more intense (Figure 5.15). In contrast, EP4 was only detected in vessels at P+0 and P+5 with a weak expression in luminal and glandular epithelium (Figure 5.14)..

Immunohistochemistry in the endometrial epithelium of natural cycles revealed that EP1 and EP3 present a weak staining in luminal and glandular

epithelium at LH+2. EP3 presented a consistent signal at LH+7 compared to LH+2 in the same compartments, EP1 was only constant in glandular epithelium, but not in luminal, as its expression increased in LH+7. The pattern of staining in EP2 was similar in LH+2 and LH+7 in stroma and glandular epithelium. However, compared to luminal epithelium, its expression was detected in both stages with stronger intensity in LH+7. EP4 was present in endometrial epithelium and vessels, luminal and glandular epithelium in both stages (Figure 5.16). In contrast, FP expression increased significantly in LH+7 in stroma, luminal and glandular epithelium (Figure 5.17). These results point to EP2 and FP as the main receptors implicated in the receptive phase of the human menstrual cycle.

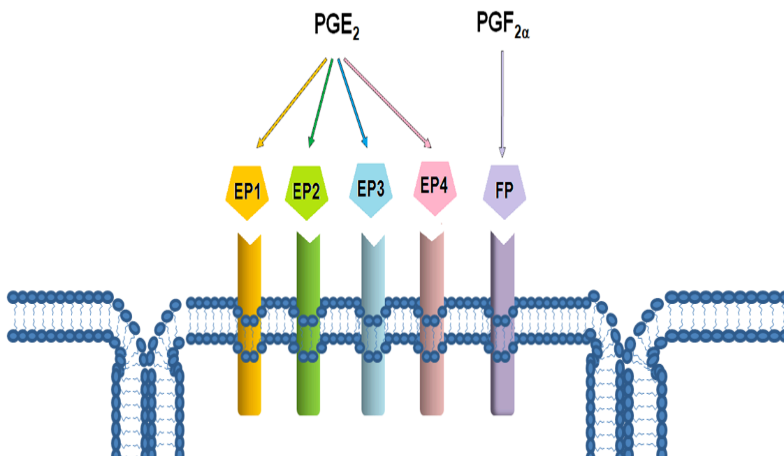


Figure 5.13. Schematic depiction of PGE_2 and $PGF_2\alpha$ /receptors

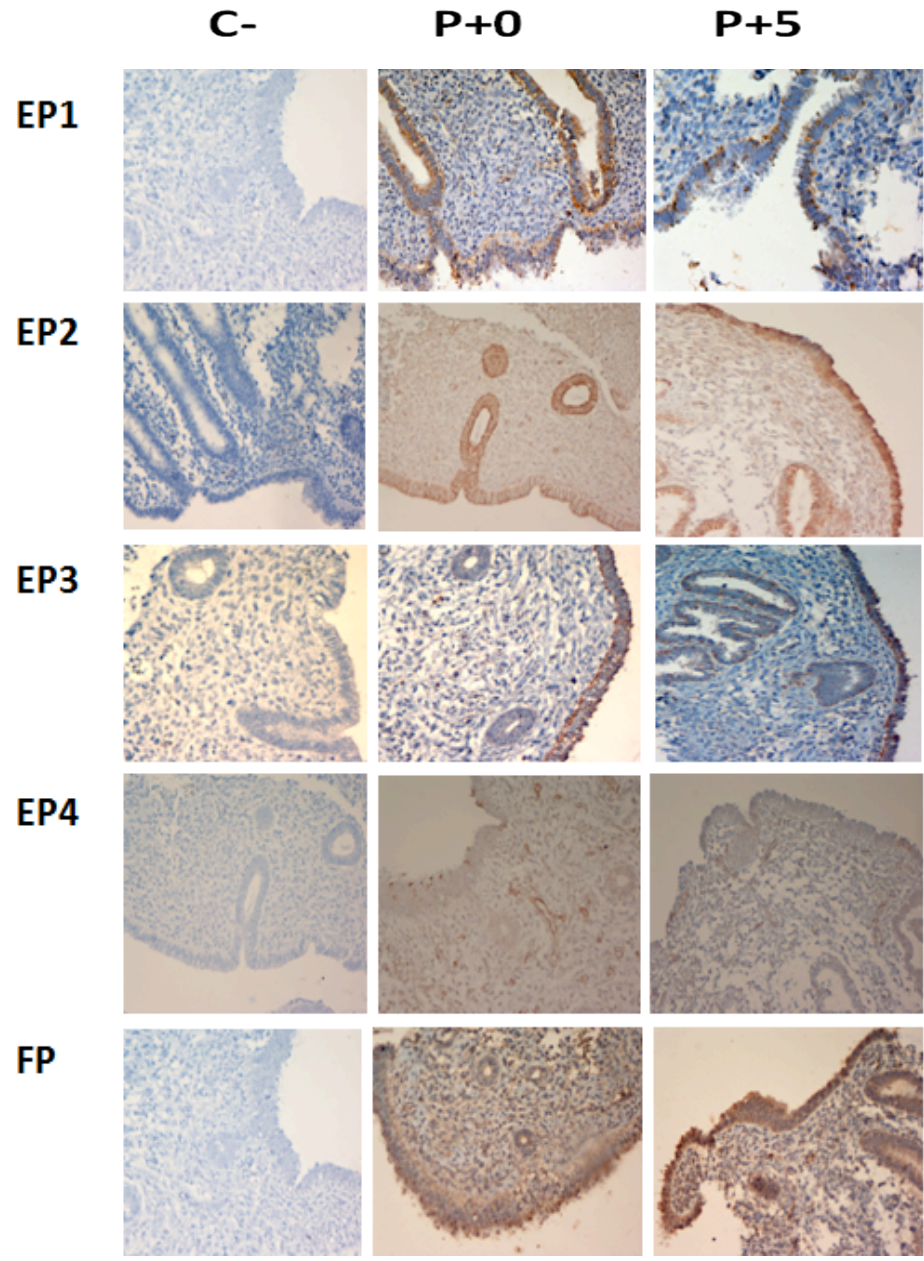


Figure 5.14. Immunohistochemical detection of of PGE_2 receptors (EP1, EP2, EP3 and EP4) and $PGF_2\alpha$ receptor (FP) proteins in the endometrial epithelium in HRT cycles of non-receptive (P+0) versus receptive (P+5) endometrium.

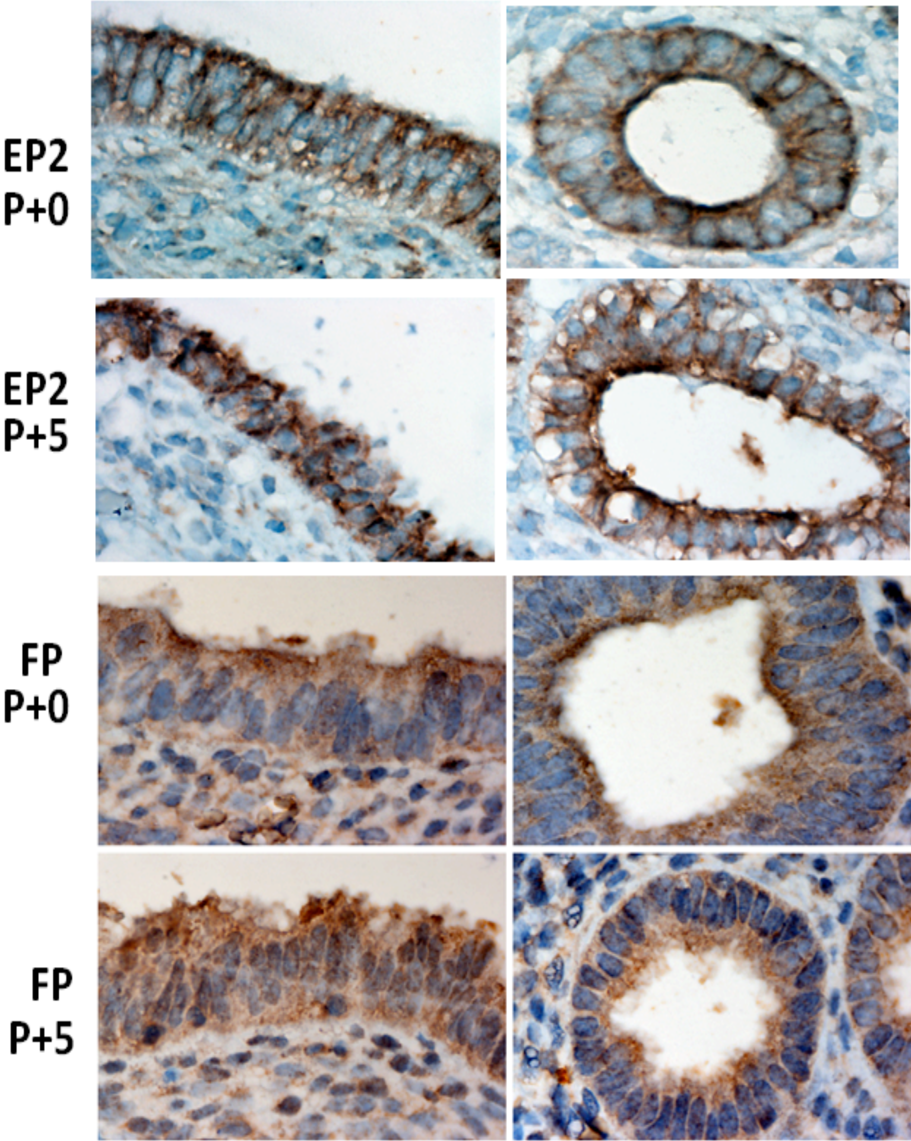


Figure 5.15. Localization of EP2 and FP protein in luminal and glandular epithelium in HTR cycles of P+0 and P+5 endometrium. Representative photomicrographs at 100X magnification

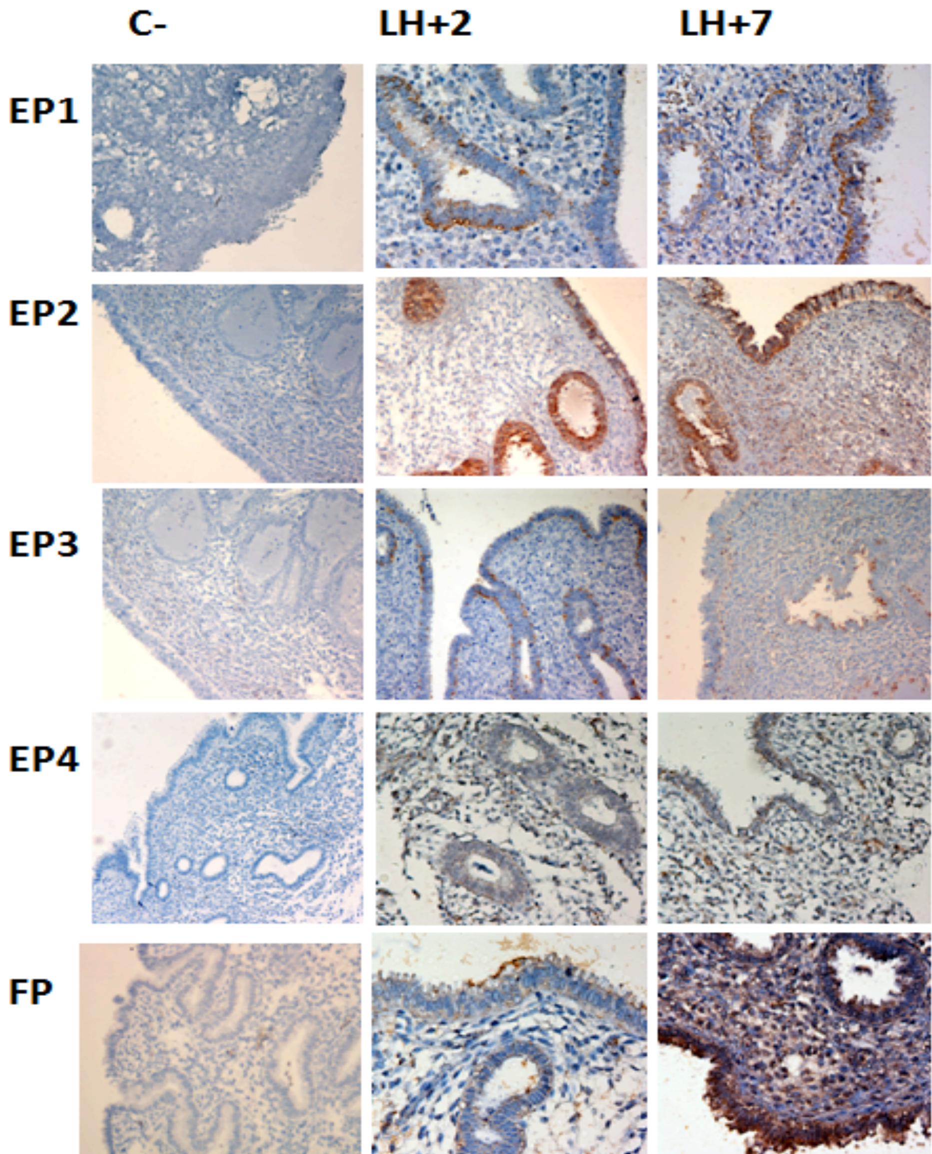


Figure 5.16. Immunohistochemical detection of of PGE_2 receptors (EP1, EP2, EP3 and EP4) and $PGF_2\alpha$ receptor (FP) proteins in endometrial epithelium in natural cycles of pre-receptive (LH+2) versus receptive (LH+7) endometrium.

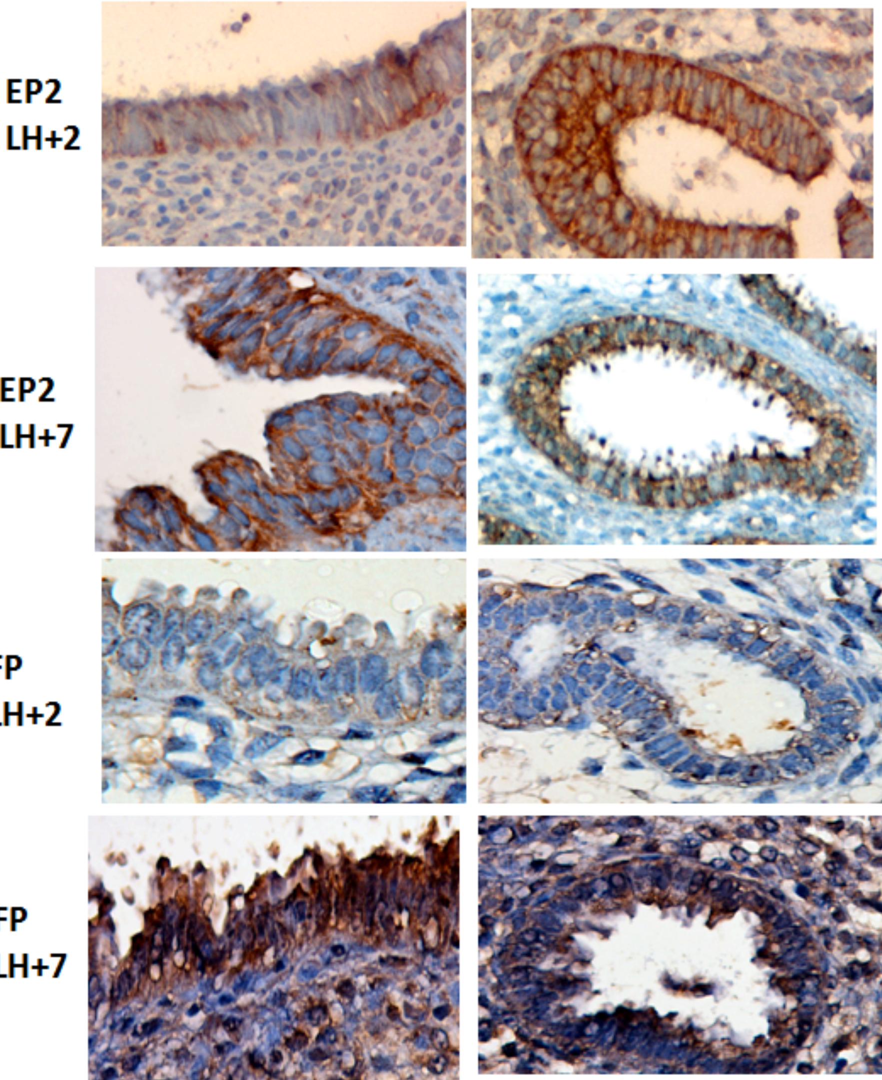


Figure 5.17. Localization of EP2 and FP proteins in luminal and glandular epithelium in natural cycles of LH+2 and LH+7 endometrium. Representative photomicrographs at 100X magnification

3.2 Identification and localization of PGE_2 and $PGF_2\alpha$ receptors in human endometrial cell lines and mouse embryos

RT-PCR was used to study whether PGE_2 (EP1-4) and $PGF_2\alpha$ (FP) receptors were expressed in both human endometrial cell lines (adhesive RL95-2 and non-adhesive HEC-1-A) and primary human epithelial cells. Cell lines and epithelial cells were grown in monolayers, representing *in vitro* the layer in which the embryo first interact with the endometrium during its adhesion process.

After 40 cycles of PCR no PGE_2 and $PGF_2\alpha$ receptors product was expressed in RL95-2 cells and HEC-1-A, with the exception of FP in HEC-1-A. These results indicate that both cell lines were not the best model to be used in further adhesion experiments with PGs. However, in primary hEEC all PGs receptors were expressed at the pre-receptive (day 15), receptive (day 20), and post-receptive (day 25), stages of the menstrual cycle. Neverthless, EP3 and EP4 showed lower expression in the three stages compared to the other receptors. JAR cells only presented expression for EP1 and EP2 receptors. For normalization, we used the level of the housekeeping GAPDH (Figure 5.18).

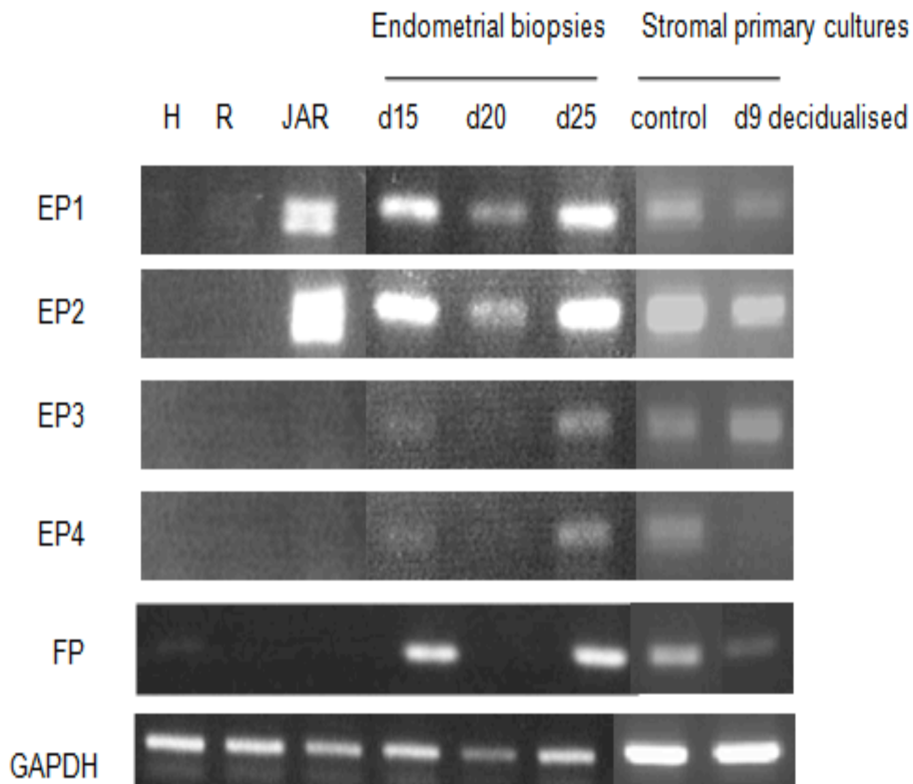


Figure 5.18. Semi-quantitative RT-PCR analysis of mRNA expressions of PGE_2 (EP1-4) and $PGF_2\alpha$ (FP) receptors in RL95-2 (adhesive), HEC-1-A (non-adhesive), JAR cells (throphoblast properties), and human endometrial epithelial cells obtained from biopsies in day 15, day 20, day 25. GAPDH was used as housekeeping gene.

Immunofluorescence on hEEC monolayers for all EP1-4 and FP was positive. While EP2 and FP presented the highest signal intensity, EP1 was almost undetectable. However, FP showed an increased intensity in cell to cell contact sites, compared to PGE receptors, and no staining was observed in control experiments (Fig.. 5.19A). On the other hand, in JAR cells only EP1 and (especially) FP presented positive staining (Figure 5.19B).

In mouse blastocyst, all receptors were expressed in trophoectoderm and inner cell mass. However, their cell localization and intensity varied among receptors. While EP2, EP3 and FP were clearly detected in the cytoplasm and in the cell membrane of the trophoblast and inner cell mass of the embryo, EP1 was only observed in the nucleus and EP4 in the cytoplasm. EP2 also showed a weak signal in the nucleus, while fluorescence signal of EP2 and FP in blastocysts was significantly stronger than the other receptors, especially FP (Figure 5.20).

A B

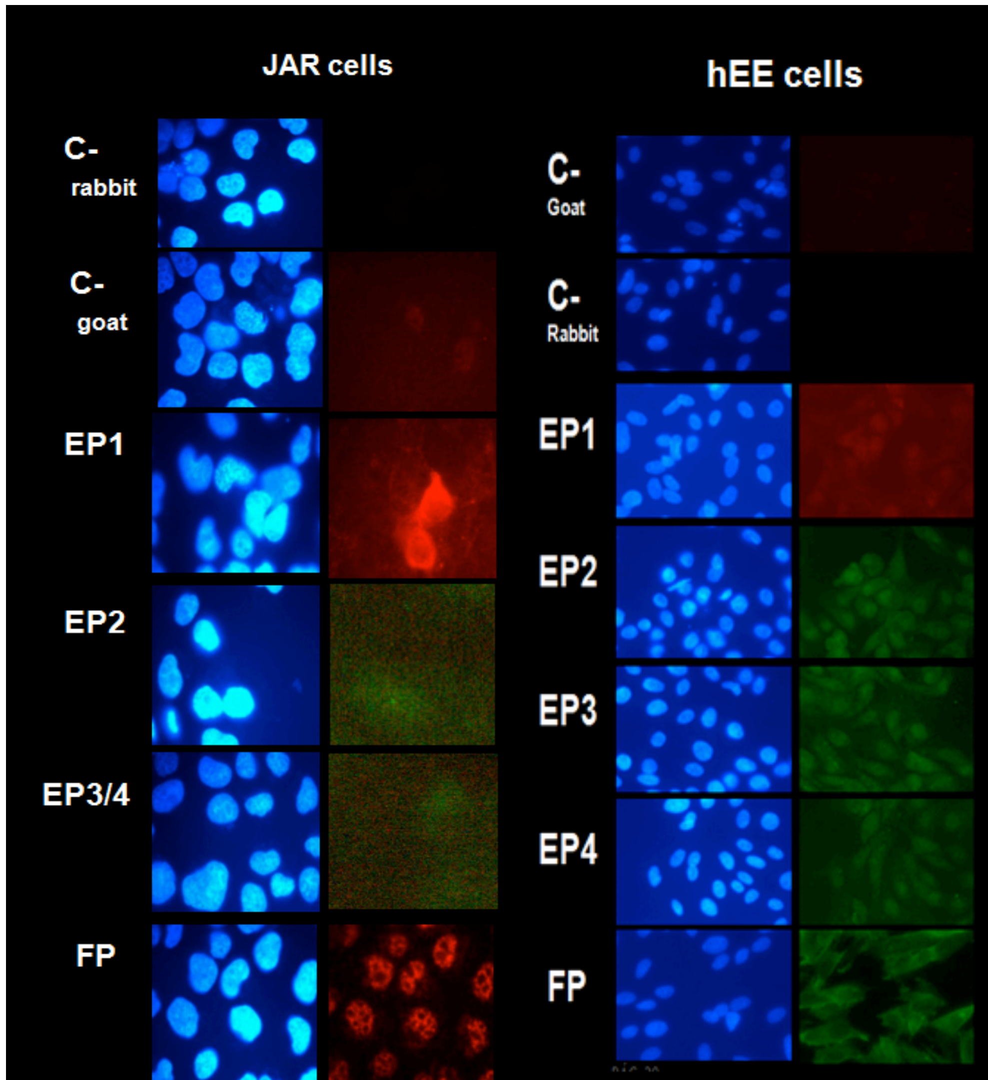


Figure 19. Immunofluorescence analysis of EP1-4 and FP receptors in hEEC and JAR cells. DAPI was used to stain the cell nuclei (blue). (A) Positive staining in EP1 and FP. (B) Positive staining in all receptors.

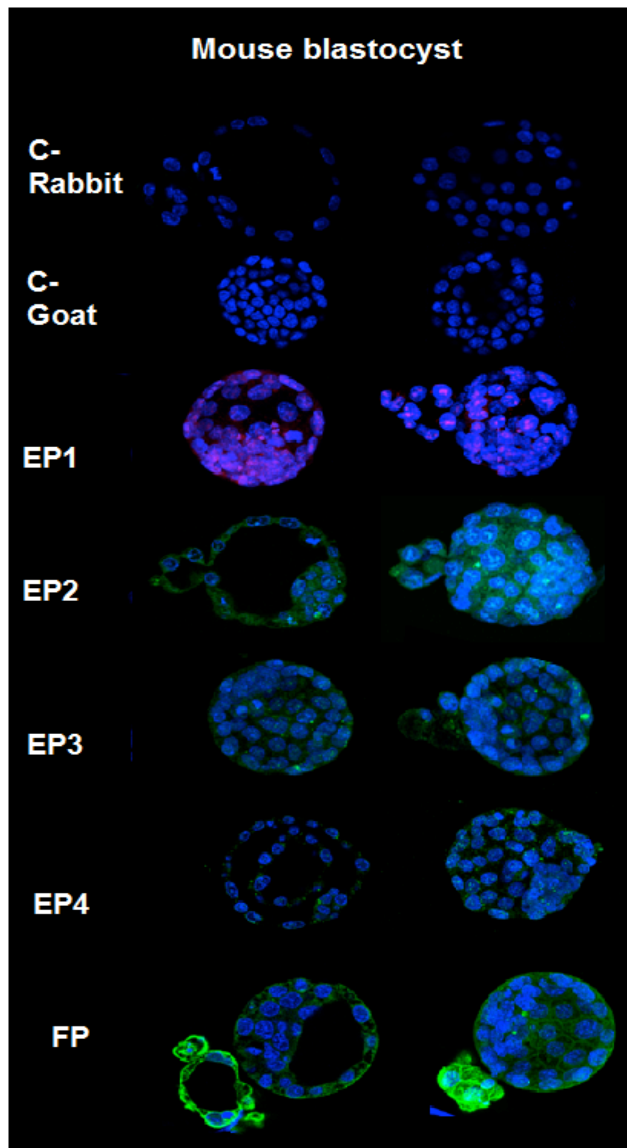


Figure 5.20. Immunofluorescence analysis of EP1-4 and FP receptors in mouse embryos. DAPI was used to stain the cell nuclei (blue). (A) Single confocal optical section fluorescence overlay on cell membrane, cytoplasm, and some (EP1& EP2) on the nucleus of inner cell mass (ICM) and throphoblastic cells of blastocyst stage embryo.

4. Functional relevance of PGE_2 and $PGF_2\alpha$ in an *in vitro model* of embryo adhesion

To investigate the functionality of PGE_2 and $PGF_2\alpha$ in embryo adhesion, we tested the inhibition of these biomarkers in an established *in vitro* model of mouse embryo adhesion to primary EEC cultures.

First, EECs were pretreated with indomethacin (50µM), CAY10607 (30nM), cyclopentane (1µM) or bimatoprost (50µM) during 48h in order to inhibit PGs production, then washed and devoid of inhibitor by addition of fresh media. After that, mouse embryos were added to pre-treated EEC and cultured together. The percentage of embryonic adhesion was assessed by counting under microscope the blastocysts attached after culture plates were moved along a 3cm diameter circular path at a speed of one rotation per second for about 10 seconds (Martin *et al.* 2002).

EECs pre-treated with indomethacin as the strongest PGs inhibitor, induce a decrease of 70% PGs secretion by hEECs in a time dependent fashion. This treatment induced a 96% in embryo adhesion compared to the control vehicle after 32h. Moreover, after pretreatment with CAY10607, an 80% decrease was obtained, and with bimatoprost, the reduction of adhesion was 70% approximately. However, no changes where observed with cyclopentane (Figure 5.21). These results show that inhibition of each prostaglandin $F_2\alpha$ synthases (AKR1C3 and CBR1) in hEEC impacts embryo adhesion as the levels of PGE_2 and $PGF_2\alpha$ are affected.

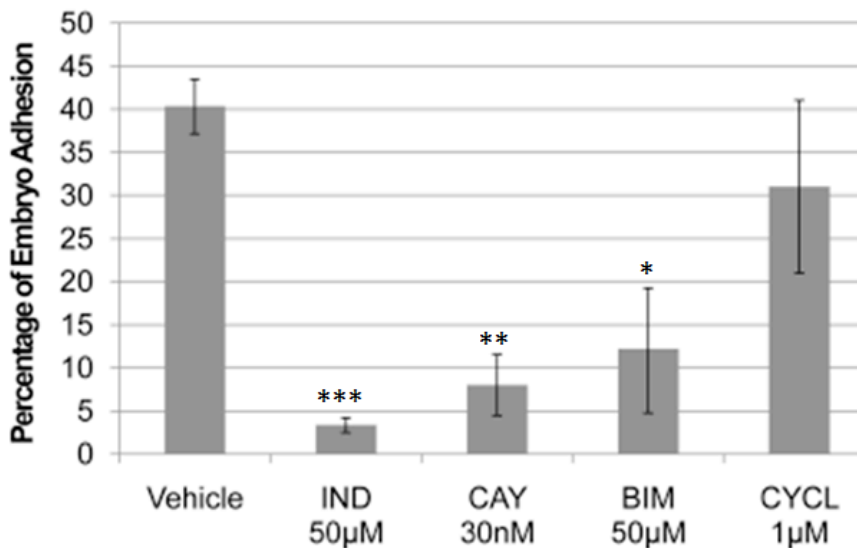


Figure 5.21. Graphs represent the percentage of adhesion measured after 32 hours of cells, pretreated after 48h with indomethacin (IND), CAY10607 (CAY), cyclopentane (CYCL), or bimatoprost (BIM) that inhibits COX, CBR1, AKR1C1, or AKR1C3, respectively. The statistically significant decrease is expressed as *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's t-test).

To further confirm the functional relevance of each PGE_2 and $PGF_2α$ on embryo adhesion, we added-back these molecules in the same *in vitro* model of embryo adhesion used.

EECs were pretreated for 48h with 50μM of indomethacin, and PGE_2 (0.01μM, 0.1μM, 1μM, 10μM) or $PGF_2α$ (1nM, 10nM, 100nM, 1000nM) were then added. The same process described before in the *in vitro* adhesion assay was used to measure the percentage of embryonic adhesion after 1h, 2h, 4h, 24h and 32h of PGs add-back treatment, and the toxicity effect was tested in the EEC monolayer with all concentrations.

Results demonstrated that adding back PGE_2 and $PGF_2α$ completely recovered blastocyst adhesion in a dose-dependent manner. However, a different percentage of embryo adhesion was observed depending on the time and PGs added. While adding PGE_2 concentrations, the percentage ranged from 0% (10nM, 100nM at 1h and 100NM at 2-4h) to 54% (100nM at 32h), different observations were obtained with $PGF_2α$, in which it ranged from 0% (1nM, 10nM, 100nM and 1μM at all hours tested) to 63% (10nM at 32h) versus their controls (Figure 5.22A &5.22B). The most stricking results were observed at 32h after PGE_2 and $PGF_2α$ were added. In the control group, embryo adhesion was around 45% as well as when PGs were added. Although embryo adhesion for most of PGE_2 concentrations was similar to their respective controls, we observed an increment of 12% at 32h at a concentration of 100nM (Figure 5.22A). Meanwhile, different results were obtained with $PGF_2α$, with a highest increment (25%) of 10nM at 32h (Figure 5.22B).

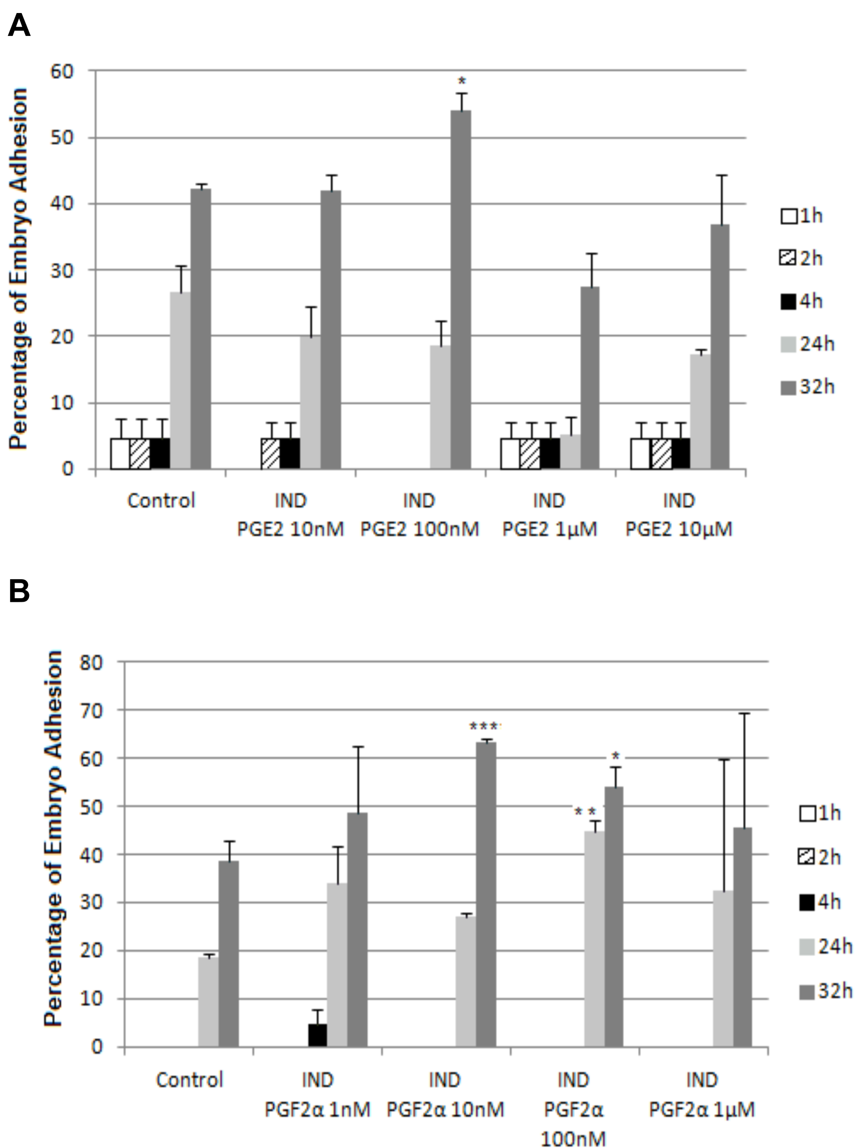


Figure 5.22. Percentage of embryo adhesion in different times (1h, 2h, 3h 4h, 24h, 48h) after PGE_2 or $PGF_2\alpha$ were added to hEECS and pretreated for 48h with indomethacin. A. Concentrations tested for PGE_2 (10nM, 100nM, 1μM, 10μM). (B) Concentrations tested for $PGF_2\alpha$ (1nM, 10nM, 100nM, 1000nM). Statistically significant increase is expressed as *p<0.05, **p<0.01, ***p<0.001 (Student's t-test).

This experiment was repeated (n=3) with the concentration of PGE_2 and $PGF_2\alpha$ at 10nM, 100nM. The percentages of embryo adhesion were consistent with our previous results (Figure 5.23). Adding back PGE_2 at 100nM, induce an increase of adhesion of 12% compared to the controls. Similarly, adding back $PGF_2\alpha$ at 10nM or 100nM after indomethacin pretreatment resulted in an increase of 20% and 12%, respectively, in embryo adhesion versus controls (Figure 5.23).

This *in vitro model* demonstrates that $PGF_2\alpha$ and PGE_2 are functionally relevant for embryo adhesion, and the depletion of these PGs seriously impairs the interaction between the embryo and EEC.

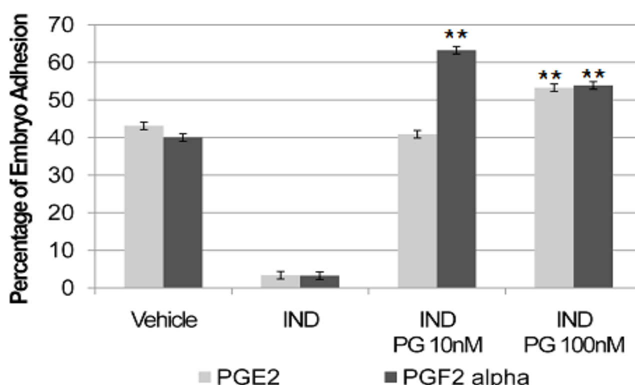


Figure 5.23. Percentage of embryo adhesion measured 32 hours after the EECs were pretreated for 48 h with indomethacin (IND) and later supplemented with PGE_2 or $PGF_2\alpha$ at different concentrations (10nM and 100 nM). The statistically significant increase is expressed as **$p<0.01$ (Student's t-test).

4.1 Agonists/ Antagonist of PGE_2 and $PGF_2α$ receptors on embryonic adhesion assay

With respect to our previous studies, we observed that PGE_2 and $PGF_2α$ effect in EEC-blastocysts coculture increased adhesiveness. To explore which EP1-4 or FP was activated and involved in embryo adhesion we measured the responses of agonist molecules. For PGE_2 receptors we had for EP2 : Butaprost (BUT) and AS701931(AS70); for EP3 : Sulprostone (SLP); and for and EP4: AS701666 (AS7-A), AS701753 (AS7-B), AS701715 (AS7-C). For $PGF_2α$, we used an agonist for FP named Fluprostenol (FLP) and its antagonist AS604872 (AS6).

Toxicity tests of agonists and antagonists were carried out to determine the concentration to be used for further adhesion experiments. Both blastocyts and hEEC from different biopsies were independently exposed to different concentrations. Results showed that most concentrations tested were not toxic either for EEC or for the blastocyst.

Once conditions were established, two concentrations were used: the maximal concentration tested and the lowest one. We then employed the same embryonic adhesion assay as described in previous experiments using agonists for EPs and FP receptors instead of the synthethic PGs, while the antagonist was added to mouse embryos that had been previously (6h before) treated with $PGF_2α$ 10nM. The percentage of mouse blastocysts adhesion using hEEC monolayer was determined 24h and 32h after treatment. A positive control was used for embryo adhesion by pretreating the cells with indomethacin and then adding back $PGF_2α$ (10nM), and indomethacin was also used as a negative control.

The first data was collected from three different experiments in which a total of 50 mouse blastocysts were examined per agonist concentration. Some differences were observed in blastocyst adhesiveness between EP1-4 or FP agonists and FP antagonist.

The results showed that the highest percentages of blastocysts adhesion in EEC monolayers at 24h using EP2 agonists where Butaprost at 5 μM and AS70 at 150nM. Their effects on embryo adhesion were similar (24% and 25%, respectively), and significantly higher than in control (12%). However, at 32h, Butaprost 5μM increased fourfold over AS70. The other concentrations tested for this receptor did not show a significant increase in embryo adhesion (Figure 5.25A).

In cells supplemented with EP3 agonist sulprostone (10μM), it was observed that at 24h, adhesions were up to 11% times higher than those in control cells ($P<0.05$)(Figure 5.25B). However, different profiles were obtained for the three EP4 agonists: AS7-A, AS7-B, AS7-C, in which for the two concentrations tested for each agonist there was a decrease in blastocyst adhesion compared to the vehicle (Figure 5.25C). On the other hand, using the FP agonist, fluprostenol, at 4μM and 18μM, adhesion was increased by around 20% at 24h for both concentrations (Figure 5.25D).

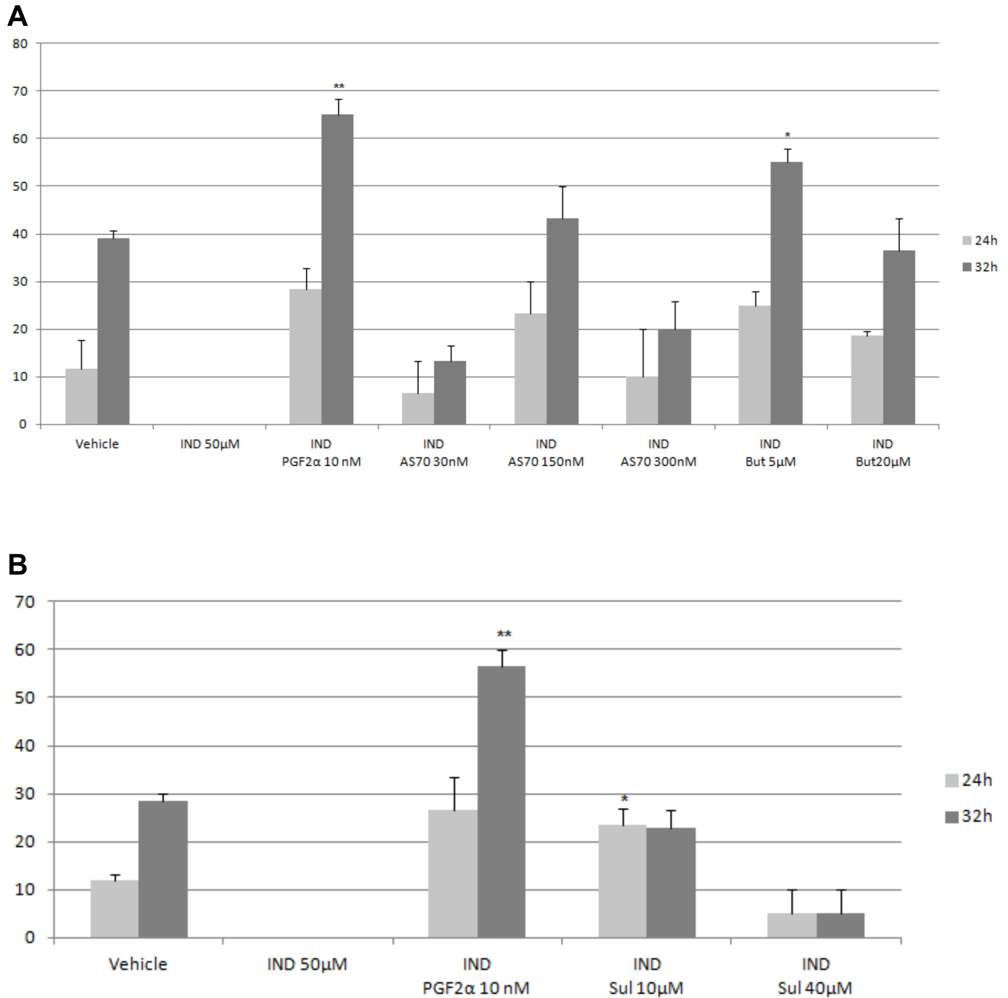
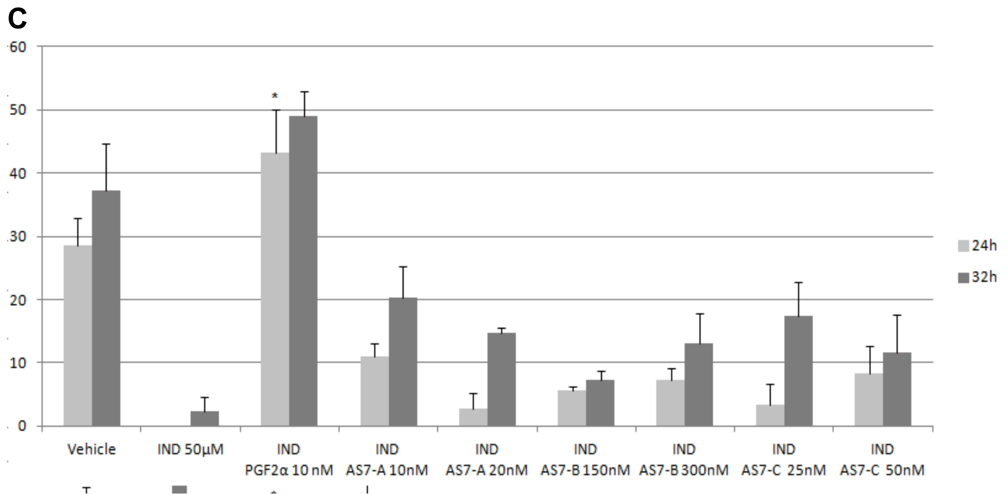


Figure 25. Percentage of adhesion measured 32 hours after EECs were treated with indomethacin (IND) and supplemented with $PGF_{2}α$ (10 nM) and their specific agonists. (A) butaprost (BUT; 5 µM, 20 µM), AS701931 (AS70; 30 nM, 150 nM, 300 nM), (B) sulprostone (SUL; 10 µM, 40 µM), Statistically significant increase is expressed as *$p<0.05$, **$p<0.01$, (Student's t-test).



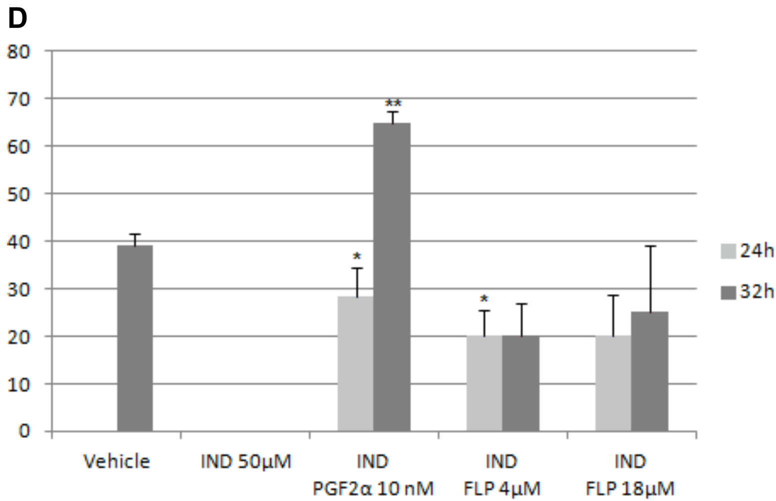


Figure 25. Percentage of adhesion measured 32 hours after the EECs were treated with indomethacin (IND) and supplemented with $PGF_{2}\alpha$ (10 nM) and their specific agonists. (C) AS701666 (AS7-A; 10 nM, 20 nM), AS701753 (AS7-B; 150 nM, 300 nM), AS701715 (AS7-C; 5 nM, 50 nM). (D) For $PGF_{2}\alpha$, we used an agonist for FP named Fluprostenol (FLP; 4 µM, 18 µM). Statistically significant increase is expressed as $*p<0.05$, $**p<0.01$, (Student's t-test).

Using the concentration obtained for the maximal embryo adhesion for each agonist and for non-embryo adhesion using antagonist receptor, the same embryo assay was repeated (n=6), in which the results presented a pattern similar to the previous experiment.

Activation of endometrial epithelial EP2 receptor with butaprost 5μM and AS70 10μM had an increment of 18% and 20%, respectively, in embryo adhesion at 24h. However, at 32h the adhesion of butaprost was 5-fold the AS70. For EP3 receptor, values changed when the number of experiments was duplicated using Sulprostone 10μM. Results showed only an increment of 3% of adhesion at 24h, and therefore not significant. On the other hand, similar results were again obtained in this second experiment using AS7-A 10nM as agonist of EP4 receptor. There was no increase but, on the contrary, a diminished embryo adhesion at both times, in agreement with the previous results. Likewise, fluprostenol 25nM as FP agonist showed only at 24h a maximum increment of adhesion (23%) compared to the positive control $PGF_2\alpha$ (21%). On the other hand, blockage with the AS604872 175nM as antagonist for FP receptor showed a decrease in embryo adhesion similar to the one obtained with the negative control, demonstrating the importance of this receptor (Figure 5.26).

These results indicate that EP2 and FP receptors are responsible for embryo adhesion induced by the presence of PGs. No embryo adhesion modifications were observed with the use of EP3 and EP4 agonists.

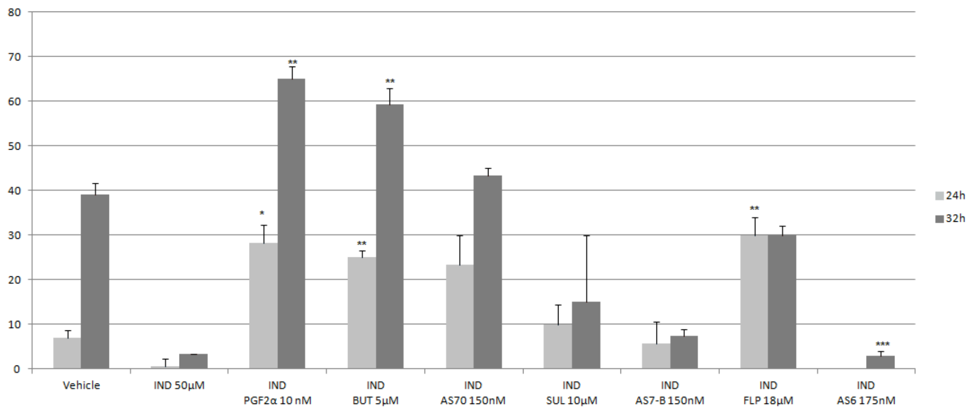


Figure 5.26. Percentage of adhesion measured 24 and 32 hours after EECs were pre-treated for 48 hours with indomethacin (IND) and then supplemented with: PGF2α (10 nM), butaprost (BUT; 5 µM), sulprostone (SUL; 10 µM), AS701666 (AS7-B; 150 nM), Fluprostenol (FLP; 18 µM), AS604872 (AS6; 175 nM), or PGF2α (10 nM). The increase with agonists and decrease with the antagonist is expressed as *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's t-test).

4.2 Effect of $PGF_2\alpha$, butaprost and fluprostenol agonists on embryo development

Moreover, the presence of butaprost and fluprostenol agonists, as well as synthethic $PGF_2\alpha$, showed an effect not only on embryo adhesion but also in its development. Most of the embryos that were in contact with synthethic $PGF_2\alpha$ and butaprost got attached once they had 30-50% of their volume out of the zona pellucida. However, after 24h the majority of the embryos that were not attached did not complete hatching. On the other hand, using the agonist receptor of $PGF_2\alpha$, some differences were observed: even though most embryos made complete hatching faster in comparison to the other treatments, after 24h the majority of the embryos were not able to get attached Figure 5.27.

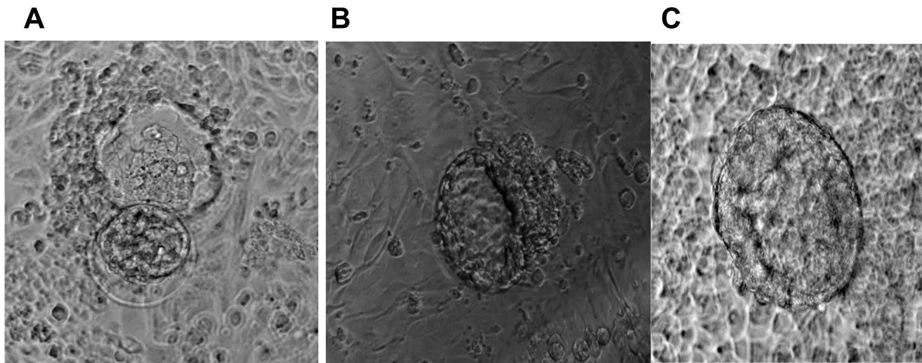


Figure 5.27. Effect of synthethic $PGF_2\alpha$ and agonists of PGE_2 and $PGF_2\alpha$ on embryo development. (A) $PGF_2\alpha$ 10 nM, (B) butaprost 5 μM, (C) fluprostenol 18 μM.

5. Analysis of PGE_2 and $PGF_2\alpha$ synthases in the EF

5.1 Detection of PGE_2 and $PGF_2\alpha$ enzymes in EF

Initially, the media obtained from hEEC monolayers was analyzed and the presence of the PGs synthases corroborated by western blot demonstrating that hEEC are able to release terminal PGs synthases to the medium, opening an exciting new possibility for the production of the PGs in the uterus.

To further confirm this observation, EF at LH+2 and LH+7 was investigated. To avoid experimental interferences albumin was removed from EF, since serum is present and 30-50% of serum is albumin.

Once albumin was removed, samples were subjected to western blot analysis to detect relative protein amounts of two enzymes, CBR1 and AKR1C1 that mediate the interconversion between PGE_2 and PGF2α. Both enzymes were expressed during the pre-receptive and receptive stages (Figure 5.28). Therefore, results obtained indicated that these enzymes are not only produced in hEECs, but also released outside the cells

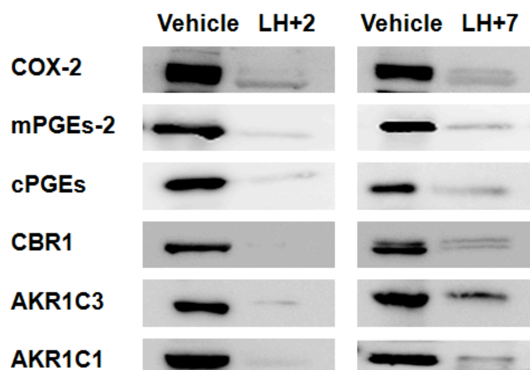


Figure 5.28. Western Blot analysis of COX-2, mPGES-2, cPGES, CBR1, AKR1C3, and AKR1C1 in EF samples from women obtained in LH+2 and LH+7.

5.2 Enzymatic activity of PGs synthases in EF

Evidence presented in the previous study suggested that the PGs synthases in EF are capable of synthesizing PGs during the complete menstrual cycle, contributing to the PGs levels in the uterine environment. To confirm the enzyme's activity in EF, PG 9-ketoreductase (CBR1) was tested at days LH+2 and LH+7.

The first step was to standardize a procedure to purify the specific enzyme (CBR1) after albumin was removed from EF. Then, because CBR1 directly converts PGE_2 into $PGF_2α$, three different concentrations (5nM, 10nM and 50nM) of synthetic PGE_2 were used as substrate and incubated with the previous purified enzyme. Finally, the final product $PGF_2α$ was measured at 15, 30, 60, 90, 120, 150, 190 minutes by an enzyme immunoassay.

Based on the equation of the standard curve, the concentration of each sample was determined by comparing the maximal binding (%B/Bo, which is inversely proportional to the amount of $PGF_2α$) to the corresponding standard concentration (Figure 5.29).

Results indicated that $PGF_2α$ (CBR1 product) was formed only in the receptive samples at LH+7, especially in the range of 60 to 90 minutes using PGE_2 at 10nM as substrate. However, from the total number of LH+7 samples (n=6), only four of them showed a specific concentration of $PGF_2α$ at 60min and only one of them at 90min (Table 5.1). Finally, no $PGF_2α$ product was detected in any condition that was established in LH+2 EF samples tested. Therefore, CBR1 is not only present in the EF but also displays activity by producing $PGF_2α$ in the presence of its substrate PGE_2.

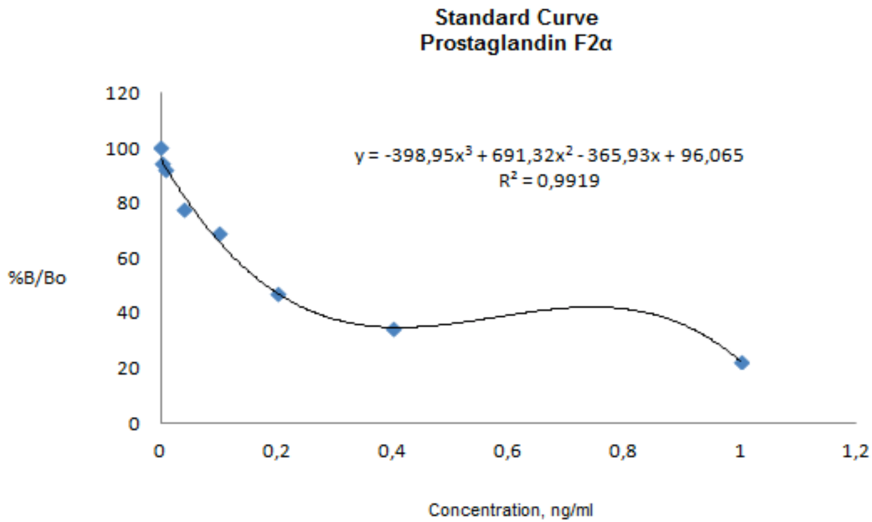


Figure 5.29. Standard Curve. The %B/Bo represents the percentage of maximal binding, which is inversely proportional to the amount of $PGF_2α$ in the sample or standard.

Table 5.1. $PGF_2α$ concentration (ng/ml) determined using as reference the standard curve in the six LH+7 (receptive) EFs.

EF Samples	$PGF_{2α}$ production ng/ml	
	60 min	90 min
LH +7 (1)	0.0011	0.0043
LH +7 (2)	0	0
LH +7 (3)	0.008	0
LH +7 (4)	0.0029	0
LH +7 (5)	0.002	0
LH +7 (6)	0	0

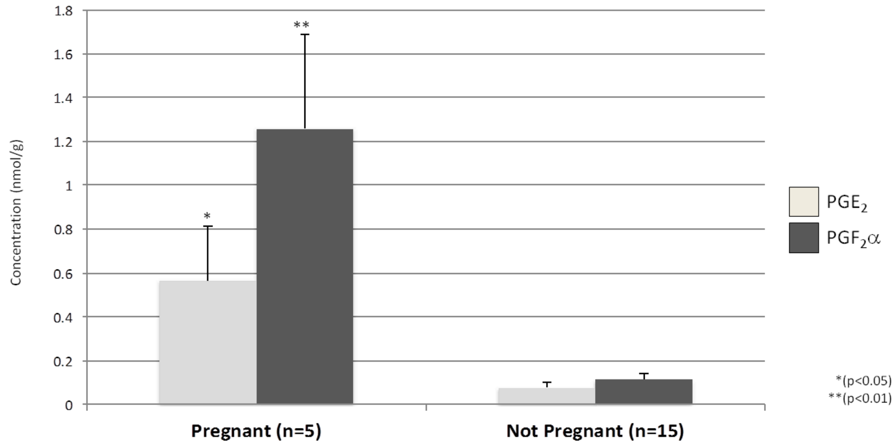
6. PGE_2 and $PGF_2α$ concentrations in EF as non-invasive biomarkers of endometrial receptivity

In the light of the results above, we hypothesized that the quantification of these two PGs in EF could serve as a non-invasive diagnostic tool to predict endometrial receptivity.

We performed a pilot study to determine the sensitivity and specificity of PGE_2 and $PGF_2α$ concentrations in EF samples obtained 24 hours prior to day-3 (n=20) or day-5 (n=17) embryo transfer. The aim was to predict endometrial receptivity and therefore successful implantation in patients undergoing either IVF or ovum donation (treated with COS or HRT, respectively). In patients undergoing day 3-embryo transfer the mean level of these PGs in cycles resulting in pregnancy was 0.56 nmol/g for PGE_2 and 1.26 nmol/g for $PGF_2α$. Interestingly, in patients in whom pregnancy was not achieved, the levels of PGE_2 and $PGF_2α$ were almost absent concretely for PGE_2 0.08 nmol/g and for PGF_2-α 0.11 nmol/g (Figure 30A). To test sensitivity and specificity we measured ROC curves for both PGs, obtaining for PGE_2 a value of 0.88 (sensitivity 80% and specificity 86.70%) and 0.973 for $PGF_2α$ (sensitivity 100% and specificity 93.30%).

In patients undergoing day-5 embryo transfer (n=17), similar results were obtained in cycles resulting in pregnancy (0.86 nmol/g and 1.47 nmol/g for PGE_2 and $PGF_2α$ respectively), while in unsuccessful transfers the concentrations of PGE_2 and $PGF_2α$ were significantly reduced (0.17 nmol/g and 0.60 nmol/g, respectively) (Figure 30B). We measured also ROC curves for both PGs, obtaining for PGE_2 a value of 0.694 (sensitivity 75% and specificity 77.8%) and 0.653 for $PGF_2α$ (sensitivity 37.50% and specificity 100%).

A.



B.

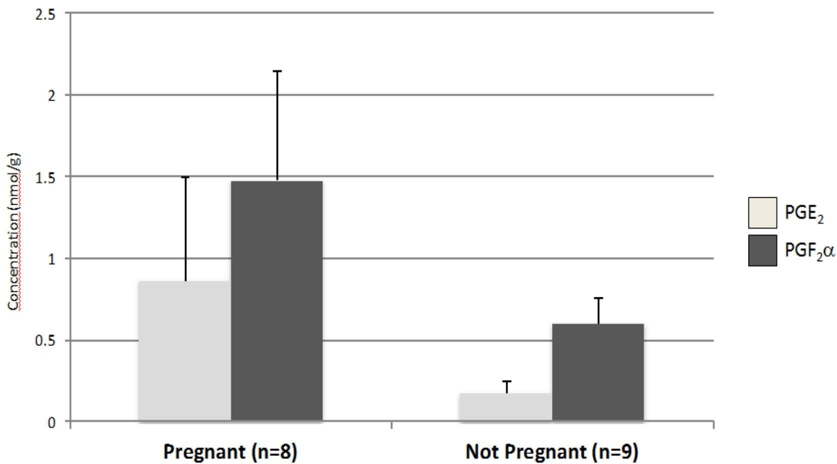


Figure 30. (A) Pilot study seeking to determine the detection sensitivity and specificity of PGE2 and PGF2α concentrations in EF obtained 24 hours prior to embryo transfer in IVF patients undergoing day-3 embryo transfer; non-pregnant (NP) versus pregnant (P). (B) In ovum recipients undergoing day-5 embryo transfer 24 hours prior to embryo transfer; non-pregnant (NP) versus pregnant (P).

VI. DISCUSSION

VI. DISCUSSION

The endometrium is a dynamic tissue throughout the menstrual cycle aiming to acquire the receptive status at due time. Moreover, the endometrial environment suffers different changes to provide the necessary nutrients and mediators that the blastocyst will need to develop and differentiate in a stage-specific manner.

Although several strategies have been proposed in order to determine the receptive stage of the endometrium to improve implantation rates in IVF treatments, the majority of them use morphological observations which involve not only an invasive method, but also a subjective diagnosis (Noyes *et al.* 1975). This clinical limitation together with the difficulty to obtain reliable samples near the implantation window has impulsed the importance of studying the metabolic process that governs embryo implantation using non-invasive methods to investigate the endometrial-embryonic communication. Because EFs are accessible in the intrauterine environment, they have been pointed out as a new tool to identify the receptive stage of the endometrium since the aspiration of EF does not affect pregnancy rates in the same cycle (van der Gaast *et al.* 2009).

However, it is crucial to understand endometrial biology. It is well known that the endometrium contains lipid compounds with important roles in reproduction (Durn *et al.* 2010; Boomsma *et al.* 2009). Defective endometrial prostaglandin (PGs) synthesis in humans has been linked to repeated implantation failure in patients undergoing IVF (Achache *et al.* 2010), indicating that lipids intervene as important mediators in embryonic implantation. Most of these studies have focused on the role of PGs in endometrium, where they cause increased vascular permeability (Bogan *et*

al. 2008) and decidualization (Chapdelaine *et al.* 2006), although their importance in influencing embryo maturation and acquisition of receptivity competence has also been suggested.

In this thesis, we investigated the human endometrial biology of prostaglandin secretions together with clinical studies with the goal to establish a non-invasive lipidomic diagnosis for endometrial receptivity.

The lipidomic profile of human EFs was investigated to elucidate which lipids appeared to be present and relevant through the menstrual cycle as well as in different endometrial preparations in ART . To our knowledge, this is the first time that human lipidomic profile in EFshas been characterized. Of the nine lipids found, most of them belong to the *N*--acylethanolamines (NAEs) familiy, which are characterized as part of a group of lipid mediators derived from a fatty acid precursor linked to an ethanolamine, the most studied is the endocannabinoid arachidonoylethanolamide (anandamide; AEA). Moreover, NAEs also comprise others non-endocannabinoids that were found in our study, such as palmitoylethanolamide (PEA), oleoylethanolamide (OEA), and stearoylethanolamide (SEA). The data obtained shows significant differences in the concentration of two specific lipids, namely prostaglandin E2 (PGE_2) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$), compared with the rest of lipids found in the lipidomic profile between days 19 to 21 of the menstrual cycle coincident with the implantation window.

Prostaglandins (PGs) are lipid mediators that are involved in many physiological and pathological processes. They have been identified as important molecules in physiological functions for the female reproductive system. In the uterus, PGs have been described to participate in processes such as implantation, control of cytokine release, cell growth, differentiation

and vascular responses (Lee & DeMayo 2004). Although extensive research in the past years provides a new perspective of the role of PGs, especially in mouse endometrium, their role in humans has not been yet elucidated.

Our results confirmed that PGE_2 and $PGF_2α$ levels fluctuate in the EF along the menstrual cycle, with highest levels at the receptive stage corresponding to the period in which the endometrium is able to receive the blastocyst. As demonstrated in other animal models, this may imply that PGs are also importantly involved in the process of implantation in the human endometrium.

As those results were obtained using the EF from patients in their natural cycles, a new study was designed to obtain the lipidomic profile of EFs in oocyte donors treated under optimal (HRT), suboptimal (COS), or refractory endometrial conditions (IUD). The goal was to confirm whether the production of those PGs was specifically dependent on the receptive stage, and therefore to corroborate that these lipids could potentially be used as endometrial biomarkers also in patients undergoing IVF or ovum donation. The resulting observation was a clear pattern for PGE_2 and $PGF_2α$ that peaked during the window of implantation in HRT, which corresponds to the 5th day of progesterone administration (equivalent to the 7th day after LH surge. Likewise, a similar pattern was observed in patients undergoing COS with a peak at hCG+7 that also matched the receptive stage of the endometrium. Furthermore, PGE_2 and $PGF_2α$ peaks obtained at P+5 in HRT were not present in refractory conditions induced by the insertion of an IUD. These results support the consistency that those two PGs could have in endometrial maturation development of the receptive state, bolstering their potential contribution to evaluate patients under IVF process for receiving and implanting an embryo.

The next step was to study endometrial biology to determine the dynamics of the production of these PGs across the menstrual cycle. Both basic and clinical research on these molecular processes are key to improve the understanding of the endometrial preparation for embryo implantation.

Initially we investigated the existence and functionality of upstream and downstream enzymes involved in the synthesis of prostaglandin in the endometrium. It is well documented that cyclooxygenase (COX-1 and COX-2) is coupled with upstream phospholipases and downstream synthases for the production of PGs (Simmons *et al*. 2004). COX-2, in particular, is one of the key enzymes in the conversion of arachidonic acid to precursors of PGs. In addition, they have been identified in the endometrium during receptive stage in a wide variety of species including mice, guinea pigs, sheep, horses, rhesus monkeys and baboons, with an essential role during the process of implantation (Lee & DeMayo 2004). It has also been identified in human endometrium during the receptive stage. However, in humans most of the attention to this enzyme has been focused on several disorderssuch as endometriosis and endometrial adenocarcinoma (Jabbour & Sales 2004).

Our immunohistochemistry studies support that the expression of COX enzymes in human endometrium have a unique expression pattern in pre-receptive and receptive endometrium. These results coincide with reports of the presence of COX-1 and COX-2 during the receptive stage (Marions & Dannielson 1999).

However, COX-2, but not COX-1, was the only PGs synthase that significantly increased in luminal endometrial epithelial cells, which is basically the first contact surface for the embryo during blastocyst implantation. Earlier studies using mice models, have demonstrated that

COX-2 is essential for blastocyst implantation (Jabbour & Sales 2004; Singh *et al.* 2011), and our results seem to pinpoint COX-2 as the main enzyme involved also in the process of implantation in humans.

To fully characterize human endometrial regulation of PGs, we decided to use two different approaches: first, we performed transcriptomic studies to assess the expression levels of the enzymes that act downstream of the pathway and are responsible for the production of PGE_2 and $PGF_2\alpha$ in the endometrium; and second, identify and localize the protein expression of these molecules in histological samples.

Q-PCR experiments of PGE_2 and $PGF_2\alpha$ synthases in the whole endometrium have not showed major general variations in the expression profiles between receptive and pre-receptive endometrium. However, as it was expected, the expression of the enzymes in the epithelial cells, which are the main source of PGs production in the endometrium, presented a higher expression compared to the whole endometrium. The results in the epithelial cells showed a higher expression level in $PGF_2\alpha$ synthases (AKR1C1, CBR1 and AKR1C3) than in PGE_2 synthases (mPGES2, mPGES1 and cPGES). These results are consistent with the concentrations of $PGF_2\alpha$ detected by lipidomics in EF during the receptive stage of the menstrual cycle.

Interestingly, a possible compensatory effect was detected between enzymes leading to the same products. These results suggest that these enzymes play an overlapping role that could balance the lack of mPGES1 and AKR1C3 during the receptive stage. Moreover, it is known that PGs synthases participate in other important processes within the cell, which

difficult the study of prostaglandin production for embryo implantation as an isolated event (Choi *et al.* 2011; Velica et al. 2009).

The next step in our study was to elucidate their morphological localization The importance of determining the differential expression of the PGs synthases in the endometrium was to learn more about the enzymes responsible for the production of PGE_2 and $PGF_2α$ in human endometrium. Besides, as described above, histology has been considered for many years the accepted method to predict the endometrial receptive state, and therefore detecting the expression of the different PG synthases in tissue samples and correlating their localization with histological changes would work as a cross-check, by comparing a classical method with the expression of the synthases. For this purpose, spatio-temporal localization of those enzymes in the transition from pre-receptive to receptive endometrium was studied. It is important to highlight that all the enzymes were expressed, but there were differences in the pattern of intensity.

Microsomal PGES1 is known to be coupled with inducible COX-2 to support deferred PGE_2 generation and to regulate immediate PGE_2 generation. Our results show that its protein expression is decreased in the luminal epithelium during the receptive stage, indicating a decline of mRNA expression in epithelial cells during the same stage in the previous studies. Moreover, mPGES1 is expressed at lower levels (both RNA and protein) during the receptive stage in the luminal epithelium in mice (Ni *et al*. 2002). Consistently, this enzyme has been associated with an important role during decidualization as it is highly expressed in the stroma but not in the luminal epithelium (Ni *et al*. 2002). Because this pattern of expression is similar in both species during the receptive stage, we suggested that, like in mouse models, mPGES1 is important but it is not the principal enzyme in

synthesizing PGE_2 during the receptive stage for embryo adhesion in humans.

Although it has been suggested that other two direct enzymes of PGE_2 production might compensate the mPGES1 deficiency, our results in humans showed that cytosolic PGES, which is constitutively expressed in conjunction with COX-1 to promote the immediate response, also decreases at the receptive stage in luminal and glandular cells. These results are consistent with those found in mouse uterus (Ni *et al.* 2003). However, even though cPGES also does not seem to be the main enzyme for PGE_2 production during the receptive stage, the predominant link with COX-1 could be associated with its contribution in maintaining homeostasis of PGE_2. Moreover, due to the similarity in the cPGES expression in humans and mouse during the receptive stage, it seems that in both humans and mice, this enzyme is involved in vascular permeability as the first stage of attachment and decidualization (Ni *et al.* 2003).

The second microsomal PGE synthase (mPGES2), that catalyzes the isomerization of PGH_2 to PGE_2, is known to be constitutively expressed with both COX-1 and COX-2. Although our results showed a uniform expression on both pre-receptive and receptive stage, its expression is slightly different from the one obseved in rat uterus, as it was highly detected in the luminal epithelium in the receptive stage (Cong *et al.* 2006). These observations suggest that while these two enzymes are important in mice and rats, other enzymes could be doing this function in humans.

In our studies, AKR1C1 and CBR1, which can modulate both PGs concentration presented higher protein expression during the receptive stage compared to those PGE_2 synthases. However, AKR1C1, AKR1C3 and CBR1

were highly expressed in the luminal epithelium, specifically during the receptive stage. Those results argue for the idea that $PGF_2α$ might be important for embryo implantation during the receptive stage, and we suggest that AKR1C3, CBR1 and AKR1C1 expression could be not only involved in the main production of $PGF_2α$ but also in modulating the PGE_2 to $PGF_{2α}$ ratio.

Our second approach to characterize human endometrial regulation of PGs consisted in studying the abundance of PGE_2 and $PGF_2α$ enzymes using western blot technique on EEC from women treated with progesterone representing two different situations, non-receptive (P0) and receptive (P5) stages. Using this method, we found that all the enzymes leading to the synthesis of $PGF_2α$ (AKR1C1, AKR1C3 and CBR1) were significantly more abundant in the receptive phase. This result is consistent with our previous observations in protein expression of these three enzymes in the luminal epithelium, suggesting their important role during the receptive stage. Moreover, AKR1C1 is a dual enzyme that enables the conversion of PGE_2 into $PGF_2α$ and *vice versa*, so high levels of $PGF_2α$ together with high levels of AKR1C1 could end up rising the production of PGE_2, what would actually reflect the sharp increase of these two PGs during the implantation window. These results are in agreement with our previous observations and reinforced our hypothesis that PGE_2 and $PGF_2α$ are important molecules for embryo implantation. As a method to demonstrate the endometrial activity of the PGE_2 and $PGF_2α$ enzymes we measured the reduction in PG release after blocking physiological enzymatic activity by using specific chemical inhibitors in EEC obtained from biopsies in luteal phase. We also tested the normal production of PGs without any inhibitor, as well as the use of indomethacin (one of the principal drugs that inhibits COX1,2 by not leading

the conversion of arachidonic acid to PGH2) that leads to the inhibition for the production of PGE_2 and $PGF_2α$.

From these results, we concluded that PGs synthases in EECs are capable of producing PGE_2 and $PGF_2α$ under normal conditions, that is, when no inhibitor is added. On the contrary, when the pathway of PGs production is inhibited, there was a significant reduction of PGE_2 and $PGF_2α$ after the cells were in contact with indomethacin. However, when specific inhibitors were used for each terminal enzyme of PGE_2 and $PGF_2α$, enzymes CBR1 and AKR1C3 were observed to play a key role in the synthesis of $PGF_2α$ in our model, as their inhibition with CAY10607 and Bimatoprost respectively showed a significant reduction in the release of $PGF_2α$ by the cells. Also, inhibition of the two enzymes that directly synthesize PGE_2 (cPGES and mPGES-1) have not induced a significant decrease in the levels of PGE_2; instead, the production of $PGF_2α$ was increased compared to normal conditions. This result suggests that PGE_2 production can be compensated by the direct enzymes from PGH2 precursor and AKR1C1 that inter-convert $PGF_2α$ and PGE_2 of the pathway. However, we suggest that high levels of $PGF_2α$ could be due to the fact that the absence of any of those enzymes will mainly increase enzymatic activity of AKR1C3 that directly produces $PGF_2α$. The produced $PGF_2α$ would then be converted to PGE_2 by the regulation of AKR1C1 to maintain natural levels.

Inhibition of AKR1C1 caused a pattern similar to cPGES and mPGES1 in the levels of PGE_2 and $PGF_2α$, although the production of $PGF_2α$ was highly increased in this case. The idea of compensating the deficiency of AKR1C1 by other enzymes seems to be similar to what was discussed above. The implication of those results is that normal levels of PGE_2 must be regulated by their direct enzymes, as no other enzymes could be associated to its

production. However, the reason of the high increase levels of $PGF_2α$ compared to the other enzymes should be also associated to the accumulation of this enzyme. This suggests that because AKR1C1 is the only enzyme that can transform $PGF_2α$ and PGE_2 and *vice versa,* its deficiency will cause the production of $PGF_2α$ by two different ways: via its direct enzyme (AKR1C3) and by PGE_2 product that is transformed by CBR1. This information indicates that the production of $PGF_2α$ by two different ways and without the regulation between the conversions of $PGF_2α$ to PGE_2 will cause an accumulation of $PGF_2α$.

These experiments highlighted the importance of the fine regulation among enzymes for the PGs production in the endometrium, demonstrating that the ones used in our study were fully functional and that all the enzymes described in this pathway are important but not essential for PGs synthesis.

After studying PGs and their synthases at the molecular and cellular levels, we investigated their localization and activity in an *in vitro* model of embryo adhesion. Because information regarding uterine sites of PGs actions in the process of human implantation is essentially non-existent currently, we decided to examine the expression of the four receptors subtypes (EP1-4) of the PGE_2 and the one receptor (FP) of $PGF_2α$ during pre-receptive and receptive stage of the endometrium. The resulting data showed that the five receptors were expressed in a temporal manner with a different localization in the endometrium suggesting that FP and EP2 could be potential mediators of PGE_2 and $PGF_2α$ actions in regulating the first steps for embryo adhesion. Both receptors are highly expressed in the luminal epithelium of endometrial epithelium in natural and HRT cycles, specifically during the receptive stage.

However, while our results indicate that EP2 and FP are the main receptors involved in the adhesion phase, this statement seems to be dependent on the species under discussion. In mouse and rats, for instance, the levels of PGI2 (that acts through the nuclear receptor PPARδ) was found to be the highest PGs in the uterus followed by PGE_2 that acts via its receptor subtype EP2 (Lim *et al.* 1999). Nonetheless, investigations in rodent models have highlighted the localization and expression in the luminal epithelium of PGE_2 (with its receptor subtype EP_2) during pre-implantation and implantation stages, suggesting them to be the main stakeholders during embryo implantation (Lim & Dey 1997; Shi *et al.* 2005). Mice results also support that EP2 is a potential mediator of PGE_2 actions, while FP was almost undetectable in rat uterus. It is important to notice that, even though we have observed some similarities between rodents and humans, there are still important differences that do not allow one to extrapolate PGs and their signaling from rodents to humans. This difference is mainly due to the fact that the process of blastocyst implantation is different among species.

Once the terminal synthases and their specific receptors of PGE_2 and $PGF_2α$ were observed during the pre-receptive and receptive stage in the human endometrium, the next step was to investigate if these PGs could mediate embryo adhesion. To examine the effect of those PGs in the endometrium, first we searched for an *in vitro* model for the interaction between the embryo and the endometrium during the process of implantation. Although some uterine cell lines (adhesive RL95-2 and non-adhesive HEC-1-A) were tested as an *in vitro* layer for embryo adhesion, PCR products indicated that these cell lines were not the best model as they did not expressed all the receptors. However, when human epithelial cells were tested all the

receptors were present. These receptors were also searched in mouse blastocysts.

Interestingly, even though PG receptors are described as transmembrane G protein coupled receptors, we have also localized them in the cytoplasm and nucleus in hEEC monolayers, and observed the EPs and FP expression in the cytoplasm and membranes of mouse blastocyts, besides some nuclear staining in EP1 and EP2. These observations are consistent with the results obtained in different cell types of the rat uterus, where EP1 and EP4 were detected in the nucleus and the rest of the EPs and FP receptors were found in the cytoplasm and cell membranes (Blesson *et al.* 2012).

There have been claims in literature of the expression of these receptors in the nucleus of the cells, suggesting that immediate effects are mediated via cell surface receptors whereas long–term responses are dependent upon intracellular receptor effects. Moreover, stimulation of isolated nuclei with PGE_2 has revealed the association of these receptors with transcriptional regulation of major genes such as COX-2 (Zhu *et al.* 2006). Those results seem to indicate that self-directed regulation of the nuclear signaling networks brings a new dimension to cellular signaling, somewhat independent from plasma membrane and cytosolic events. The whole scenario could be explained if extracellular PGs can be internalized in cells via prostaglandin transporters that will induce their intracellular receptors in the nuclei, leading to an increase in the transcription of pro-inflamatory gene expression. That means that different signaling functions and pathways are activated by plasma membrane receptors and nuclear receptors. The plasma membrane of PGs receptors could therefore trigger immediate physiological actions, whereas nuclear receptors could act through gene regulation during the process of implantation in the endometrium.

We also observed that the expression and intensity of fluorescence varied among EPs and FP receptors, especially in hEEC and mouse blastocysts. In general, a strong tendency for increased signal of EP2 and (especially) FP in the cell membrane was observed. Collectively, the results on the lipidomic profile and on the expression of the receptors in the human endometrium and in mouse blastocysts indicate that while EP2 could be a potential mediator of PGE_2, FP must be the mediator $PGF_2\alpha$ during the process of implantation.

Having confirmed the expression of the EPs and FP receptors, we tested the inhibition of these biomarkers in our model of embryo adhesion using primary hEEC cultures and mouse blastocysts. We observed a significant decrease in embryo adhesion in these experiments. Interestingly though, blastocyst development was unaffected by CBR1 and AKR1C3 inhibitors. Results showed that the exposure of the inhibitors to the mouse blastocysts significantly delayed the onset of maximal binding activity. In contrast, inhibition AKR1C1 did not affect embryo adhesion compared to CBR1 and AKR1C3. In accordance to previous results, while CBR1 and AKR1C3 inhibitors decreased the level of $PGF_2\alpha$ production, AKR1C1 inhibitor increases it, confirming that $PGF_2\alpha$ may play an important role during the process of adhesion as its low levels are directly related to a decrease in embryo adhesion.

To further understand the functional relevance of each PGE_2 and $PGF_2\alpha$ on embryo adhesion, we tested the addition of different concentrations of PGE_2 and $PGF_2\alpha$ in the same in *vitro model* used in embryo adhesion studies. Using mouse blastocysts we observed that PGE_2 and/or $PGF_2\alpha$ are necessary for embryo implantation, as the addition of PGs to the incubation media during adhesion time favoured the recovery of embryo adhesion that

was lost when pre-treating EEC with indomethacin. The results showed that the increase in embryo adhesion reached 15% in comparison to the control for PGE_2 (100nM) and a maximum of 30% of adhesion increase for 10nM of $PGF_2α$. Whether these two PGs can work independently or if they regulate each other to reach the optimal balance to accomplish their role in embryo implantation is a question that we leave for future studies.

Moreover, adhesion studies using different *in vitro* implantation models have also shown different results, especially in the times of adhesion. This data is consistent with the literature of adhesion studies with JAR spheres that has determined the adhesion over a very short time from 1-3 hours (Liu *et al.* 2011). However, different results were observed when mouse embryos were employed: they showed little or no binding prior to 48h followed by a peak of binding activity at 72h (Schultz *et al.* 1997). These results contrast with our adhesion assays, as we observed that half of the mouse blastocysts got attached at 32h. The differences in times of adhesion between mouse embryos and JAR spheres seem to indicate that both models cannot be compared. On the other hand, several studies show that some proteins that are required for adhesion (like for instance fibronectin on the apical surface of the trophectoderm) are translated during a period of 16 to 24 h of culture, (Schultz *et al.* 1997), what would explain the larger than 24h embryo attachment delay.

We also explored which of the receptors (EP1-4 for PGE_2 or FP for $PGF_2α$) was activated and involved in embryo adhesion by measuring the responses of some of their selective agonist molecules under the same adhesion assay, but substituting the synthethic PGs for agonist receptors, and also using an antagonist for FP receptor. Our results indicated that butaprost and fluprostenol (agonists for EP2 and FP receptors, respectively) enhanced

embryonic adhesion 24h after they were administrated. Although adhesion using EP2 agonist showed an increment of 30% compared to the FP agonist, arguably the most interesting result is the fact that the FP antagonist reversed the effect by reducing embryo adhesion in a similar fashion to indomethacin. These findings provide an important molecular framework to the importance of the PGE_2-EP2 and $PGF_2\alpha$-FP pathways in human endometrium during the process of adhesion at implantation.

Importantly, butaprost and fluprostenol agonists, as well as synthethic $PGF_2\alpha$ and indomethacin, changed not only embryo adhesion but also embryo development. While indomethacin significantly inhibited mouse embryo hatching, different results were seen with butaprost and synthethic $PGF_2\alpha$: both increased embryo adhesion, even though they had not released completely the zona pellucida during the first 24h. On the other hand, in presence of fluprostenol, embryos completed hatching faster than with the other treatments. Therefore, one can conclude that the presence of PGE_2 and $PGF_2\alpha$ in the uterine environment may also be needed to improve embryo hatching. A similar result has been reported elsewhere (Chida *et al.* 1986), indicating the influence of PGs, in particular with $PGF_2\alpha$, in accelerating the hatching process. This information is consistent with our observations, as it is indirectly shown that mouse blastocysts during the pre-implantation stage metabolize arachidonic acid and produce PG.

The results discussed above also suggest that while EP2 could be a potential mediator of PGE_2, FP could also act through $PGF_2\alpha$ by regulating embryo adhesion and blastocyst development. Based on that, we suggest that both PGs could serve as a marker for uterine receptivity for implantation.

Before claiming that, we worked to understand whether PGs could also be balanced in the uterine environment by the presence of their synthases, CBR1 and AKR1C: they would mediate the conversion between PGE_2 and $PGF_2\alpha$ to maintain adequate levels of those PGs for the implantation process. Our results confirmed the presence of PGs synthases in the human EF samples in pre-receptive and receptive stages of the menstrual cycle. This suggests that hEECs are able to release PGs enzymes to uterine environment, and confirmed that CBR1 was not only present, but it also displayed activity, in the EF by producing $PGF_2\alpha$ in the presence of its substrate PGE_2. These results demonstrate that the production and balance of PGs take place not only in the cells but also in the EF, which opens a new exciting field to understand the mechanisms by which the levels of PGs are regulated in the endometrium during the receptive stage for embryo adhesion.

Finally, we hypothesized that quantifying PGE_2 and $PGF_2\alpha$ in human EF could serve as a non-invasive biomarker to predict the success of embryo implantation. In particular, analyzing the levels of PGs in the EF obtained 24 hours before day-3 and day-5 of the embryo transfer correlated well with the cycle outcome. In cycles resulting in pregnancy we found levels similar to the physiological conditions reported previously in lipidomic profile, while in all embryo transfers resulting in no pregnancy with normal embryo scores, low levels in PGE_2 and $PGF_2\alpha$ were observed. These results demonstrate that PGE_2 and $PGF_2\alpha$ can potentially be used as biomarkers in the future by analyzing them in the EF, allowing for a possible non-invasive tool for the prediction of the receptive state.

In summary, we have demonstrated a specific PGE_2 and $PGF_2\alpha$ profile in the human EF which can be used to detect the WOI in natural, IVF, and

ovum recipient cycles, which is abrogated with the insertion of an IUD (i.e. in refractive endometrium). We have also shown that PG synthases required for the production of PGE_2 and $PGF_2α$ are located in the endometrial epithelium and uterine fluid that are hormonally regulated during the WOI by PG receptors located in the embryo. Using an *in vitro* model of embryo adhesion, we demonstrated that inhibition of PGE_2 and $PGF_2α$ or the PG receptors EP2 and FP prevents embryo adhesion, which can be reversed by adding back these molecules or by using EP2 and FP agonists. Finally, we showed that PGE_2 and $PGF_2α$ concentrations in EF aspirated 24h prior to embryo transfer are predictors of a successful pregnancy outcome. The overall scientific body of evidence presented in this thesis strongly suggests that PGE_2 and $PGF_2α$ concentrations in human EF can be used as non-invasive biomarkers that can be used to personalize ART treatments, offering a new diagnostic tool to assess the endometrial factor, specifically endometrial receptivity, just before replacing the embryo into the uterine cavity.

VII.- CONCLUSIONS

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The significant increase of PGE_2 and $PGF_2\alpha$ can be used to detect the WOI in natural cycles.

This lipidomic profile is consistent in the same patients in natural, HRT and COS cycles in the receptive endometrium and it is completely abrogated in refractory cycles induced by the insertion of an IUD. These observations indicate that the production of PGE_2 and $PGF_{2\alpha}$ is dependent of the receptive stage of the endometrium.

Specific PGE_2 and $PGF_2\alpha$ peaking profile in the endometrial fluid is related with their secretion by epithelial cells. However, due to the presence of PGs synthases in the endometrial fluid, these PGs production could also be regulated in the uterine cavity.

The expression and localization of the specific PGs synthases in the human endometrium suggest that CBR1 and AKR1C3 are the main PG synthases implicated in PGE_2 and $PGF_{2\alpha}$ production during the receptive phase.

The presence of PGs is essential for embryo adhesion. The chemical inhibition of this PG synthases reduces blastocyst adhesion in an *in vitro* model. These results suggest that embryonic adhesion is mediated by the presence of PGs secreted by hEECs. Clinical translation of this study may be important to understand implantation failure in IVF.

PGE_2 and $PGF_2\alpha$ have an essential role on embryo adhesion, and our studies show that they act via their EP2 and FP receptors. Such results could lead to further insights into the use of these agonists/antagonists receptors as new targets for improving clinical reproduction.

During the WOI, $PGF_2\alpha$ production may be modulated not only by endometrial epithelial cells but also by enzymatic activity in the endometrial fluid. These findings support a role for $PGF_2\alpha$ as a key modulator of endometrial receptivity.

Our pilot study demonstrates that PGE_2 and $PGF_2\alpha$ levels in human EF obtained 24 hours before embryo transfer correlates with cycle outcome.

VIII.- REFERENCES

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