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Departamento de Bioquímica y Biología Molecular
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THESIS DISSERTATION

Perfil de miRNAs secretados en el fluido endometrial a lo largo del ciclo menstrual. Caracterización funcional. Potencial uso como herramienta diagnóstica de receptividad endometrial no invasiva.

Profile of miRNAs secreted in the uterine fluid across menstrual cycle, functional characterization, and potential application as a non-invasive diagnostic tool in endometrial receptivity.

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CERTIFICA:

Que el trabajo de investigación titulado: **"Perfil de miRNAs secretados en el fluido endometrial a lo largo del ciclo menstrual. Caracterización funcional. Potencial uso como herramienta diagnóstica de receptividad endometrial no invasiva."** ha sido realizado íntegramente por Don Juan Manuel Moreno Moya 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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To everyone who has been there to help me in any moment of these four years, this is also yours.

For my heroine, keep saving me.

A todos los que alguna vez han estado ahí para ayudarme durante estos cuatro años, esto también es vuestro.

A mi heroína, sigue salvándome.

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Resumen

INTRODUCCION

La implantación embrionaria y el embarazo requieren una comunicación entre el endometrio y el embrión pre-implantatorio.

El endometrio humano posee dos funciones principales: la adquisición temporal de un fenotipo adhesivo que favorezca la implantación embrionaria, también denominada "receptividad endometrial", y una vez se produce implantación, ejerce un segundo papel en la invasión, placentación, desarrollo fetal y finalmente el parto. Para ello, el endometrio dialoga de forma activa con el embrión mediante mecanismos de señalización que pueden ser secretados de forma paracrina hacia el fluido endometrial que nutren y regulan el correcto desarrollo embrionario.

Actualmente, no existe consenso en los marcadores diagnósticos para determinar el estadio de receptividad endometrial a nivel clínico, únicamente los criterios de "Noyes" surgidos en 1975 han sido ampliamente empleados. Sin embargo, dado que consisten en criterios histológicos, ha sido ampliamente discutida la elevada variabilidad inter-observador. Desde entonces, se han hecho grandes esfuerzos en la búsqueda de alternativas moleculares cuantitativas, como por ejemplo los estudios transcriptómicos, proteómicos, lipidómicos y metabolómicos, e incluso se ha conseguido desarrollar una herramienta predictora que está siendo actualmente usada a nivel clínico, denominada "array de receptividad endometrial", basada en los perfiles transcriptómicos analizados a partir de biopsias tomadas del endometrio en día 19-21 de ciclo menstrual natural, coincidente con el estadio de receptividad endometrial.

Otra de las limitaciones se debe al uso de biopsias endometriales, consideradas un método disruptivo que implica realizar transferencia embrionaria en un ciclo posterior.

En los últimos años, un nuevo concepto denominado "secretómica", basada en el estudio de las moléculas presentes en las secreciones, ha introducido nuevas posibilidades en el ámbito del estudio endometrial. Diferentes tipos de moléculas tales como proteínas, lípidos y metabolitos han sido hallados en secreciones obtenidas de la cavidad endometrial. Además, no se han observado defectos en las tasas de implantación tras aspiración de pequeñas muestras de secreciones previa a la transferencia embrionaria en el mismo ciclo.

Por otro lado, hace dos décadas se descubrieron un nuevo tipo de ácido nucleico, los miRNAs. Dichas moléculas poseen alrededor de 19-22 nucleótidos y funcionan como reguladores negativos de la expresión génica, para ello bloquean la traducción de RNA mensajeros mediante la hibridación por complementariedad de secuencia y secuestro por el complejo RISC. Se han descrito miles de miRNAs en el genoma humano, cada uno capaz de reconocer cientos de genes diana, lo que supone una gran variedad funcional dependiendo del contexto biológico. Además, estas moléculas han demostrado ser capaces de acomplejarse con lípidos/proteínas, ser secretadas en exosomas u otro tipo de vesícula y sobrevivir largos periodos de tiempo sin degradarse. Por estos motivos, se han propuesto como un potencial biomarcador en diferentes patologías humanas.

Diferentes trabajos han recogido la expresión de miRNAs en el tejido endometrial durante la fase de receptividad, pero ninguno de ellos ha explorado las secreciones endometriales a lo largo del ciclo menstrual, ni su función más allá de la predicción bioinformática. Recientemente se ha demostrado la capacidad de diversos tipos celulares de internalizar miRNAs a través de diferentes mecanismos que aún no han sido completamente descritos, como endocitosis y/o receptores específicos de membrana.

La hipótesis de la presente tesis afirma que los miRNAs están presentes en las secreciones endometriales, que se expresan con un patrón determinado a lo largo del ciclo menstrual, y que poseen la capacidad de alcanzar al embrión-preimplantatorio y regular la implantación embrionaria a un nivel no descrito hasta ahora.

OBJETIVOS

- Objetivos generales:

Determinar la presencia y patrones de miRNAs en las secreciones endometriales humanas a lo largo del ciclo menstrual, enfocándonos en la ventana de implantación para hallar potenciales biomarcadores de receptividad endometrial.

Determinar si las secreciones endometriales, así como el cultivo in vitro de células epiteliales endometriales primarias están secretando activamente exosomas con presencia de miRNAs.

Determinar la capacidad embrionaria para incorporar miRNAs vehiculazados o libres.

Determinar la regulación génica y efectos fenotípicos de los miRNAs incorporados por el embrión.

- Objetivos específicos:

Estudiar los efectos transcriptómicos y proteómicos de hsa-miR-30d en las células epiteliales endometrial.

Determinar cambios en los patrones de metilación derivados de altos niveles de hsa-miR-30d en las células epiteliales endometriales.

Demostrar la producción de exosomas in vitro en el medio de cultivo de células epiteliales endometriales primarias.

Demostrar la presencia de miRNAs en formas libres o vehiculizadas

METODOLOGÍA

Tomamos muestras de secreciones endometriales a lo largo del ciclo menstrual dividido en 5 grupos (fase proliferativa temprana, proliferativa tardía, fase secretora temprana, secretora media (o ventana de implantación) y secretora tardía), realizamos extracción de RNA total, microarrays de miRNAs, PCR cuantitativa, analisis bioinformático, aislamiento de exosomas, microscopía electrónica, co-cultivo de embriones murinos junto con exosomas o miRNAs, microscopía confocal y microscopía electrónica de barrido.

Por otro lado, tomamos muestras de biopsias endometriales y realizamos aislamiento y cultivo primario de células epiteliales endometriales, transfecciones transitorias con hsa-miR-30d, extracción de RNA total, de proteína total y de DNA total. Análisis transcriptómico mediante microarrrays de expresión génica, análisis proteómico mediante iTRAQ, analisis epigenético mediante MeDIP, PCR cuantitativa, western-blot e inmunohistoquímica, aislamiento de exosomas producidos por el cultivo in vitro y análisis bioinformático.

RESULTADOS

Mediante microarrays hallamos 19 miRNAs diferencialmente expresados en las secreciones endometriales a lo largo del ciclo menstrual con respecto a la ventana de implantación, momento durante el cual entran en contacto con el embrión pre-implantatorio. En concreto, hallamos altamente up-regulado hsa-miR-30d, que correlaciona con estudios previos sobre tejido endometrial. Además, exploramos los efectos transcriptómicos (176 genes diferenciales), proteómicos (108 proteinas diferenciales) y epigenéticos de la transfección de hsa-miR-30d en las células epiteliales endometriales “in vitro”. De forma relevante hallamos infra expresado el gen asociado a impronta H19, lo que puede deberse a una sobre-expresión de la proteína para el gen DNMT1, que está implicado en metilación del DNA, que resultó confirmado al hallar hipermetilada la región promotora para el gen H19. Por otro lado, el

conjunto de miRNAs endometriales son secretados de forma libre o asociados a vesículas tipo exosomas e incorporados por las células del trofectodermo embrionario, induciendo modificaciones transcripcionales y funcionales asociadas con el fenotipo adhesivo “in vitro” del embrión murino en el caso de hsa-mir-30d.

CONCLUSIONES

1. Los miRNAs están presentes en las secreciones endometriales a lo largo del ciclo menstrual.
2. El perfil de miRNAs durante la ventana de implantación muestra importantes diferencias en comparación al resto de fases del ciclo menstrual, lo que apoyaría su uso como nuevo biomarcardor de receptividad endometrial.
3. La expresión ectópica de hsa-miR-30d en células epiteliales endometriales humanas induce modificaciones transcriptómicas relacionadas con ciclo celular, proliferación y desórdenes endocrinos que podrían influenciar la receptividad endometrial.
4. El receptor de estrógenos es un regulador de los efectos asociados con hsa-miR-30d.
5. La expresión ectópica de hsa-miR-30d en las células epiteliales endometriales primarias humanas induce modificaciones proteómicas relevantes para la fisiología endometrial y el estatus epigenético.
6. Existe un incremento en el estatus de la metilación de la región diferencialmente metilada del gen H19 en las células epiteliales endometriales primarias tras el tratamiento con hsa-miR-30d
7. Las células epiteliales endometriales secretan exosomas tanto en el fluido endometrial como en los medios de cultivo “*in vitro*”.
8. Los exosomas y los miRNAs libres pueden ser internalizados por el trofectodermo de los blastocistos murinos eclosionados.
9. Hsa-miR-30d está presente en las secreciones durante el periodo de receptividad endometrial y puede modular la expresión génica y el fenotipo de adhesividad *“in vitro”* de los embriones murinos eclosionados y listos para implantar.

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Abstract

Embryonic implantation and pregnancy require communication between the maternal endometrium and the preimplantation embryo. During this process, the blastocyst actively regulates the endometrium, whereas the endometrial fluid, secreted by the endometrial epithelium, nurtures and regulates the development of the embryo. In the present dissertation, we show that maternal miRNAs secreted by the endometrial epithelium into the endometrial fluid act as transcriptomic regulators of the preimplantation embryo. Assessment by microarrays revealed the presence of specific maternal miRNAs in the endometrial fluid that are associated with the window of implantation and are in direct contact with the preimplantation embryo; specifically, we explored endogenous effects of hsa-miR-30d, the most prominent miRNA during endometrial receptivity in the epithelial endometrial cells. These endometrial miRNAs are secreted either freely or as exosome-associated molecules and are then taken up into the embryo via the trophectoderm, where they induce embryonic transcriptional and functional modifications. Our results offer the possibility for development of a novel non-invasive tool to predict the receptivity status of endometrium based on miRNAs profiles secreted in endometrial fluid. Furthermore, we demonstrates a model in which endometrial maternal miRNAs function as transcriptomic regulators during early embryo development, thus offering a new perspective on the cross-talk during implantation and pregnancy, and potentially on the developmental origins of certain adult diseases.

TABLE OF CONTENTS

COVER LETTERS .... **III**

ACKNOWLEDGMENTS .... **VIII**

RESUMEN .... **IX**

ABSTRACT .... **XIV**

TABLE OF CONTENTS .... **XV**

LIST OF ABREVIATIONS .... **XVII**

I.INTRODUCTION **1**

1. THE ENDOMETRIUM 2
1.1. Definition 2
1.2. Anatomy 2
1.3. Menstrual cycle 5
2. ENDOMETRIAL RECEPTIVITY 6
2.1. Definition 6
2.2. Morphological evaluation 8
2.3. Single molecule approach 10
2.4. Novel molecular characterization:"Omics" 10
2.5. Non-invasive "Omics" 14
3. MICRORNAS 15
3.1. Introduction 15
3.2. Types of RNA and miRNA biogenesis 15
3.3. Nomenclature 17
3.4. The role of miRNAs in cell biology 19
3.5. MiRNA detection methods 20
3.6. The functional characterization of miRNAs 23
3.7. miRNAs as biomarkers 25
3.8. miRNAs as pharmacological agents 26
3.9. Intercellular communication 26
4. EMBRYO DEVELOPMENT 27
4.1. Early embryo development 27
4.2. The effects of growth factors on human embryo development 28
4.3. Main differences with mouse embryo pre-implantation development 29
4.4. Human embryo implantation stages 29

II. HYPOTHESIS **33**

III. OBJECTIVES **37**

1. General objectives 39
2. Specific objectives 39

IV. MATERIAL AND METHODS **41**

1. SAMPLE COLLECTION AND PROCESSING 43
1.1. Ethics statement 43
1.2. Endometrial samples 43
1.3. Epithelial and stromal separation and primary culture 43
1.4. Endometrial secretion aspirations 44
1.5. Exosome isolation and labeling 45
2. NUCLEIC ACID ANALYSIS 46
2.1. Total RNA extraction 46

TABLE OF CONTENTS

2.2. Retrotranscription 47
2.3. Quantitative Real-Time PCR 47
2.4. RNAse treatment 47
2.5. MiRNA microarrays 48
2.6. MRNA microarrays 49
2.7. MiRNAs transient transfections 49
2.8. Methylated DNA Immunoprecipitation 49
2.9. Primer design 50
3. PROTEIN ANALYSIS 51
3.1. Total protein extraction 51
3.2. Western-blot 52
3.3. Immunohistochemistry 52
3.4. Isobaric tag for relative and absolute protein quantitation (iTRAQ) 53
4. MICROSCOPY 54
4.1. Confocal microscopy 54
4.2. Transmission electron microscopy 55
4.3. Scanning electron microscopy 55
5. MOUSE EMBRYOS 55
5.1. Day 1.5 mouse embryo collection 55
5.2. Embryo culture 56
5.3. Adhesion assays 56
6. BIOINFORMATIC ANALYSIS 56
6.1. Microarrays data analysis 56
6.2. Proteomic data analysis 57
6.3. Statistics represented 58

V. RESULTS 59

1. Human EF contains secreted miRNAs of maternal origin 61
2. MiRNAs of maternal origin display differential miRNAs profile during the window of implantation (WOI) 62
3. MiR-30d is expressed in the epithelial compartment of the receptive endometrium 65
4. The transcriptomic effect of miR-30d transfection in primary human endometrial epithelial cells 66
5. Bioinformatic analysis of genes regulated by miR-30d in human endometrial epithelial cells 67
6. The proteomic effect of miR-30d transfection in primary human endometrial epithelial cells 70
7. The methylation status of the H19 gene 72
8. Exosomes are secreted by the endometrial glands 73
9. Maternal miRNAs are transported as free- and exosome-associated molecules 75
10. Embryo ability to uptake miRNAs 76
11. Embryo ability to uptake exosomes derived from hEEC 77
12. Transcriptomic changes after hsa-miR-30d uptake by the murine embryo *"in vitro"* 78
13. Phenotypic changes after hsa-miR-30d uptake by the murine embryo *"in vitro"* 79

VI. DISCUSSION 81

VII. CONCLUSIONS 93

VIII. REFERENCES 97

IX. ANNEXES 115

LIST OF ABREVIATIONS

2D-DIGE: Bidimensional differential gel electrophoresis
ALDH2: Aldehyde dehydrogenase 2
ART: Assisted reproduction treatment
CD63: Cluster of differentiation 63
circRNA: Circular RNA
COS: Controlled ovarian stimulation
DNMT1: DNA (cytosine-5)-methyltransferase 1
E2: Beta-estradiol
ECM: Extracellular-matrix
EEC: Endometrial Epithelial Cell
EF: Endometrial Fluid
EGA: Embryonic genome activation
EP: Early proliferative
eRNA: enhacer RNA
ES: Early secretory
ESR1: Estrogen receptor 1
FDR: False discovery rate
FFPE: Formalin-fixed paraffin embedded
FSH: Follicle stimulating hormone
GE: Gene expression
hCG: Human chorionic gonadotropin
hEEC: Human endometrial epithelial cell
HPLC: High-performance liquid chromatography
ICAT: Isotope-coded affinity tag
ICM: Inner cell mass
ISH: In situ hybridization
ITRAQ: Isobaric tag for relative and absolute quantitation
LH: Luteinizing hormone
LNA: Locked nucleic acid
lncRNA: Long non-coding RNA
LP: Late proliferative
MCS: Multicloning site
MeDIP: Methylated DNA immunoprecipitation
miRNA: MicroRNA
MMP: Matrix-metalloproteinase
MS: Mid secretory
NTS: nucleotides
ORF: Open reading frame
P4: Progesterone
PCA: Principal component analysis
piRNA: piwi RNA
qPCR: Quantitative polymerase chain reaction
RISC: RNA-induced silencing complex
RNA: Ribonucleic acid
RNA-seq: RNA sequencing
Rnase MRP: Ribonuclease MRP

LIST OF ABREVIATIONS

RNAseP: Ribonuclease P
rRNA: ribosome RNA
SAM: Significance analysis of microarrays
SEM: Scanning electron microscopy
siRNA: Small interference RNA
snoRNA: Small nucleolar RNA
SRP RNA: Signal Recognition Particle RNA
StEM: Standard error mean
TE: Trophectoderm
TEM: Transmission electron microscopy
TIMP: Tissue inhibitor metalloprotease
tRNA: Transfer RNA
uNK: Uterine natural killer
UTR: Untranslated region
WOI: Window of implantation
ZGA: Zygotic genome activation

I.INTRODUCTION

I. INTRODUCTION

Reproduction is a key function for our existence; the European IVF-monitoring program determined that one in six couples has fertility problems, and half of these couples remain subfertile or need assisted reproductive treatments (ART), such as In-Vitro Fertilization (IVF). Indeed, in Europe in 2008, an estimated 1.6 % of births have been derived from this type of ART and this number keeps increasing due to the tendency of women to postpone their motherhood beyond their best fertile age (Ferraretti *et al*. 2012, Wright *et al*. 2008).

Since its beginning in 1978 (Steptoe and Edwards 1978) much effort has been done to improve ART, although pregnancy rates remain around 30% per embryo transfer, suggesting that correct embryo implantation is the limiting step for success (Figure 1) (Koot *et al*. 2012). Multiple embryo transfer has been extended to achieve higher pregnancy rates but at the expense of multiple gestations, which has generated an epidemic of multiple births (21% of deliveries in ART) (Ferraretti *et al*. 2012), with serious adverse obstetrical outcomes (Verberg *et al*. 2007).

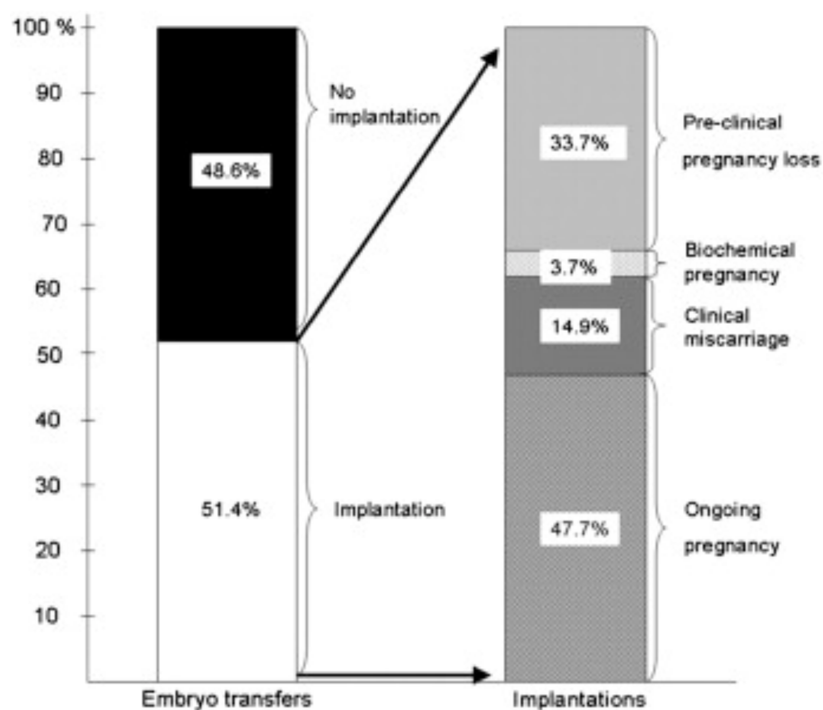


Figure 1. Treatment outcome after embryo transfer. Reproductive outcome (left bar) and pregnancy (right bar) in women who collected urine samples 9–19 days after oocyte retrieval (n = 179). Adapted from (Koot et al. 2012).

1. THE ENDOMETRIUM

1.1. Definition

The human endometrium is the mucous membrane that coats the inner part of the uterus in mammals (Figure 2). In humans and higher primates, it is hormonally regulated and it changes dynamically during the menstrual cycle. These changes are necessary in preparation for the receptive stage that is essential for embryo adhesion, implantation and gestational development to keep nurturing and protecting the allografic fetus.

1.2. Anatomy

The uterus consists of two anatomically different sections: the uterine body (corpus uteri with the uterine cavity), which contains a smooth muscle layer, also called the myometrium, coated by a tunica mucosa named endometrium, and the cervix, which is also coated with the endocervix (Figure 2).

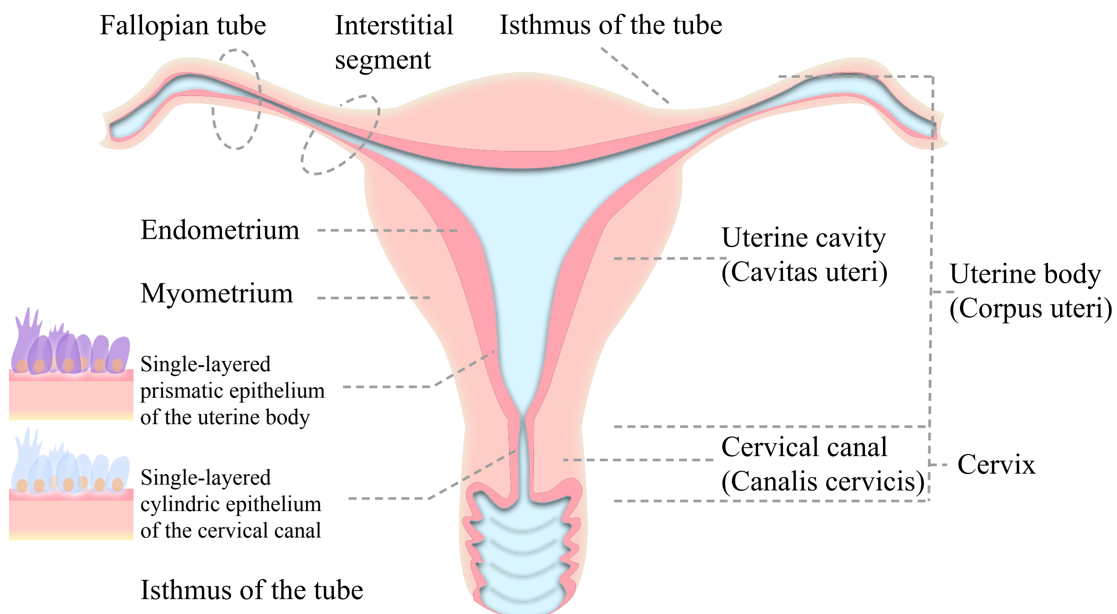


Figure 2. Schematic representation of the uterus.

The human endometrium consists in epithelial, stromal and vascular compartments, and also immune resident cells. All these four compartments are located in two regions named “functionalis”, which is regenerated each month and “basalis”, which is not released and is the resource for the cyclic regeneration of the endometrium (Figure 3).

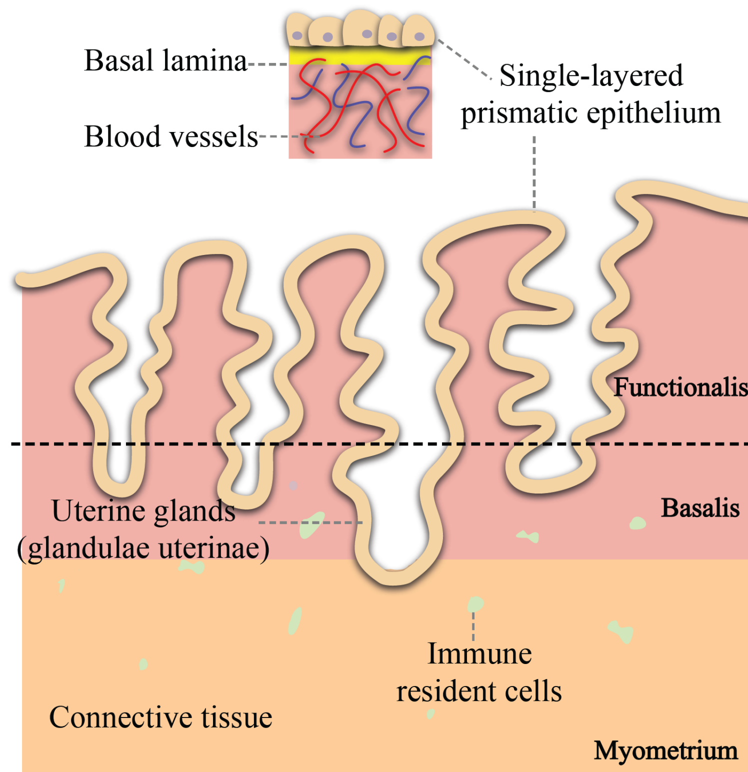


Figure 3. Schematic representation of the endometrium.

The epithelial compartment is a monolayer of polarized cuboid cells oriented towards the lumen of the uterus formed by two components, the luminal and the glandular epithelia. The luminal compartment modifies its morphology across the menstrual cycle (Murphy and Shaw 1994). These changes are induced in response to estrogens and affect the plasma membrane, cytoskeleton, tight junctions and microvilli, which are reduced during secretory stage, while the apical protuberances originated by endometrial fluid endocytosis (Kabir-Salmani *et al*. 2005), also called “pinopodes”, increases. The glandular compartment contains epithelial cells that proliferate during secretory phase forming large glands producing and secreting molecules to nurture the implanting blastocyst, other features are glycogen accumulation in the subnuclear cytoplasm and giant mitochondria (Dockery *et al*. 1988).

The endometrial stroma is a connective tissue formed by cells and extracellular matrix. The principal cell type is the fibroblast, which is involved in matrix remodeling across the menstrual cycle, principally during the decidualization process in the luteal phase. Several morphological and biochemical changes characterize this process in response to estrogen and progesterone exposure. Morphologically, it is characterized by elongated fibroblast-like Endometrial Stromal Cells (ESCs) transformed into enlarged round cells with specific ultrastructural modifications, accompanied by the secretion of specific markers such as prolactin (PRL) and the insulin-like growth factor binding protein-1 (IGFBP-1) (Wahlstrom and Seppala 1984), in addition to extracellular matrices such as laminin, type IV collagen, fibronectin and heparin sulphate proteoglycan as part of their differentiation program (Garrido-Gomez *et al*. 2011). The onset of this process in the ESCs surrounding the terminal spiral arteries marks the end of the window of implantation.

The vascular compartment is a complex network starting at myometrium. The uterine arteries form the arcuatus arteries that originate the radial arteries, which cross the myometrium and reach the endometrial-myometrial junction where they differentiate into basal arteries that give rise to the spiral arteries, which are the support for the basalis region. Basalis arteries ramify in the functional layer and each one can support a 4-8 mm^2 of endometrial surface (D. Neill *et al*. 2006). In the human endometrium there are three main angiogenesis events: during menstruation to repair the vascular compartment, during the fast grow in proliferative phase and during the secretory phase when the number of spiral arteries increases (Gargett and Rogers 2001).

The immune resident cells consist in uterine natural killer cells (uNKs), macrophages and T cells which main function is to protect the genital tract from infections and avoid the immune rejection during embryo implantation. The leucocyte population found in normal endometrium accounts for 10-15% of the total stromal cell population, and it is the highest during the late secretory and premenstrual stages (Bulmer and Johnson 1985).

1.3. Menstrual cycle

The menstrual cycle is exclusive from primates and humans, as the rest of mammals posseses an estrous cycle characterized by the endometrial reabsorption. In the menstrual cycle the endometrium is expelled and renewed it in each menstruation (Jabbour *et al*. 2006). The cyclic regulation of human endometrium is due to the effects of the ovarian steroids, estrogen and progesterone (E2 and P4 respectively), implying the coordination between menstrual and ovarian cycles (Critchley *et al*. 2006). There are three main phases during menstrual cycle: menstrual, proliferative, and secretory (Noyes *et al*. 1975) (Figure 4).

Menstruation phase: it starts with initiation of menses (day 0) and takes 3-5 days. The reducing levels of E2 and P4 induce the detachment of the functionalis layer and the endometrial shedding. After this phase, the endometrium is thin and only the basalis layer is not released.

Proliferative phase: it goes from the end of menstruation untill the day of ovulation (day 14). The stromal and epithelial cells from the functionalis layer proliferate and regenerate it in response to increasing levels of estrogens, secreted from the ovary. The glandular epithelium acquires linear shapes and vascularization. The endometrial thickness increases from 4 to 7 mm approximately.

Secretory phase: it takes from the ovulation (day 15) till menstruation (day 28). After the ovulation, the corpus luteum starts to secrete high amounts of E2 and P4. The estrogens induce a slight proliferation, and progesterone promotes decidualization. Between days 19-21, the endometrial epithelium becomes receptive (Aplin 2000). When there is no implantation, the corpus luteum degenerates, E2 and P4 descends, the endometrium becomes ischemic, and glandular secretion stops. The final result is the elimination of the functionalis layer and the start of a new cycle (Hawkins and Matzuk 2008).

2. ENDOMETRIAL RECEPTIVITY

2.1. Definition

The human endometrium has two main functions: temporal acquisition of an adhesive phenotype to allow for the embryo to implant, also called “endometrial receptivity”, and its active participation in the initial dialogue with the embryo will direct the invasion, placentation, fetal development and finally parturition (Finn and Martin 1974).

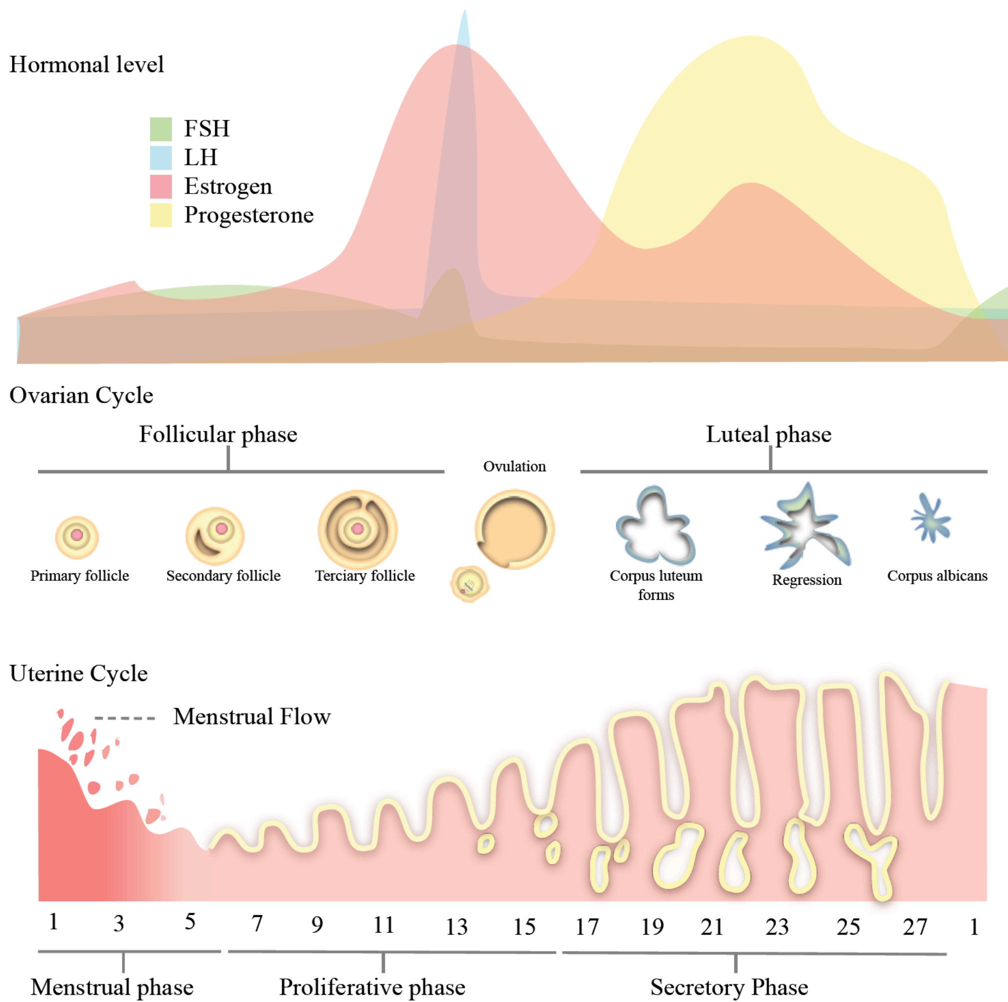


Figure 4. Schematic view of the human menstrual and ovarian cycles.

The term "window of implantation" (WOI) corresponds to the period of time in which the endometrium remains receptive for an embryo and occurs in response to the presence of endogenous or exogenous progesterone and after appropriated stimulation with 17β-estradiol. When the luminal endometrial epithelium acquires a receptive status it experiments several changes such as "plasma membrane transformations", pinopode formation, and tight lateral junctions (Murphy and Shaw 1994).

2.2. Morphological evaluation

Morphological evaluation is based in the cyclic histological changes reported by Noyes (Noyes *et al*. 1975) from 8,000 endometrial biopsies across menstrual cycle that were sectioned and stained with hematoxilin-eosine. It has been considered as the gold standard in endometrial evaluation and is based in eight basic histological features: glandular mitosis, nucleus pseudostratification, basal vacuoles, secretion, edema from stroma, pseudodecidual reaction, stromal mitosis and leucocyte infiltration (Figure 5). The accuracy in the endometrial dating depends on several factors such as the ovulation day, time of biopsy, quality of biopsy, absence of endometrial lesions, fixation technics and the pathologist interpretation. Therefore, ovarian stimulation alters the endometrial maturation process. As a consequence, nowadays the endometrial status is not routinely assesed in infertility clinics worldwide due to the absence of objective and reliable diagnostic tests that can inform clinicians about the status of endometrial receptivity.

Other morphological biomarkers that have been proposed for determine receptivity is the pinopodes formation (Nikas 1999); however, the pinopodes are present in the post-receptive endometrium (Quinn and Casper 2009), and the technique requires visualization by scanning electron microscopy (SEM) that is too complex technic to be routinely used in clinical practice.

A third non-invasive morphological evaluation of the receptivity is the transvaginal ultrasound. Several parameters have been related with the size, thickness and vascular perfusion of the endometrium. However, they do not correlate well with endometrial receptivity nor gestation prediction after ART (Dickey *et al*. 1992, Garcia-Velasco *et al*. 2003, Remohi *et al*. 1997).

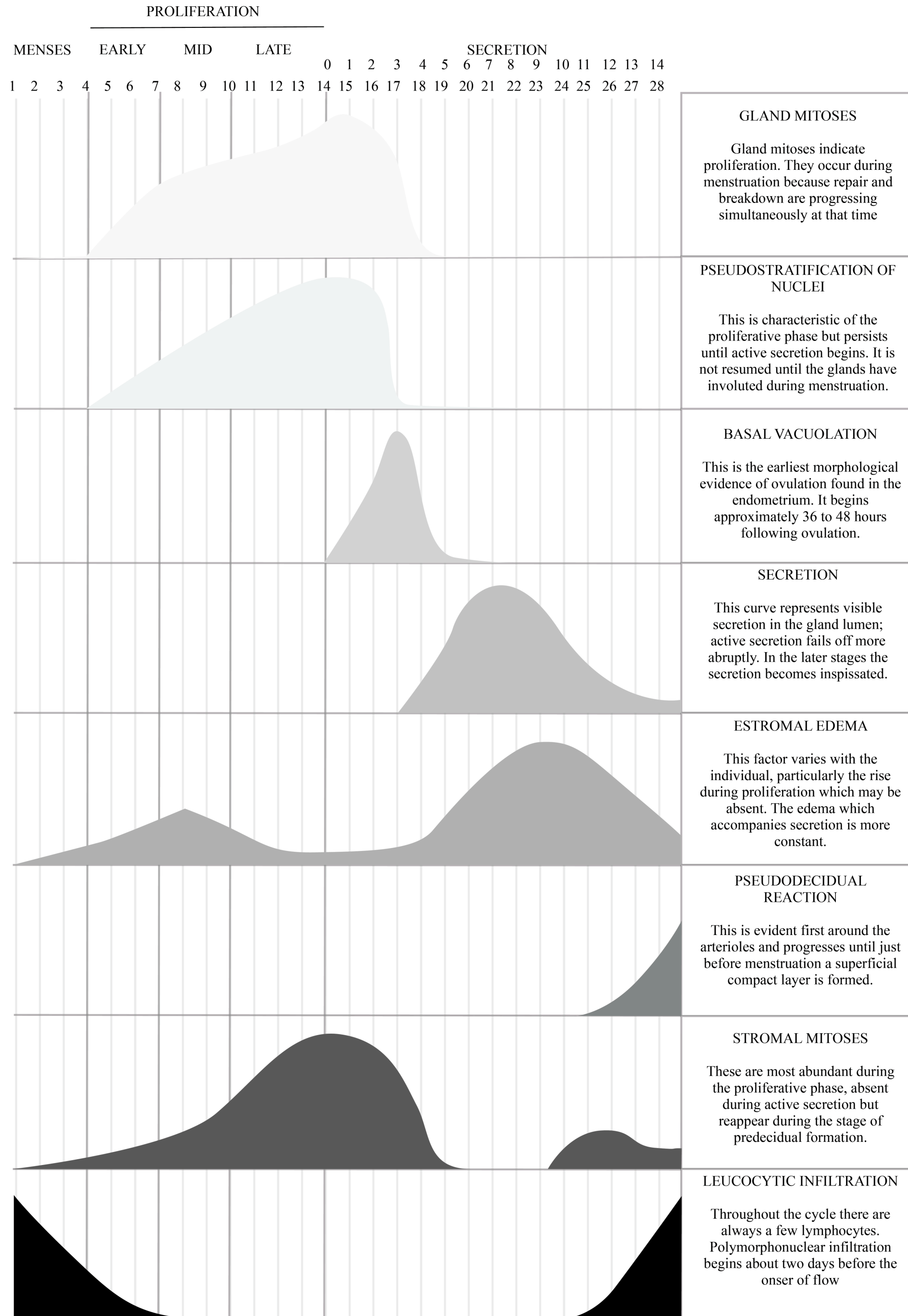


Figure 5. Noyes hystological criteria.

2.3. Single molecule approach

Several groups have studied the expression of specific molecules in human endometrium, at the different cell compartments during the different stages of menstrual cycle (Aghajanova *et al*. 2008), for example, Integrins are a family of cell adhesion receptors that ligates with extracellular matrix components to trigger specific cell signaling. Some authors have determined that Integrins β3, α4 and α1 were indicators of endometrial receptivity (Lessey 2004). Mucins are other type of molecules studied; such as Mucin 1, which acts as a barrier for adhesion but is cleaved specifically by the embryo (Meseguer *et al*. 1998, Meseguer *et al*. 2001). The osteopontin is a receptor for integrins that has been found increased in endometrial glands and secretions during receptivity (Borthwick *et al*. 2003, Carson *et al*. 2002, Riesewijk *et al*. 2003). Nevertheless, none of them has been show to be clinical predictors of human endometrial receptivity.

2.4. Novel molecular characterization: "Omics"

The new technologies that allowed the consecution of the human genome project (Lander *et al*. 2001) and recently of the ENCODE (Encyclopedia of DNA Elements) project have changed significantly the possibilities of research in the field, from single molecules to the whole genome of a cell in a single experiment (ENCODE Project Consortium *et al*. 2012).

The term "Genomic" consists in the study of DNA and chromosomes, while "Transcriptomics" (or "Functional genomics") studies the global mRNA gene expression. The basis of functional genomics resides in the fact that all the cells contain the same genetic information but depending on tissue, cell type or biological process in play, the cells selectively express the genetic material. For transcriptomics, the most common evaluation techniques are the gene expression microarrays and RNA sequencing (RNA-seq). Several works that have explored the functional genomics during the window of implantation in humans, and they have determined the most important molecules at the normal receptive status of endometrium (Borthwick *et al*. 2003, Carson *et al*. 2002, Diaz-Gimeno *et al*. 2011, Diaz-Gimeno *et al*. 2012, Horcajadas *et al*. 2004, Horcajadas *et al*. 2005, Horcajadas *et al*. 2008, Kao *et al*. 2002, Riesewijk *et al*. 2003). Researchers at IVI Foundation, has developed a predictive tool for the

assessment of the endometrial receptivity based on a subset of these differentially expressed genes, creating a customized microarray named “Endometrial Receptivity Array (ERA)” (Diaz-Gimeno *et al*. 2011, Diaz-Gimeno *et al*. 2012, Ruiz-Alonso *et al*. 2012). This tool has shown a significant improvement in helping patients with repeated implantation failure, in those cases that ERA predicted endometrium as “pre-receptive”, their embryos were transferred two days later than the usual day of embryo transfer in the next cycle, resulting in higher percentages of pregnancies and babies at home (Ruiz-Alonso *et al*. 2014). However, since ERA requires a biopsy to obtain the endometrial sample, same-cycle embryo transfer is not possible.

Proteomic explores the existence of translated proteins and their relative quantity; the most used technics are the “bi-dimensional differential gel electrophoresis” (2D-DIGE), the “Isotope-coded affinity tag” (ICAT), and the “Isobaric tag for relative and absolute quantitation” (iTRAQ) that are based in liquid chromatography. The principal disadvantage of the proteomics in general is that requires very high protein concentrations and a mass spectrometry system to identify each peptide (Wu *et al*. 2006). The proteome of receptive versus non-receptive endometrium of healthy donor patients by 2D-DIGE was studied and several molecules, such as Annexin-A2 and Stathmin-1 were found up-regulated (Dominguez *et al*. 2009). A different group performed ICAT between proliferative and secretory endometria and found five proteins with a consistent differential expression (DeSouza *et al*. 2005), such as NMDA receptor subunit zeta 1 precursor and FRAT1. The proteomic of *“in vitro”* decidualization process was also described (Garrido-Gomez *et al*. 2011).

	Transcriptomic studies						Proteomic studies	
Name	Carson (2002)	Riesewijk (2003)	Mirkin (2005)	Talbi (2006)	Haouzi (2009a)	Díaz-Gimeno (2011)	Li et (2006)	Domínguez (2009)
ANXA4	-	4	6.5	4.9	2.6	4.7	2.1	1.9
ANXA2	-	4	5.6	2	-	-	-	2.1
MAOA	-	15	-	-	9.9	8.4	-	3.4
TAGLN	-	6	-	-	5.9	-	-	1.7
LCP1	-	-	2.6	1.6	-	-	-	1.6
PGRMC1	-	-	-	-1.8	-	-	-	-2.4
STMN1	-	-	-3.2	-	-	-	-	-2.2
APOL2	-	-	-	-	2.4	-	-	3.7
ALDH1A3	-	-	-	-	16.5	-	-	1.8
S100A10	-	-	-	-	3.5	-	-	4.8

Table 1. Relation between transcriptomic and proteomic results for endometrial receptivity.

In Table 1 (Haouzi *et al*. 2012), summarizes the most common molecules identified during the receptivity phase in the different studies at transcriptomic and proteomics levels. This scheme exemplifies how biologically mRNA transcription and the protein translation levels are not always correlated. Figure 6 shows that the results differ among the six mentioned transcriptomic studies. Alternatively, this observation could also be explained by the patients' characteristics and the day of biopsies.

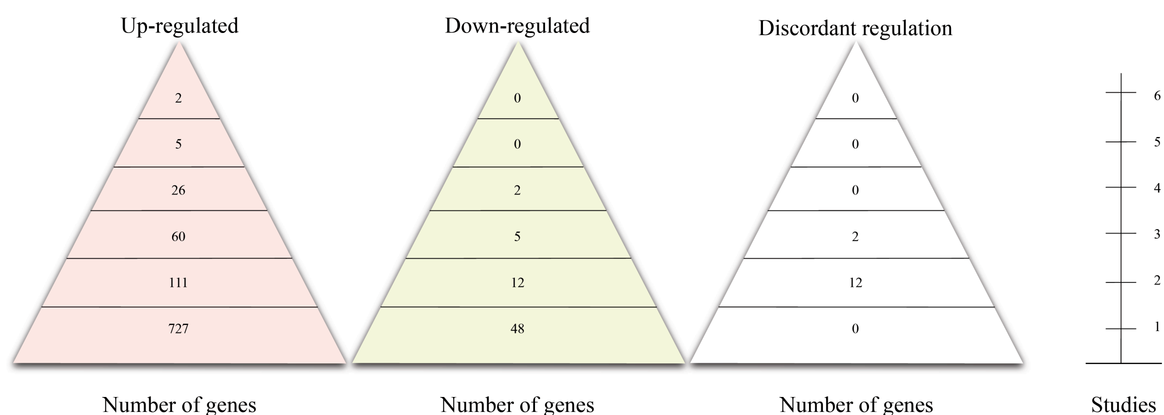


Figure 6. Number of genes involved in endometrial receptivity common to the microarrays studies.

Lipidomics is a novel term assigned to the study of global lipid profile in different type of samples. Some recent reports have applied lipidomics to the study of reproductive function by assessing the lipid content of endometrial biopsies during pregnancy (Durn *et al*. 2010). The technique used for this kind of metabolite evaluation is the high-performance liquid chromatography (HPLC) to fractionate, separate, and identify the composition and expression levels of lipids at single level (Vilella *et al*. 2013).

The miRomics is a novel concept for the –omics that has been assigned to the study of global profiles of miRNAs (miRNAs). MiRNAs are short non-coding RNAs that we will further describe in the next section. Thousands of them have been identified, and they can be quantified using the same strategies as for mRNA, that is RNA-seq and microarrays. They have been proposed as novel biomarkers in the endometrium to study receptivity: Kuokkannen *et al*. using microarrays technology explored the miRNA's profile in the endometrial epithelial cells

isolated from endometrial biopsies of women at late proliferative and mid secretory stages of the menstrual cycle. This study obtained 12 up-regulated miRNAs during the mid-secretory stage that would target genes related to cell cycle at this stage, what would confirm the role of these miRNAs in the control of cell proliferation (Kuokkanen *et al*. 2010). In other study, a Genome-wide identification of miRNAs by deep sequencing technology revealed the differential miRNA expression between endometrial biopsies in pre-receptive (LH+2) and receptive stages (LH+7) of natural cycles and from hCG+4 (equivalent to pre-receptive) and

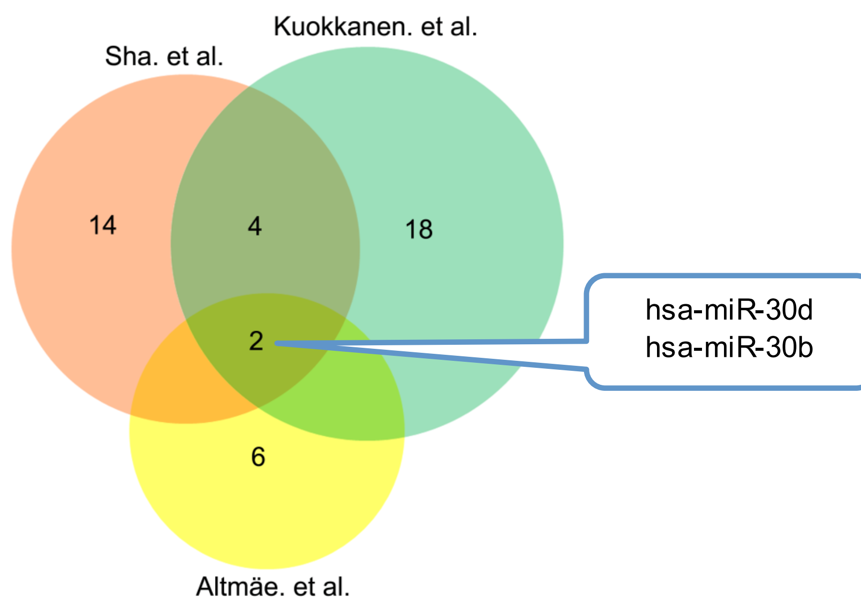


Figure 7. Venn's diagram showing the number shared miRNAs in the three studies of endometrial receptivity.

hCG+7 (equivalent to receptive) in stimulated cycles (Sha *et al*. 2011). From this study, 22 miRNAs were significantly deregulated comparing natural vs. stimulated cycles at receptive stages, and 20 in receptive vs. pre-receptive in natural cycles. Moreover novel miRNAs not previously described were sequenced for the first time. A similar study explored miRNAs between pre-receptive and receptive stages in proven fertility women by microarrays and four differentially expressed miRNAs were reported (Altmae *et al*. 2013). The Venn's diagram in Figure 7 shows how miRNAs were correlated among the different studies mentioned.

2.5. Non-invasive "Omics"

There is also a trend towards the non-invasive omics in the study of the endometrial receptivity, looking forward not to damage the uterine context or interfere with conception in the same menstrual cycle. For example, the "secretomics", or study of uterine secretions would potentially replace biopsies as a non-disruptive technic. This concept uses the same technology as transcriptomics, proteomics, miRomics, and lipidomics. It has been shown that aspiration or flushing of fluid may be performed using an embryo transfer catheter immediately before embryo transfer in IVF cycles without negatively affecting implantation rates (van der Gaast *et al*. 2003).

The endometrial secretome is constituted by secreted mediators that can modulate endometrial receptivity, the maintenance and nurturing of ascending spermatozoa, and the early development of preimplantation embryo. The components of uterine fluid are derived from the luminal epithelium and glands, proteins selectively transudated from blood, and contributions from the tubal fluid. The uterine cavity is very tight and for that reason the volume of uterine fluid is low: It is difficult to retrieve more than 10 μL from a woman (Salamonsen *et al*. 2013).

The primary components are proteins, aminoacids, electrolytes, glucose, urea, cytokines, growth factors, metalloproteinases and their inhibitors, immunoglobulins, α-1 antitrypsin precursor, haptoglobin, and transferrin (Boomsma *et al*. 2009).

The lipidomics of endometrial secretions are characterized by presence of triglycerides, eicosanoids (prostaglandins (PGs), thromboxane and leukotriene), endocannabinoids and sphingolipids, which play a central role in the biology of reproduction (Berlanga *et al*. 2011). A significant increase in the concentration of two specific lipids, PGE2 and PGF2α was found between days 19–21 of the menstrual cycle, coincident with the window of implantation (Vilella *et al*. 2013).

The miRomic of endometrial fluid across menstrual cycle will be described for the first time in the present doctoral thesis; the only similar approach in the literature was carried out recently by Salamonsen and collaborators, who observed *"in vitro"* the existence of small

secreted vesicles which contained miRNAs in the conditioned media of endometrial epithelial cell lines (Ng *et al*. 2013).

3. MIRNAS

3.1. Introduction

MicroRNAs, also called miRNAs, were first described two decades ago when Ambros and colleagues identified a specific twenty-two nucleotides-long RNA derived from the lin-4 gene in C. elegans. This molecule was able to repress lin-14 gene translation, but not its transcription, by binding to its complementary 3'-UTR region (Lee *et al.* 1993, Wightman *et al.* 1993). At first this small RNA seemed to be restricted to nematodes, but seven years later homologs of the LET7 gene, which is associated with developmental timing, were found in humans and other vertebrates (Pasquinelli *et al.* 2000). Since then, the numbers of miRNAs known and the interest in their mechanisms of action and functions have exponentially increased (Ambros 2004, Bartel 2004).

3.2. Types of RNA and miRNA biogenesis

Ribonucleic acid (RNA) is a cell-synthesized molecule that is principally known for its role in exporting genetic information from the nucleus into the cytoplasm, where it is translated into proteins. However, new types of RNAs with novel roles, termed 'non-coding RNAs', have recently been uncovered, which are summarized in Table 2 (Moreno-Moya *et al.* 2013).

MiRNAs are transcribed by RNA polymerase II and/or RNA polymerase III as long 100-1000 nts primary, or *pri-miRNAs*, which are usually capped at the 5'-end and are poly-adenylated at the 3'-end. Pri-miRNAs are sequentially processed by the ribonucleases Drosha and Pasha (DGCR8) to produce 60-70 nt pre-miRNAs, which have a 5'- phosphate and a 2 nts overhang at the 3'-end. These products are then exported to the cytoplasm by Exportin-5, where they bind to the ribonuclease Dicer and are processed to yield a double strand 'miRNA:miRNA'. Finally, a helicase unwinds the duplex into mature miRNAs (Zhang *et al.* 2007).

RNA function	RNA name	Detailed role in the cell
Protein translation	**Messenger RNA** (mRNA)	Codifies the information to translate DNA into protein. Pre-mRNA is transcribed from DNA by RNA Pol II, and maturation process involves removal of introns, addition of a 5' methylguanisine cap, and polyadenylation of the 3' end of the RNA molecule. The mRNA is exported to the cytoplasm and translated into proteins at ribosomes. The genetic code is based in codons, that corresponds to three consecutive nucleotides that identify the amino acid sequence of the protein.
	Transfer RNA (tRNA)	It is the responsible to bring the amino acid corresponding to a specific mRNA codon. tRNA are composed of: an anticodon (a 3nt complementary to the mRNA codon), an amino acid binding-site, and a binding-site for the aminoacyl-t-RNA synthetase, an enzyme which links an amino acid to a tRNA.
	Ribosomal RNA (rRNA)	The RNA component of the ribosome. For translation into protein, the ribosomes align the anticodon of tRNA with mRNA codon and a peptidyl transferase activity links amino acids together. Eukaryotes have 4 types of rRNA: 18s rRNA in the small ribosomal subunit, and 28s, 5.8s, and 5s rRNA in the large ribosomal subunit.
Protein function	**Signal recognition particle RNA** (7SL RNA or SRP RNA)	This type of RNA is part of the SRP that links to the ribosomes and delays protein translation until is associated with the SRP receptor located in the membrane. Once associated, the SRP is released and the ribosome continues the protein translation crossing the plasma membrane.
RNA function and maturation	**Small nuclear RNA** (snRNA)	It is part of the spliceosome, a complex that removes introns from pre-mRNA. It has been described 5 types of small nuclear RNAs (snRNAs).
	Small nucleolar RNA (snoRNA)	Involved in modifications of other RNAs. There are two main clases: H/ACA box snorRNAs (direct conversion of uridine to pseudouridine), and C/D box snoRNAs, (fir addition of methyl groups to RNAs).
	Ribonuclease P (RNAseP)	The RNA component of a ribozyme which cleaves and generates the mature tRNA but also is required for RNA Polymerase III transcription of various noncoding RNA genes (tRNA, 5s rRNA, SRP RNA, and U6 snRNA genes).
	Y RNA	Part of the RoRNP ribonucleoprotein complex. Y RNA may be important in resistance to UV irradiation and in DNA replication. It is required for increased proliferation of cancer cell lines.
	Ribonuclease MRP (Rnase MRP)	The RNA component of Rnase MRP, a ribozyme that is essential for mitochondrial DNA replication. In the nucleus, Rnase MRP participates in precursor rRNA processing.
	Circular RNA (circRNAs)	Act as molecular 'sponges', binding to and blocking miRNAs.
Regulatory RNAs	**Long non-coding RNA** (lncRNA; includes lincRNA and long RNA pseudogenes)	Non-protein coding transcripts from 200 nt to 100 kb in length. They appear to function in diverse areas including epigenetics, alternative splicing, and nuclear import. For example, lncRNA XIST is responsible for the X-chromosome inactivation.
	Enhancer RNAs (eRNA)	A type of RNA that regulates epigenetically affecting enhancers functions in the promotor region of genes altering its transcription
Telomere synthesis	**Telomerase RNA**	The RNA component of telomerase, which extends the ends of DNA preventing its loss during replication. The protein component of the telomerase has reverse transcriptase activity and the RNA component serves as a template for the telomere repeat.
RNA intereference (RNAi)	**MicroRNA** (miRNA)	Short RNAs (19-25 nucleotides) typically involved in the downregulation of gene expression. Biogenesis and mechanism will be further described in the next epigraph.
	Small (short) interfering RNA (siRNA)	Exogenous double-stranded, short RNA molecules (21-23 nt) that silence the expression of specific genes. Following transfection of siRNAs an RNA-induced silencing complex (RISC) is assembled. The siRNAs unwind and a single strand of the siRNA remains bound to RISC. Then the complex targets and cleaves mRNA transcripts that have complementary sequences to the bound siRNA. Physiologically siRNAs are also generated after RNA virus infections.

Piwi-interacting RNA (piRNA)	Short (23-32 nt) RNA that are part of riboprotein complexes active to ensure germ-line stability by silencing transposoons within germ cells. piRNA are found in clusters encoding 10 to thousands of different piRNAs throughout the mammalian genome.

Table 2. The RNA Universe (Moreno-Moya *et al.* 2013).

Mature miRNAs are incorporated to the RNA-Induced Silencing Complex (RISC) and bind to the complementary 3'-UTR of its specific target mRNA. This either results in inhibition of mRNA translation or promotes its degradation and leads to post-transcriptional gene silencing (PTGS). Additionally, the RNA-induced transcriptional silencing (RITS) complex, which uses AgoI instead of AgoII in its effector complex, was described almost a decade ago. This complex exerts DNA/histone modifications (e.g. methylation) on the genome, and therefore triggers transcriptional gene silencing. Although RITS has been identified in many species so far, it is yet to be confirmed in humans (Castanotto *et al.* 2005, Jackson and Standart 2007, Nilsen 2007).

In most cases, the 'seed region' (the 7-8 bases after the first or second base of the 5' end of the miRNA) matches exactly the corresponding target-mRNA sequence. Nucleotide base pairing also occurs at the 3' region of the miRNA, although this is thought to be less important than 5' pairing. Duplex mismatches between miRNA:mRNAs cause the formation of bulge structures in the central region which may be useful for mRNA regulation (Figure 8). Because of the short recognition elements, the same miRNA can recognize hundreds of gene targets and, at the same time, each gene can be targeted by several miRNAs. Unfortunately, the combined complexity of these interacting elements makes the understanding of miRNA biology relatively difficult.

3.3. Nomenclature

As previously mentioned, the miRNAs are sequentially processed from a long primary miRNA transcript (pri-miRNA), to a hairpin (pre-miRNA), and finally, once exported into the cytoplasm, to the single stranded mature miRNA. Experimentally confirmed miRNAs follow a standard-nomenclature system (Griffiths-Jones *et al.* 2006). The prefix 'mir' is followed by a dash and a number, the latter often indicating the order of naming: capitalized 'miR-' refers to the mature form of the miRNA and 'mir' refers to the pre-miRNA. MiRNAs with almost identical sequences are annotated with an additional lower case letter; for example, miR-30b is

almost identical to miR-30d. Pre-miRNAs that generate two nearly identical mature miRNAs but which have different genomic origins are indicated with an additional dash-number suffix, for example, the pre-miRNAs mir-194-1 and mir-194-2 are located in different regions of the genome but they both generate miR-194. Species are designated with a three-letter prefix, e.g., hsa-miR-30d is a human (Homo sapiens) miRNA whereas mmu-miR-30d is a mouse (Mus musculus) miRNA. When two mature miRNAs originate from opposite arms of the same pre-miRNA, they are denoted with a -3p or -5p suffix. When the relative expression levels are known, an asterisk following the name indicates a miRNA that is found at low levels relative to the miRNA in the opposite arm of the pre-miRNA hairpin. For example, hsa-miR-30d and hsa-

miR-30d* share a pre-miRNA hairpin, but hsa-miR-30d is the predominant form found in the cell.

3.4. The role of miRNAs in cell biology

MiRNAs are expressed in all tissues, and regulate a wide spectrum of processes such as cellular differentiation, proliferation, and apoptosis (He and Hannon 2004), and the roles exerted by miRNAs can be very different from each other. In cancer, for example, several miRNAs have been thoroughly characterized and classified as oncogene regulators

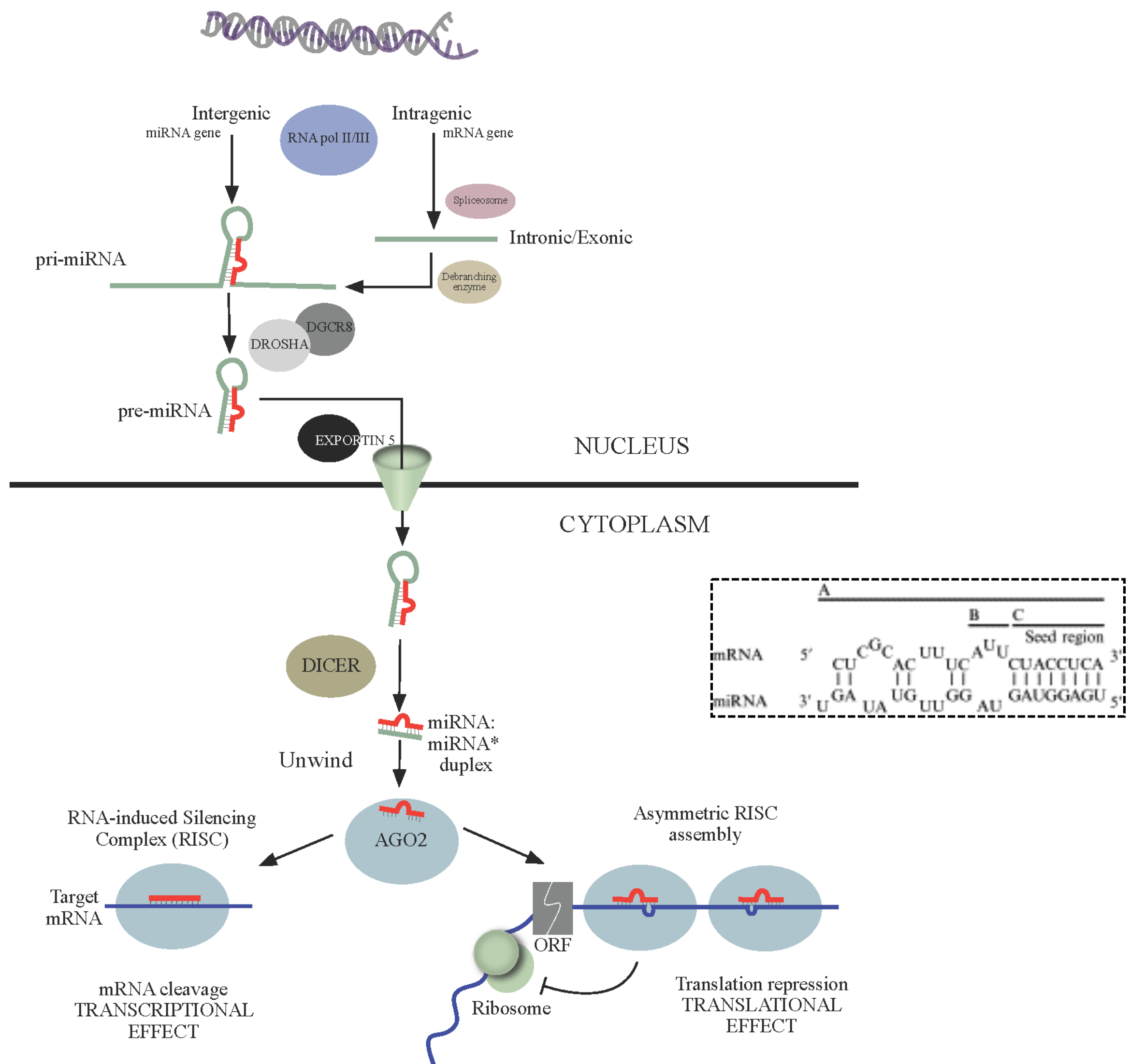


Figure 8. The miRNA biogenesis and mechanisms of action.

(oncomiRs), while, on the contrary, others have been described as tumor suppressors (e.g., the let-7 family), which is able to target oncogenes such as RAS, MYC, HMGA2, and cell cycle check points (Zhang *et al.* 2007).

There is a growing trend towards screening miRNAs for diagnostic purposes in reproductive biology, although most studies do not investigate their functional roles but rather consider only *in silico* predictions. The main conditions studied that are relevant to reproductive biology are endometriosis (Wang *et al.* 2013), endometrial cancer (Gilabert-Estelles *et al.* 2012), endometrial receptivity (Altmae *et al.* 2013), decidualization (Estella *et al.* 2012), pre-eclampsia (Yang *et al.* 2011), and ectopic pregnancies (Zhao *et al.* 2012). MiRNAs may eventually be found at the origins of idiopathic pathologies in reproductive biology, but for that intense and focused basic research to elucidate the specific effects that different miRNAs have on the different cell types comprising the endometrium is required.

These molecules are present not only in tissues but also in a variety of biological samples (e.g., whole blood, serum, plasma, urine, saliva, etc.). Given the small size of miRNAs, specific RNA extraction methods ensuring their recovery must be used, what led to the development of column-based kits by some companies to achieve an optimal recovery yield. Correct purification of the small RNA fraction should be confirmed by gel electrophoresis.

3.5. MiRNA detection methods

Traditional *in situ* hybridization (ISH) has been adapted to visualize miRNAs inside cells by using specific miRNA probes and hybridizing them directly on the samples. This technology has been developed to work with formalin-fixed paraffin-embedded (FFPE) tissues, cryosections (using Exiqon technologies), and/or cell cultures (using Panomics technologies); the latter also uses fluorescent *in situ* hybridized probes (FISH).

Microarray is currently the most popular miRNA detection method. It consists in hybridizing fluorescent-labeled miRNA samples onto glass-printed probes, scanning them, and processing the data. This technique requires between 30 ng to 5 µg of total RNA depending on which platform is chosen, e.g. Agilent (the most economical), Affymetrix (which has the

widest range of probes/miRNAs), and Exiqon (the most sensitive to low amounts of input RNA (Guerau-de-Arellano *et al.* 2012). Since they consist of short length recognition sequences, each melting temperature (Tm) is different, which negatively affects the recognition specificity and/or sensitivity. However, this issue has recently been overcome by using new 'locked nucleic acid' (LNA) probes that allow Tm standardization (Castoldi *et al.* 2006). This method enables the simultaneous detection of a large number of miRNAs, and it also allows for microarray customization.

TaqMan-based arrays or PCR arrays entail real-time qPCR amplification, which first requires a reverse transcription step using stem-loop primers; this reduces the risk of detecting genomic DNA and improves the detection efficiency and sensitivity. Although nanograms of input material can be amplified, several array cards are required to cover the human miRome (Mestdagh *et al.* 2008), and therefore this must be taken into consideration when designing experiments.

Next generation sequencing is also becoming a viable option for assessing the miRome because its cost is gradually declining (Rothberg and Leamon 2008). This technique requires the generation of a small RNA library in which 5' and 3' RNA adaptors are ligated to either end of the miRNAs. The 3' adaptors bind to the mature miRNAs or other small RNAs that carry the 3' hydroxyl group (which is usually generated by the enzymatic cleavage catalyzed by Dicer). Reverse transcription is then followed by PCR amplification. There are two principal sequencing methods: pyrosequencing and sequencing by ligation. These types of methodologies are useful for discovering novel miRNAs but their main disadvantage is that between 1 and 5µg of RNA is required to perform the assay.

Technology		Basic principle	RNA quantity	disadvantages	advantages
Microarrays		Hybridizing fluorescent-labeled miRNA samples onto glass-printed probes, scanning them, and processing the data	Between 30 ng to 5 µg of total RNA	Specificity and/or sensitivity, complex data processing and further qPCR validation	Simultaneous detection of a large number of miRNAs. Can be customized. Different platforms: Agilent (open), Affymetrix (closed) and Exiqon (LNA probes)
In situ Hybridization		miRNA probe which hybridizes in fixed tissue or cell samples and can be visualized and quantified under the microscope	Single-molecule detection	Complex method and not fully quantitative	Cellular localization of the miRNA. Fluorescent labelled probes lets to plex and co-localize signals.
TaqMan-based arrays or PCR arrays		Real-time qPCR amplification	Nanograms	Several array cards required to cover the human miRome	No need of further validation
Next generation sequencing (NGS)	**Synthesis**	Fluorescent nucleotides are ligated to reverse terminators, allowing the addition and measurement of a single nucleotide at each step	1 and 5µg	Requires the removal of each terminator sequence, Sanger validation and complex data processing	
	Pyrosequencing	The addition of each nucleotide releases inorganic pyrophosphate that activates luciferase-coupled activity and produces light	1 and 5µg	Complex data processing and requires Sanger validation	Fastest sequencing method
	Sequencing by ligation	A di-nucleotide fluorescently-labeled primer has to be bound by complementarity to the template, which is then ligated to the sequencing primer	1 and 5µg	Complex data processing and requires Sanger validation	Improvement in sequencing accuracy
Nanostring nCounter		A probe specific for each miRNA, called a miRTag, is ligated with the 3' end of the miRNA. A bridge sequence which is complimentary to the miRNA and miRTag is used during the ligation step and is subsequently removed. The miRNA-miRTag can then bind to an identifying barcode and later to a biotinylated capture probe which binds to a streptavidin-coated slide in order to allow the barcodes to be digitally counted with a scanner.	Single-cell	Number of different barcodes	It does not require amplification or reverse transcription, and the statistical analysis is easy because the counts are measured digitally.

Table 3. Summary of miRNA detection methods.

A different technology, the Nanostring nCounter uses synthetic RNA segments labeled with different fluorochromes to create specific molecular barcodes (Geiss *et al.* 2008). Each probe, called a miRTag, is specific for each miRNA and ligates to the 3' end of the miRNA. A bridge sequence complimentary to the miRNA and miRTag is used during the ligation step and is subsequently removed. The miRNA-miRTag then binds to an identifying barcode, and later to a biotinylated capture probe; after the ligation to a streptavidin-coated slide the scanner counts digitally the barcodes. The advantage of this technology is that it does not require amplification or reverse transcription, and because the counts are measured digitally it is possible to measure one RNA copy per cell or at the single cell level. A summary of all miRNA methods can be seen in Table 3.

3.6. The functional characterization of miRNAs

Functionally characterizing miRNAs relies on identifying the biologically relevant target mRNAs that they regulate. Therefore, several bioinformatic and experimental approaches have been developed to identify miRNA target genes. Many computer programs, including *Targetscan* (http://www.targetscan.org/), *Pictar* (http://pictar.mdc-berlin.de/), *Mirò* (http://ferrolab.dmi.unict.it/miro/), *Miranda* (http://www.microrna.org/microrna/home.do), *Mirmap* (http://mirmap.ezlab.org/), *Microcosm* (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), and *Diana Lab* (http://diana.cslab.ece.ntua.gr/) predict target genes based on 3'-UTR complementarity sequences.

However, *in silico* predictions also require *"in vitro/vivo"* confirmation, and various strategies have been developed in this direction: e.g., artificially synthetized oligonucleotides; miRNA 'mimics', oligonucleotide-miRNA duplexes with passenger strands designed to target specific mRNAs; 'antimiRs or antagomiRs', oligonucleotides complementary to endogenous miRNAs which are designed to bind and inhibit their function; 'target protectors', oligonucleotides complementary to a specific section of an mRNA target gene which are used to protect it from a given miRNA; and finally, miRNA 'sponges', an open reading frame (ORF), linked to a 3′UTR with multiple miRNA-specific binding sites, which acts as a competitive miRNA binding inhibitor. Importantly, all of these types of molecules can be transfected into cells and can therefore be investigated by western-blot or qPCR (Small and

Olson 2011) (Figure 9). 'Scramble' miRNA is commonly used as a transfection negative control.

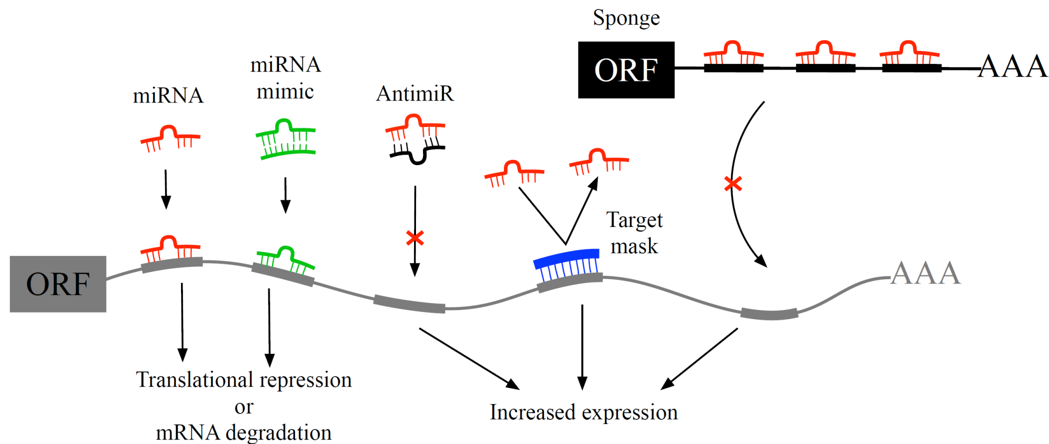


Figure 9. Artificial oligonucleotide manipulation at the miRNA function.

Direct miRNA gene targeting can be explored with luciferase assays by combining systems using the 'firefly' and 'renilla' luciferase genes. Most 3'-UTR regions of target genes can be cloned in the multicloning site (MCS) within the firefly luciferase. When synthetic oligonucleotides (scramble, mimic, or antimiRs) and the vector(s) are co-transfected into a mammalian cell line, the vector constitutively expresses both the renilla and firefly luciferases, however if translation repression is triggered by a specific miRNA in the 3'-UTR of the firefly luciferase mRNA its luminescence signal (but not that of renilla luciferase) decreases (Figure 10) (Guo *et al.* 2013).

Once miRNA target genes are predicted and confirmed, functional characterization requires a deeper understanding of the biological functions underlying those genes. Therefore, further experiments must still be designed to determine parameters such as proliferation, cytoskeleton modifications, cell invasion and migration properties, and their differentiation and dedifferentiation processes.

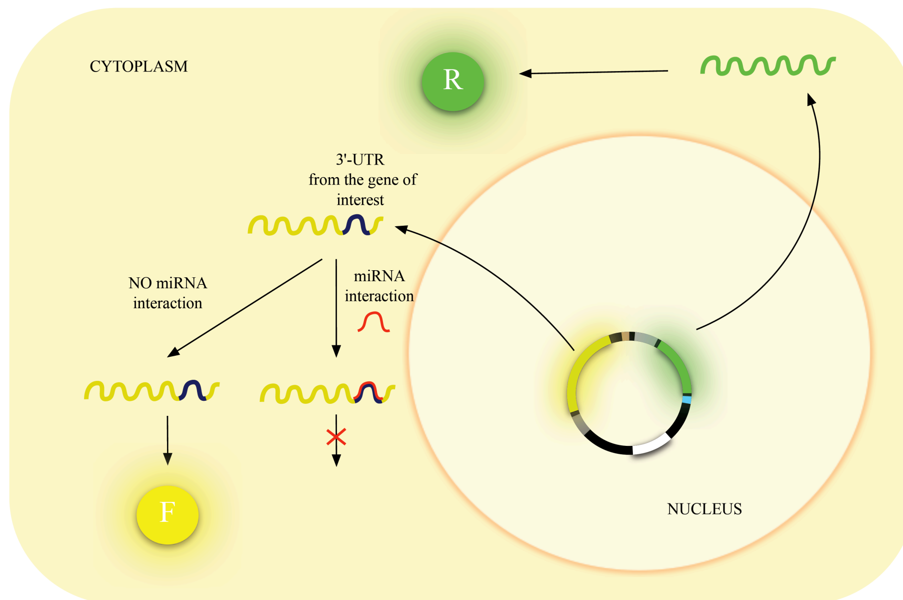


Figure 10. Scheme of the pmiRGLO Luciferase vector assay

3.7. miRNAs as biomarkers

Many miRNAs present in serum predict or correlate with disease status and prognosis in several types of malignancies. Furthermore, compared to the proteomic or the transcriptomic approaches, it is easier to adapt their use for the clinic because there are only approximately 1500 detectable human miRNAs (Cortez *et al.* 2011). However, an important barrier to the use of miRNAs as non-invasive biomarkers is the conflicting data published in relation to the same pathologies or conditions. This might be explained by the lack of common methodological standardization in the field, e.g., in sample collection or qPCR normalization techniques. Regarding the former, it is important to control or avoid hemolysis as well as the use of EDTA because it blocks the polymerase reaction during PCR. Regarding the latter, the consistency of results could be improved by adding synthetic miRNA 'spikes' (derived from different organisms) to the qPCR mix, e.g., ‘cel-miR-39’ from C. elegans (Mitchell *et al.* 2008).

3.8. miRNAs as pharmacological agents

There are currently two major barriers to the use of miRNAs as pharmacological treatments: firstly, one miRNA can target several genes at once. Fortunately, active research is focused on improving the stability of miRNAs *"in vivo"* and on directing their action to target specific cells or organs, e.g., by conjugating miRNAs to carrier molecules such as lipids (de Antonellis *et al.* 2013), polymers (Klimenko and Shtilman 2013) or peptides (Jarver *et al.* 2012). Secondly, unmodified miRNAs can trigger unspecific interferon responses in tissue culture and *"in vivo"*. In response to the presence of siRNAs, the dsRNA-dependent protein kinase R (PKR) induces interferon beta upregulation, activating the Jak-Stat pathway and leading to the expression of IFN-stimulated genes (Sledz *et al.* 2003).

Many pharmaceutical companies have recently started investing in developing miRNAs for the treatment of human diseases. Most of them, including *Santaris Pharmaceuticals*, *Rosetta Genomics,* and *Regulus Therapeutics* have focused on developing treatments for liver cancers; *Mirna Therapeutics* has focused on lung, prostate and blood cancers, whereas *miRagen Therapeutics* has focused on cardiovascular and muscle diseases.

3.9. Intercellular communication

Little is known about the potential "hormonal" role of miRNAs present in plasma/serum and the effects of these molecules in distant sites of the body (Cortez *et al*. 2011). Recipient cells can internalize miRNAs transported by HDL using a pathway involving nSMase2 (Vickers *et al*. 2011). Recently it has been described small sized vesicles ranging from 20-100 nm, also called exosomes, that are released from cells through ceramide dependent secretory machinery (Kosaka *et al*. 2010). These exosomes containing miRNAs can be uptaken by the recipient cells by endocytosis. Recent studies demonstrated that 90% of plasma and serum miRNAs are not encapsulated by vesicles, but co-fractionated with protein complexes, mainly Ago2-miRNA complexes (Arroyo *et al*. 2011, Zhu and Fan 2011)(Figure 11).

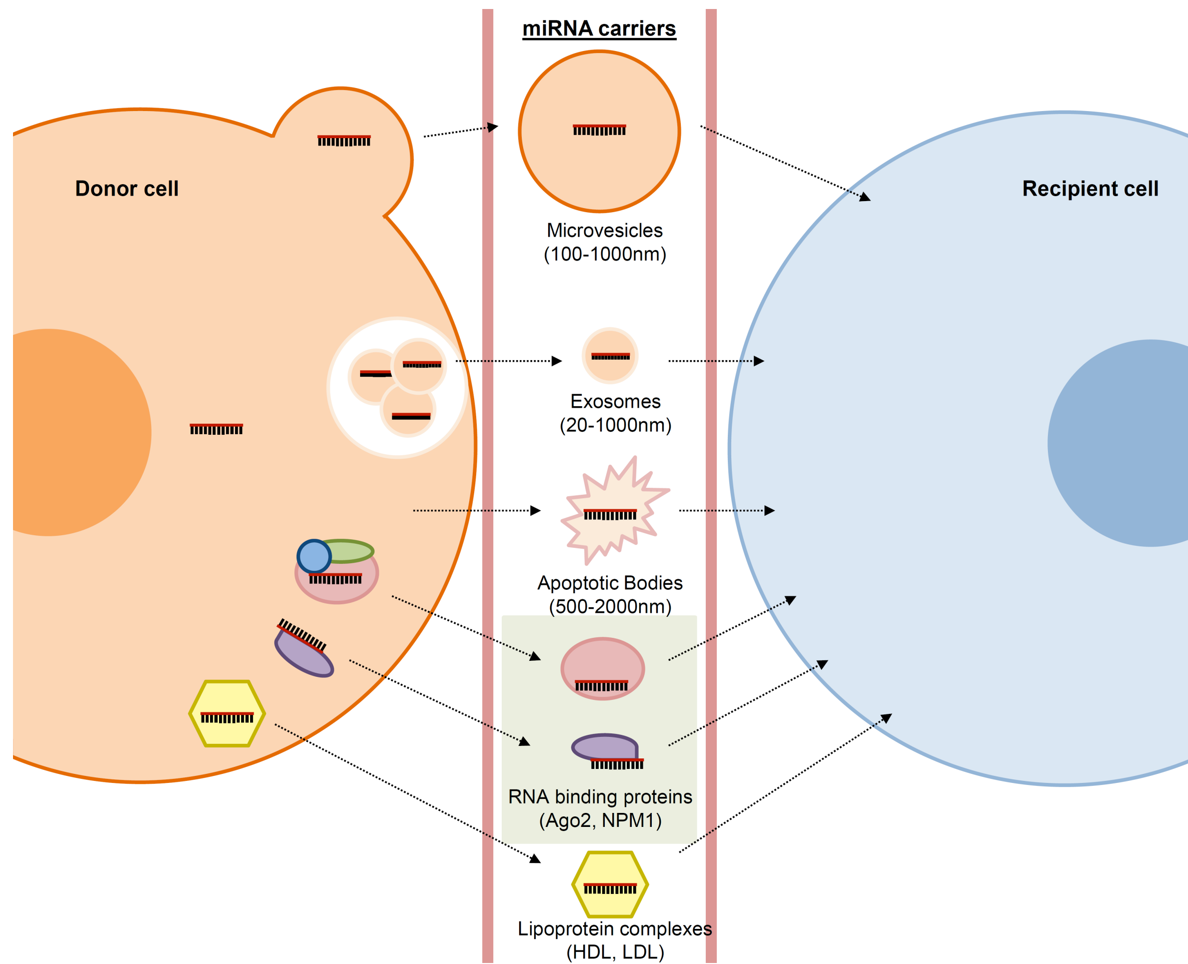


Figure 11. MiRNA-mediated intercellular communication.

4. EMBRYO DEVELOPMENT

The human zygote has to experience huge transformations to become a healthy implanted embryo. Each step is crucial for the success and the correct development. For this goal the embryo is not alone and the mother's womb has to control the new life growing inside.

4.1. Early embryo development

The first description of pre-implantation embryos comes from mid-50s in voluntary patients that had their uteri dissected after hysterectomies, and a two-cell stage embryo and another embryo in gastrulating stage were observed. Since then, our understanding of human early embryo development relies on *"In Vitro* Fertilization" (IVF) techniques.

The human embryo development changes from a transcriptional silenced state that includes the fusion of egg and sperm, migration, pronuclear fusion, genetic and epigenetic reprogramming, and a series of cell divisions. When an embryo cleavages from 4- to 8- cell stage at day 3, it begins a major wave of embryonic genome activation (EGA) that was reported for the first time in 1988, when the inhibition of transcription by α-amanitin had no effect in embryos prior 4-cell stage (Braude *et al*. 1988). More recently, EGA has been determined to occur at day 3 independently of cell number (Galan *et al*. 2010). These findings differ from those observed in mouse, in which zygotic gene activation (ZGA) is initiated after 26-29 hours post-fertilization (Potireddy *et al*. 2006). After EGA, the embryo undergoes compaction and forms the morula. Subsequent cell divisions lead to compaction and cavitation to form a blastocyst that comprises the trophectoderm (TE) delimiting a cavity fluid-filled and an inner cell mass (ICM). After that, the blastocyst hatches from its zona pellucida and begins the implantation process at day 5-6, but just before implanting, the ICM diverges into early epiblast and primitive endoderm cells. Since a successful pre-implantation development can be predicted by using time-lapse prior to EGA, it is likely that human embryo is in large part influenced by the inherited maternal and paternal factors involved in the RNA metabolism/translation and cytokinesis (Cruz *et al*. 2011, Wong *et al*. 2010).

4.2. The effects of growth factors on human embryo development

It has been observed that the consumption of growth factors by the embryos lead to the "quiet embryo hypothesis" in which the quiet embryos are more viable than those active (Baumann *et al*. 2007). This means that a hyperactive metabolism, result of culture stresses, would increase the expression of genes involved in glycolysis, glucose transport and lactate metabolism. However, this hypothesis has only been demonstrated in bovine and it remains unclear whether there are cell-specific or general survival mechanisms that correlate with unique growth factor receptor expression in human ICM or TE cells.

4.3. Main differences with mouse embryo pre-implantation development

While human and mouse embryos are similar at the pre-implantation stage, there are several molecular differences that would generate significant differences later in the developmental timing. These differences include gene expression patterns, programs of epigenetic modification, genetic instability, and a longer transcriptional silence period in human embryo relative to mouse. Therefore, human embryos undergo one additional round of cell division prior to implantation (256-cell stage in human compared to 164- cells in murine blastocysts) (Niakan *et al*. 2012).

4.4. Human embryo implantation stages

a) Apposition

Prior to implantation, the blastocyst migrates towards the upper third part of the uterus and the ICM rotates to face the luminal epithelium. This stage takes place at 5-6 days after ovulation.

b) Adhesion

During adhesion, the trophoblast and endometrium surfaces experiment molecular changes to allow cell-to-cell interaction between the two cell systems. These changes are led by steroids (Aplin 2000, Bazer *et al*. 2009) and/or by the embryo (Simon *et al*. 1998), leading to the expression of adhesive molecules in the luminal epithelial cell surface during the Window of Implantation (WOI).

Integrins and cadherins are transmembrane proteins able to interact with cell receptors or with extracellular matrix proteins (ECM), mediating adhesion and migration processes. For example, L-selectin is expressed in the trophectoderm surface and binds to receptive epithelial endometrial cells (EECs). Other proteins described in the trophectoderm such as ICAM-1, N-CAM and V-CAM-I or E-cadherin also have also been associated with the adhesion process (Zygmunt *et al*. 1998).

In this stage, the importance of a series of molecules with autocrine-paracrine actions such as cytokines, LIF, CSF-α and interleukins (Simon *et al*. 1996) has also been described. The blastocyst controls its adhesion through receptor-ligand interactions with the EEC surface and by the removal of molecular barriers such as MUC1 in the implantation sites (Meseguer *et*

al. 2001). Another mechanism involved in the embryo adhesion is the apoptosis induction in EECs, mediated by the Fas ligand exposure by the embryo, and recognition by Fas receptor in the EECs that triggers the apoptotic pathway (Galan *et al*. 2000). This process is activated together with the secretion of TGFβ by the embryo (Kamijo *et al*. 1998), allowing the blastocyst to initiate the invasion process.

c) Invasion

Once the blastocyst has attached to the luminal epithelia, it starts an invasive process in which it will reach gradually the uterine stroma. On day 10 post-fertilization, approximately, the blastocyst is completely embedded in the stromal tissue, the epithelium has grown over the implantation site and the trophoblast is still growing. At this time there is also an important process that gets the trophoblast in direct contact with the uterine circulation, establishing an utero-placental circulation to support the growing fetus.

Progesterone is the principal hormone responsible for the invasive process. Also, the control of the invasion is mediated by several components such as, integrins, serine-proteases, collagenases, plasminogen, and metalloproteases (MMPs) of the ECM (Strickland *et al*. 1976). In particular, MMP-2 and MMP-9 are secreted by the human trophoblast (Cohen *et al*. 2006), and their action is counter-balanced by the tissue inhibitor metalloproteases (TIMPs) secreted by stromal cells (Irwin *et al*. 2001).

Embryo invasion has been compared to the invasive process of tumoral cells (although the former is controlled spatial and temporarily) and two different mechanisms have been proposed to explain how it is controlled. The first one asserts that the human chorionic gonadotropin (hCG) secreted by the embryo negatively regulates *"in vitro"* the protease activity of the urokinase uPA, which would reduce the embryo invasiveness (Chou *et al*. 2003). The second mechanism is attributed to the stromal barrier. The stromal differentiation triggered by the endometrium to generate a highly dense ECM, also known as "decidua" (Lala *et al*. 1983), generates a local context that promotes the anchorage rather than an invasion by the trophoblast (Figure 12).

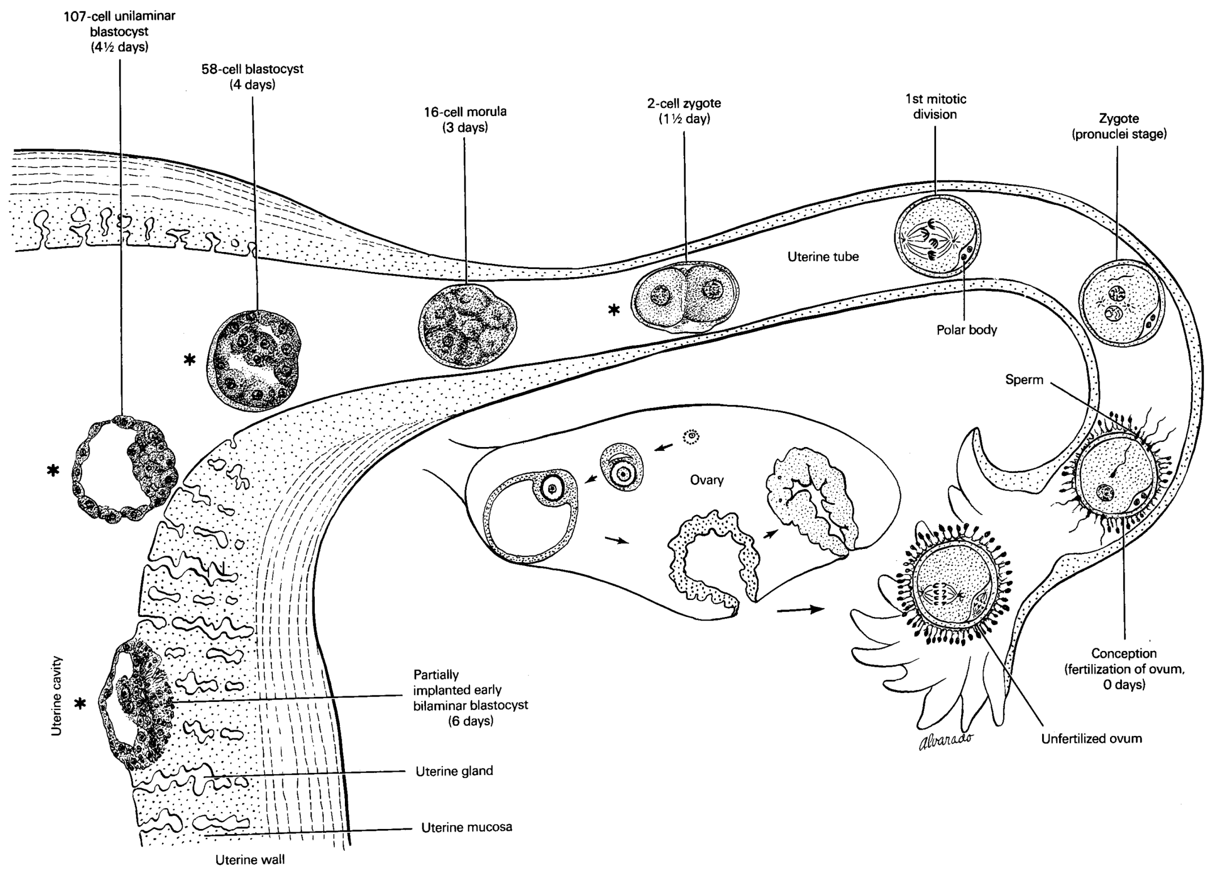


Figure 12. The stages of development during the first week and the approximate location of each stage in the uterine tube or uterus (Gasser).

II. HYPOTHESIS

II. HYPOTHESIS

The human endometrium actively secretes in the lumen several types of molecules with paracrine/endocrine effects to the endometrium and the upcoming embryo. We hypothesize that miRNAs are present among these molecules, that they show a specific pattern across the menstrual cycle, and that they have the capacity to reach the pre-implantation embryo in a new form of intercellular communication.

III. OBJECTIVES

III. OBJECTIVES

1. General objectives

To determine the presence and pattern of miRNAs in human endometrial fluid across the menstrual cycle focusing on the window of implantation phase.

To find whether the endometrial fluid and the *"in vitro"* cultured endometrial epithelial cells are actively secreting exosomes containing miRNAs.

To determine the uptaking ability of the embryos for free and/or exosome-vehicled forms of miRNAs.

To determine gene regulation and phenotypic effects of miRNAs uptaken by the embryo.

2. Specific objectives

To study the transcriptomic and proteomic effects of hsa-miR-30d in endometrial epithelial cells.

To determine changes in methylation patterns derived from high levels of hsa-miR-30d in endometrial epithelial cells.

To demonstrate the *"in vitro"* production of exosomes in the medium of cultured primary endometrial epithelial cells.

To demonstrate the presence of miRNAs in free or exosomes vehicled forms.

IV. MATERIAL AND METHODS

1. SAMPLE COLLECTION AND PROCESSING

1.1. Ethics statement

This study was approved by the Institutional Review Board and Ethics Committee of the Instituto Universitario-Instituto Valenciano de Infertilidad (Universidad de Valencia, Spain) [1204-C-102-FV-F]. Informed written consent was obtained from each patient prior to tissue and endometrial fluid collection.

1.2. Endometrial samples

Endometrial biopsies were obtained at day LH+0 from 18 to 35-year-old women with regular menstrual cycles (n = 15) (n = 4 for microarrays; n = 4 for iTRAQs; n = 4 for qPCRs; n = 3 for western-blots). Patients have normal karyotypes, good general health, a BMI of 19-29 Kg/m2 undergoing controlled ovarian stimulation (COS) for oocyte donation. Three additional biopsies were obtained at LH+7 from women under natural cycle. Patients diagnosed with endometriosis and/or endometritis were excluded. All patients signed informed consent prior to entering the study.

The COS protocol was carried out by following a GnRH-agonist long protocol with 200 IU recombinant follicle stimulating hormone (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 the ovarian response as judged by serum estradiol concentrations and ultrasound scans every three days.

1.3. Epithelial and stromal separation and primary culture

The endometrial samples were processed to separate the epithelial and stromal fractions by collagenase digestion and gravity sedimentation, as previously reported by our group (Simon *et al*. 1997). The purified hEECs were plated at 10-20% confluence and maintained in culture until the experiments were performed.

1.4. Endometrial secretion aspirations

Endometrial fluid (EF) samples (n=20) were divided into five groups (n=4 per group) according to the stage of the cycle obtained. EF was obtained from healthy subjects with no underlying endometrial pathologies and who had regular menstrual cycles of 25 to 33 days. None of these women received hormonal treatment in the 3 months preceding biopsy and collection of EF. Briefly, the subject lay in the lithotomy position, a speculum was inserted, the cervix was cleansed, a flexible catheter (Wallace; Smiths Medical) was gently introduced, and approximately 20-50 μL of endometrial secretion was aspirated. Endometrial fluids were classified in five different groups: Early Proliferative (EP, day 0-8, n=4); Late Proliferative (LP, day 9-14, n=4); Early Secretory (ES, day 15-18, n=4), Mid-Secretory or Window of Implantation (MS or WOI, day 19-23, n=4) and Late Secretory (LS, day 24-28, n=4).

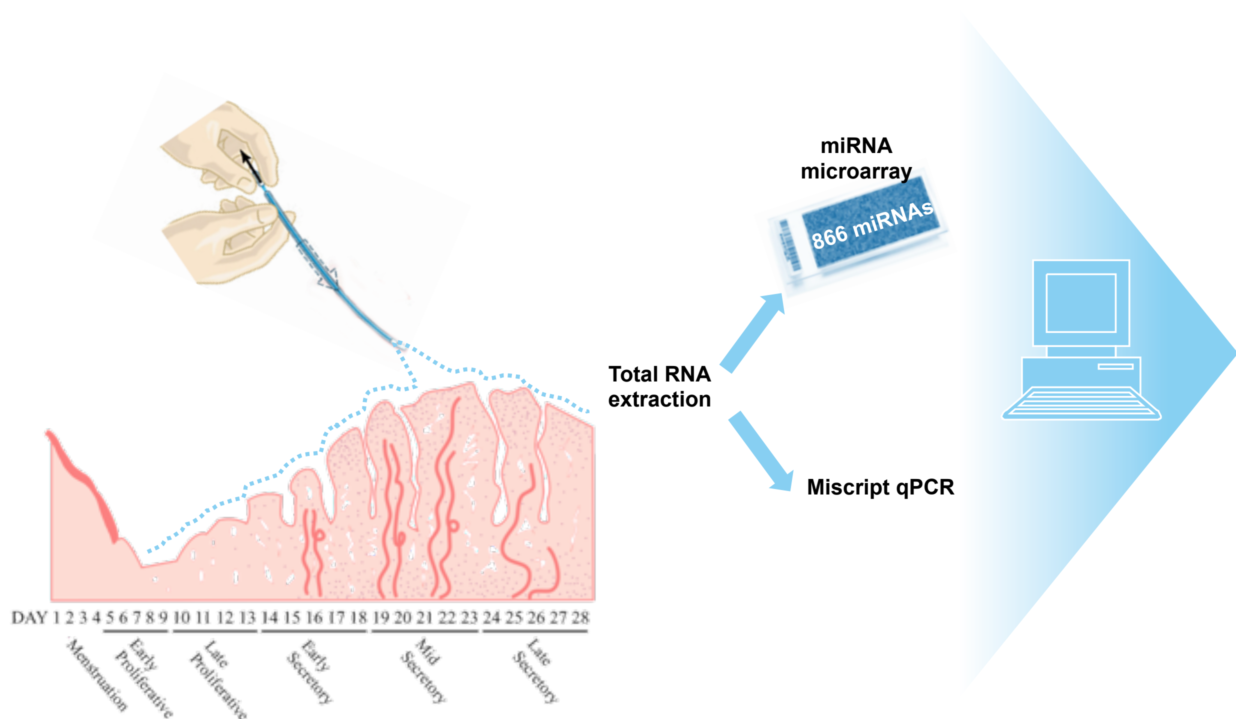


Figure 13. Experimental design for assessment of miRNA's profile across menstrual cycle

1.5. Exosome isolation and labeling

Isolation of exosomes from EF. EF samples were diluted in PBS (Life Technologies S.A., Madrid, Spain), vortexed vigorously, and filtered using a 0.22μm-syringe filter (Pall Corporation, UK). The filtrates were then centrifuged at 300 × *g* for 10 min to remove whole cells. The supernatant was subjected to a second centrifugation at 2,000 × *g* for 10 min to remove dead cells, and the supernatant was centrifuged again at 10,000 × *g* for 30 min to remove cell debris. The supernatants were refiltered with a 0.22μm-syringe filter (Pall Corporation) and ultracentrifuged at 120,000 × *g* for 70 min. Pellets containing exosomes were used for electron microscopy, embryo uptake assays, RNA extraction, and qPCR.

hEEC cultures and exosome isolation. Endometrial samples obtained from healthy donors were processed to separate the epithelial and stromal cell fractions by collagenase digestion, as previously reported by our group (Simon *et al.* 1997), and the purified cells (hECC) were plated out into 24-well plates (Falcon; Becton Dickinson Inc.). When cultures reached confluence they were washed with DMEM (Gibco) to remove FBS-contaminated exosomes and cultured in DMEM. After 48 h, conditioned medium was collected, and exosomes were isolated. Briefly, approximately 35 mL of primary hEEC-conditioned medium was centrifuged at 300 × *g* for 10 min to remove whole cells; the supernatant was centrifuged at 2,000 × *g* for 10 min to remove dead cells and centrifuged again at 10,000 × *g* for 30 min to remove cell debris. The supernatant was then filtered with a 0.22-μm syringe filter (Pall Corporation) and ultracentrifuged at 120,000 × *g* for 70 min. Pellets containing exosomes were used for electron microscopy, embryo uptake assays, Western blot analysis, RNA extraction, and qPCR.

Previously isolated exosomes were incubated with 5 μM fluorescent Vybrant DiO (Life Technologies) at 37°C for 30 min. After labeling, exosomes were collected and ultracentrifuged to wash off the excess dye, added to hatching embryos, and incubated for 12-24 h.

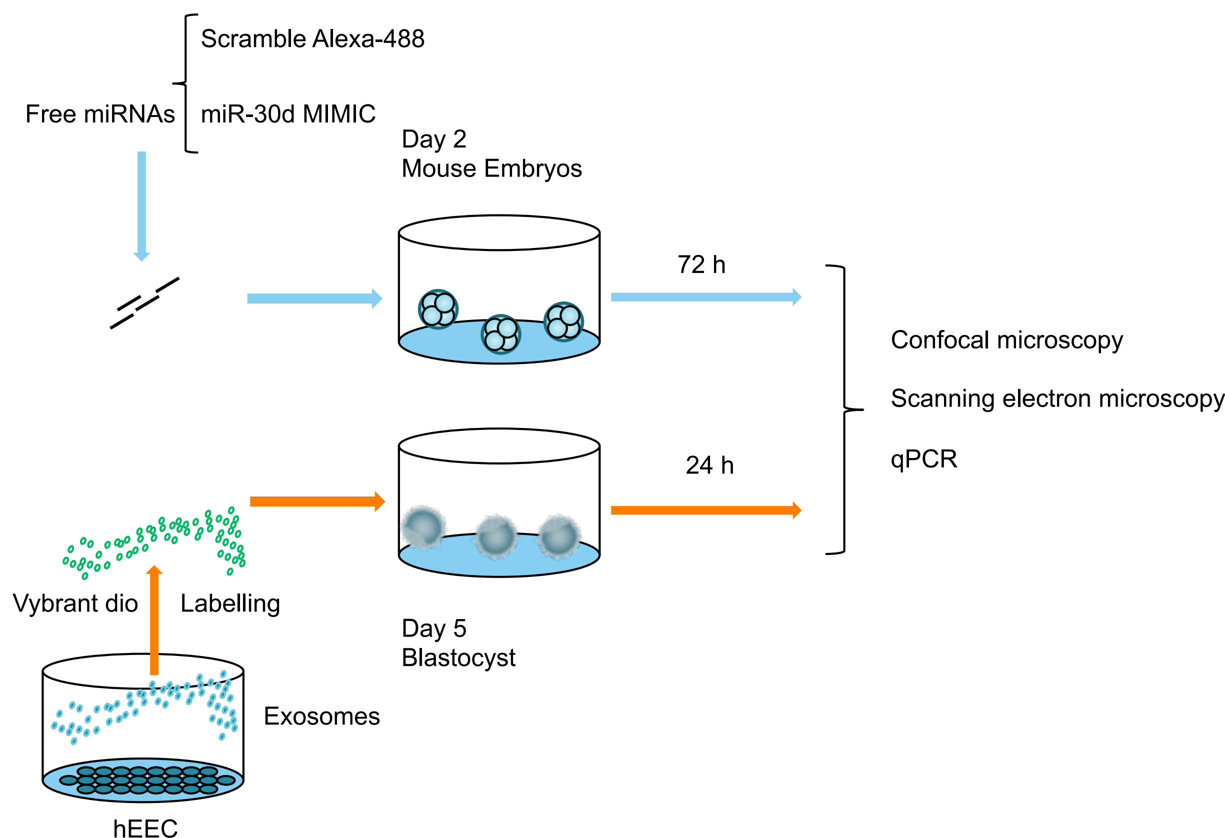


Figure 14. Experimental design for assessment of miRNA's uptake by murine embryos.

2. NUCLEIC ACID ANALYSIS

2.1. Total RNA extraction

For hEECs and EF samples, to ensure that the miRNA fraction was recovered we performed RNA extraction using the miRNeasy Kit (Qiagen, Valencia, CA, USA). The RNA extracted was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., MA, USA) and the quality of RNA samples was assessed using a Nano LabChip BioAnalyzer 2100 (Agilent Technologies Inc., DE, USA).

For mouse embryos, to ensure that small amounts of messenger RNA are suitable for transcriptomic analysis and/or PCR, the total RNA was extracted using the Arcturus PicoPure RNA isolation Kit (Applied Biosystems, CA, USA). The RNA extracted was quantified using

a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., MA, USA) and the quality of RNA samples was assessed using a Pico LabChip BioAnalyzer 2100 (Agilent Technologies Inc., DE, USA).

2.2. Retrotranscription

Since it is desirable to perform miRNA and mRNA quantification in the same samples, we used a strategy that allows retrotranscription of both types of RNA in the same reaction. For this purpose we used the miScript reverse transcription kit (Qiagen, Valencia, CA, USA) with using the HiFlex Buffer protocol according to the manufacturer's recommendations.

2.3. Quantitative Real-Time PCR

Once RNA has been converted to cDNA, relative quantitative PCR was performed using LightCycler FastStart DNA Master SYBR green I in a LightCycler 480 (Roche Applied Science, USA) and the fold-change was estimated using the -2ΔΔCt formula.

2.4. RNAse treatment

To test whether smallRNAs (20-200nts) contained in exosomes were protected from degradation, total RNA extraction was performed on exosomes isolated by using the miRNeasy Kit (Qiagen). The extracted RNA was treated with 10 ng/μL of RNase A (Sigma-Aldrich, Madrid, Spain) at room temperature for 30 min. RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and the proportion of miRNAs was evaluated using the Pico RNA LabChip BioAnalyzer 2100 (Agilent)

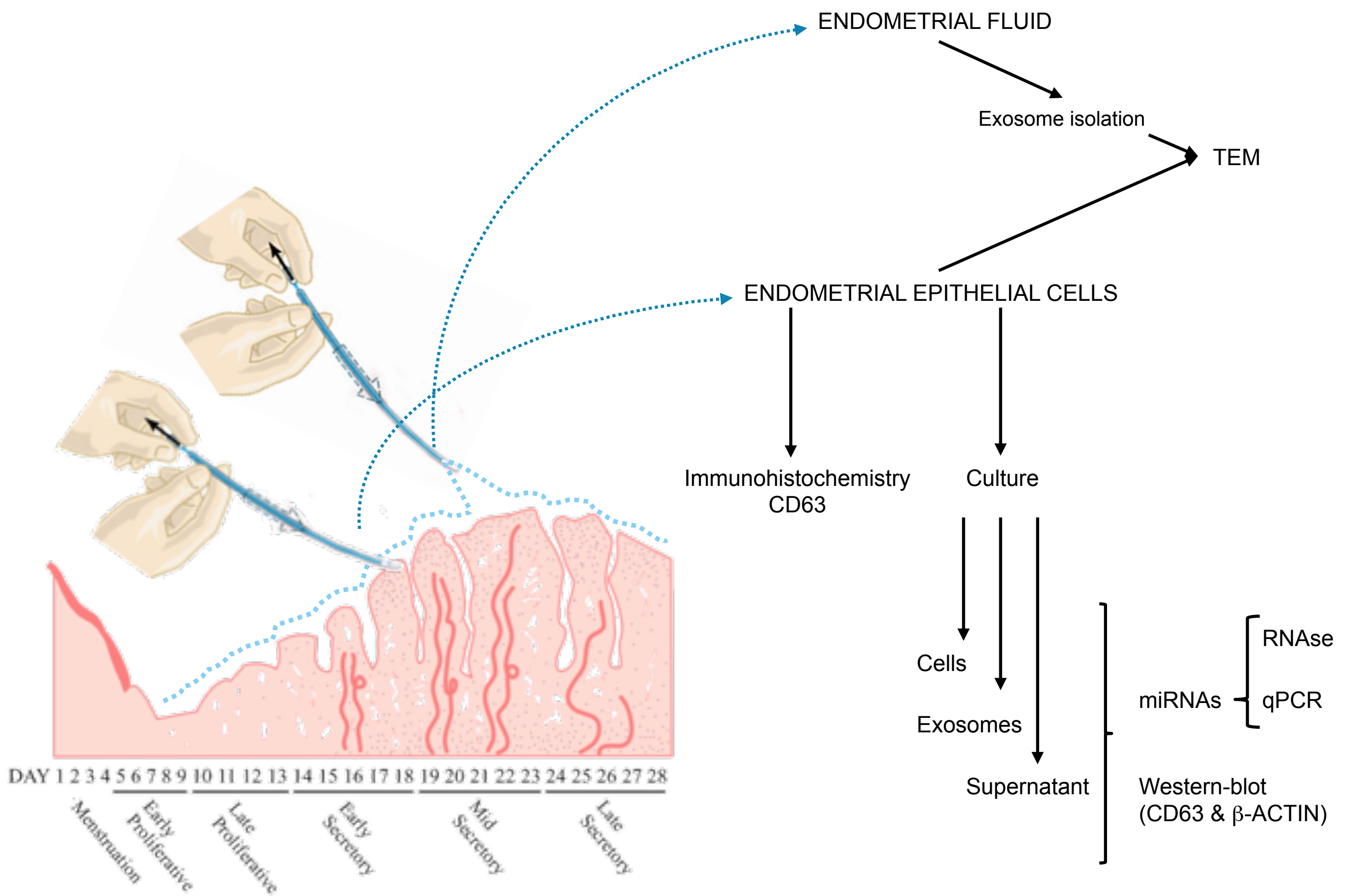


Figure 15. Experimental design for detection of miRNAs secreted exosomes and/or as other type of soluble forms

2.5. MiRNA microarrays

The grouped EF cohorts were analyzed using human miRNA v3.0 8x15K microarrays (Agilent), which evaluate the expression of 866 human miRNAs. Total RNA from each sample was processed according to the manufacturer's instructions, and then scanned. Raw data are available in the Gene Expression Omnibus (GEO) database with the accession number GSE44558.

2.6. MRNA microarrays

Total RNA from each sample were analyzed with Agilent GE 4x44K Human v2 and Mouse v3 microarrays, processed according to the manufacturer's instructions and then scanned. Raw data for the study of embryos treated with miRNAs is available in the GEO database with the accession number GSE44730.

The raw data for the hEEC transcriptome, transfected under two different conditions (scramble or mimic; n = 4) is available in the GEO database with the accession number GSE46721.

2.7. MiRNAs transient transfections

When hEEC cultures reached 50% confluency the cells were transiently transfected with 50 nM of either miR-30d mimic or scramble miRNA using HiPerfect, following the manufacturer's instructions (Qiagen, Valencia, CA, USA); after 72 hours RNA was extracted from the cells.

2.8. Methylated DNA Immunoprecipitation

For MeDIP assay we used the Methylated DNA Immunoprecipitation kit (Abnova Corporation, Taiwan). The genomic DNA was extracted from three different scramble- or mimic-30d-treated hEECs, sonicated into fragments ranging in size from 200 to 1000 bp, and divided into input (non-immunoprecipitated) and immunoprecipitated portions as recommended by the manufacturer. The immunoprecipitated DNA was incubated with anti-5-methylcytosine monoclonal antibody to bind methylated DNA. Methylated DNA was subjected to quantitative real-time PCR as previously mentioned for the H19 DMR region (paternally methylated and maternally expressed) and the UBE2B (typically unmethylated) genes (Movassagh *et al*. 2010). To evaluate the relative enrichment of target sequences after MeDIP, we estimated the fold-change using the -2ΔΔCt formula, normalizing against input DNA.

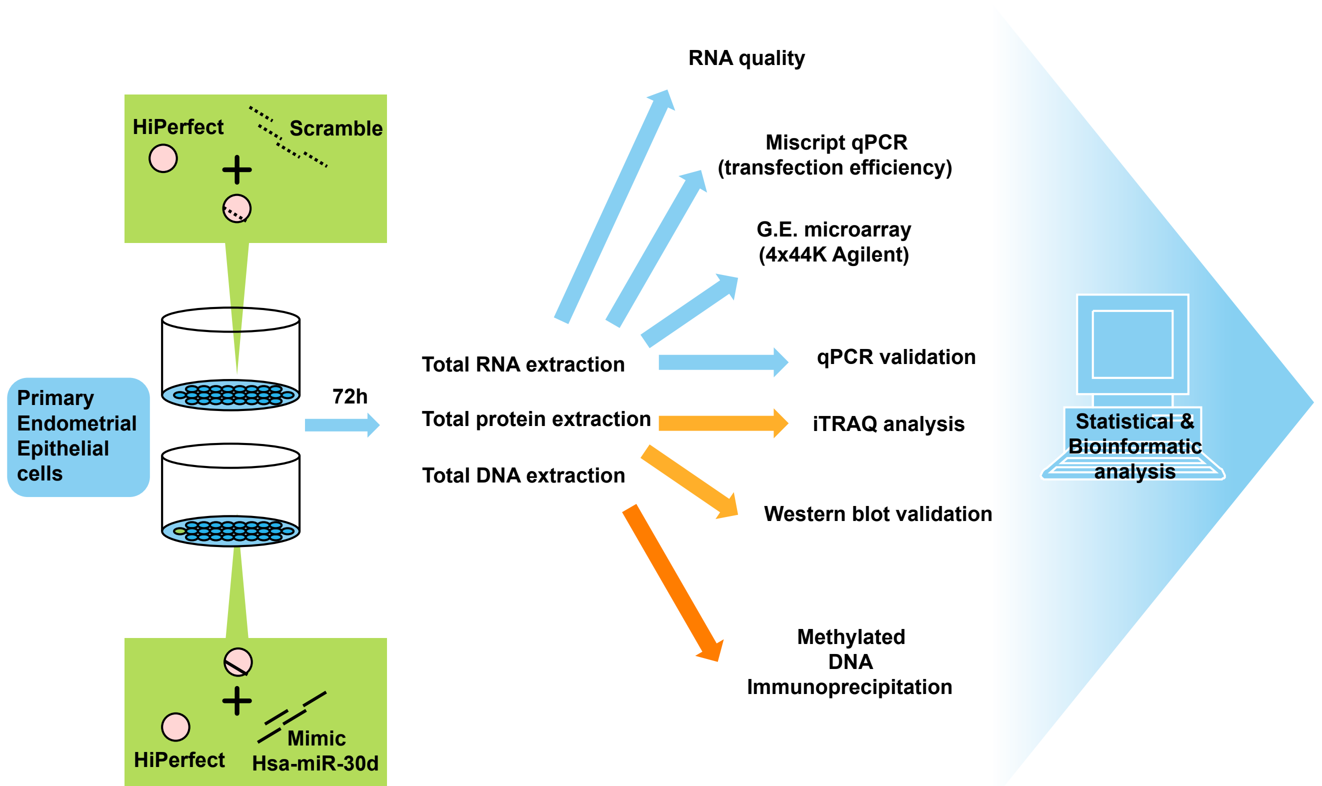


Figure 16. Endometrial epithelium study workflow

2.9. Primers design

Appropriate primers for PCR were designed to span exon-exon boundaries to the PCR template sequence. Initially, the primer melting temperature (Tm), were calculated. Primers were constructed based on the optimal length (20-22 bases) and a melting temperature in the range of 60ºC. To avoid regions of homology all primers were tested using NCBI Primer Blast software. For the different experiments we used several primers that are enlisted in Table 4.

Mouse gene primers

Gene		Sequence
Cdh5	Forward	ATGGCAGGCCCTAACTTTCC
	Reverse	GTGCGAAAACACAGGCCAAT
Itgb3	Forward	GGGTACCAAGTTGGCCTCTC
	Reverse	ATCTCGATTACGGGACACGC
Gapdh	Forward	AACTCGGCCCCCAACACT
	Reverse	CCTAGGCCCCTCCTGTTATTATG

Human gene primers

Gene		Sequence
GAPDH	Forward	GAAGGTGAAGGTCGGAGTC
	Reverse	GAAGATGGTGATGGGATTTC
H19	Forward	GTGGACTTGGTGACGCTGTA
	Reverse	CACCATCCTCCCTCCTGAGA
BMP4	Forward	GGAGCTTCCACCACGAAGAA
	Reverse	GGAAGCCCCTTTCCCAATCA
FOS	Forward	GGGGCAAGGTGGAACAGTTA
	Reverse	AGGTTGGCAATCTCGGTCTG
>For MeDIP assays		
DMR_H19	Forward	GAGCCGCACCAGATCTTCAG
	Reverse	TTGGTGGAACACACTGTGATCA
UBE2B	Forward	CTCAGGGGTGGATTGTTGAC
	Reverse	TGTGGATTCAAAGACCACGA

miRNA primers

Gene	Distribuitor	Nº Ref
hsa-miR-30d-5p	QIAGEN	MS00009387
SNORD96A	QIAGEN	MS00033733

Table 4. List of primers used

3. PROTEIN ANALYSIS

3.1. Total protein extraction

For total protein extraction, pellet of cells and or exosomes were lysed in ice-cold buffer containing 300 mM NaCl, 20 mM Tris, 10 mM EDTA, 2% (v/v) Triton X-100, pH = 7.3 supplemented with a protease inhibitor cocktail (Roche Applied Science, USA), and 10 mM PMSF (Sigma-Aldrich, USA). Protein quantification was performed using the Bradford assay.

3.2. Western-blot

For immunoblotting, 25 μg/lane of protein extracts from the cell lysates were separated on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (BIO-RAD, USA, CA) and incubated overnight at 4° C with antibodies specific to human CD63 (Abcam, UK, MEM-259), DNMT1 (Abcam, UK, ab19905), ALDH2 (Abcam, UK, ab54828), WNT1 (Abcam, UK, ab15251), PGRMC1 (Santa Cruz, sc-271275), and β-ACTIN as a housekeeping control (Santa Cruz, sc-47778). After washing three times, the blots were incubated with diluted horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, St. Cruz, CA) for 1 hour at r.t. Blots were then washed extensively and developed using SuperSignal West Femto Chemiluminescent kit (Thermo Fisher Scientific). Densitometry analysis of the gels was carried out using the ImageJ software (http://rsbweb.nih.gov/ij/links.html) and each western blot was normalized to the housekeeping protein band to correct for differences during sample loading.

3.3. Immunohistochemistry

Formalix-fixed and paraffin-embedded endometrial biopsies were sectioned with a thickness of 5 microns and mounted on glass slides coated with Vectabond TM (VectorLab, 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%(5 min), 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) and primary antibody CD63 (Abcam, UK, MEM-259). The primary antibody was 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 with gentle movement. Secondary antibodies were included in the kit and are valid for rabbit, mouse, and goat origin primary antibodies. Immunostaining was then visualized with 200uL of 2.30-

diaminobenzidine (DAB) chromogen. After counterstaining with hematoxylin and washing with distilled water, slides were mounted with entellan euKit (Merck, Darmstadt, Germany) and analyzed with a Nikon Eclipse 80i microscope. For negative controls, primary antibody were omitted and samples were incubated in DAKO Antibody Diluent.

3.4. Isobaric tag for relative and absolute protein quantitation (iTRAQ)

Primary hEEC samples under Mimic-30d or Scrambled experimental conditions were lysed and 100 µg of each isolated protein mixture were loaded onto a 1D PAGE gel without resolving the mixture. Each lane was cut and digested overnight (o.n.) at 37° C with sequencing-grade trypsin (Promega) as previously described (Shevchenko *et al*. 1996). The reaction mixtures were dried in a speed vacuum. Each sample was re-dissolved in 80 µL of TEAB-ethanol solution (3/7; v/v), were sonicated for 10 minutes, added to the appropriate iTRAQ Reagent vial, and vortexed thoroughly. Each sample vial was immediately rinsed with an additional 20 µL of TEAB-ethanol solution and incubated at room temperature (r.t.) for three hours. All samples were combined into a single tube and dried by vacuum centrifugation. 200 µg of the peptide mixture was dissolved in 225 µL of 7 M urea/2 M thiourea/1.6% ampholytes. One IPG strip (GE; 11 cm, pH 3-11 NL) was hydrated with the peptide solution o.n. at r.t. and the peptides were isoelectrofocused with 5000-25000 Vh. After focusing, the strip was washed with milliQ-grade water, and cut into 11 equal pieces. The peptides were extracted with 100 µL each of three solutions (5% ACN, 0.1% TFA; 50% ACN, 0.1% TFA; 100% ACN 0.1% TFA). All the peptide fractions were combined, dried by vacuum centrifugation, and re-dissolved in 20 µL of 2% CAN, 0.1% TFA. The final peptide concentration was determined by measuring the absorbance at 280 nm using a NanoDrop instrument.

For the colored peptide solutions an exploratory liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed in order to determine the optimum amount to be injected into the column. LC-MS/MS was carried out as follows: 5 µL of each sample was loaded onto a trap column (Nano LC Column, 3 µ C18-CL, 75umx15cm; Eksigent) and desalted with 0.1% TFA at 3 µL/min for 5 min. The peptides were then loaded onto an

analytical column (LC Column, 3 μ C18-CL, 75 μm x 12 cm, Nikkyo Technos) equilibrated in 5% acetonitrile and 0.1% FA (formic acid). Elution was carried out with a linear gradient of 35% B in A for 90 min, (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nL/min. Peptides were analyzed in a nano-ESI QqTOF mass spectrometer (5600 TripleTOF, ABSCIEX). The triple time of flight (TOF) was operated in 'information-dependent' acquisition mode, in which a 0.25 s TOF-MS scan from 350–1250 m/z was performed, followed by 0.075 s product-ion scans from 100–1500 m/z on the 25 most intense 2-5 charged ions. ProteinPilot's default parameters were used to generate a peak list directly from 5600 TripleTOF wiff files, and their Paragon algorithm was used to search the Expasy protein database with the following parameters: iTRAQ QUANTITATION, trypsin specificity, cys-alkylation (IAM), taxonomy restricted to 'human', and the search-effort set to 'rapid'. To avoid using the same spectral evidence in more than one protein, the identified proteins were grouped based on MS/MS spectra by the ProteinPilot Pro Group algorithm. Thus, proteins sharing MS/MS spectra are grouped regardless of the peptide sequence they are assigned. The protein within each group that can explain more spectral data with confidence is shown as the primary protein in the group. Only proteins in the group for which there is individual evidence (unique peptides with enough confidence) are also listed with a different list number, usually towards the end of the protein list.

4. MICROSCOPY

4.1. Confocal microscopy

Viable non-fixed embryos in suspension incubated with either exosomes or fluorescent Scramble miRNA at different stages were incubated with DAPI [40,6-diamino-2-phenylindole] for nucleus staining and analyzed under a confocal laser microscope (Zeiss, Germany), equipped with fluorescence optics and appropriate filters and taking approximately 100 hundreds image sections that were combined in a single high resolution image of embryos.

4.2. Transmission electron microscopy

To assess the presence of exosomes in primary hEEC using TEM, cells and isolated exosomes, samples were fixed in Karnovsky's solution (Doughty *et al.* 1997). Briefly, freshly isolated primary hEEC were post-fixed in osmium tetroxide, washed, and stained with uranyl acetate. Samples were dehydrated, embedded in epoxy resin, ultrasectioned, transferred to carbon-coated grids, and observed using a JEM-1010 transmission electron microscope (Jeol Korea Ltd.) at 100,000 kV.

Isolated exosomes were resuspended in 50 μL of Karnovsky's solution, incubated for 1 h on a Formvar carbon-coated grid, and contrasted with uranyl acetate.

4.3. Scanning electron microscopy

For scanning electron microscopy (SEM), post-fixed embryos in Karnovsky's solution were subjected to critical point dehydration, gold-coated and observed in an S-4100 scanning electron microscope (Hitachi) at 10,000 kV.

5. MOUSE EMBRYOS

5.1. Day 1.5 mouse embryo collection

The B6C3F1 mouse strain was purchased from Charles River Laboratories. Female mice aged 6-8 weeks were primed to ovulate by administering 10 IU of pregnant mare serum gonadotropin (Sigma-Aldrich, Irvine, UK), followed by administration of 10 IU of human chorionic gonadotropin (Sigma-Aldrich) 48 h later. Females were housed overnight with male studs and examined the following morning for the presence of a vaginal plug (classified as day 1 of pregnancy). On day 2 of pregnancy, mice were euthanized by cervical dislocation, and embryos were flushed from the oviduct with PBS using a 30-gauge blunt needle.

5.2. Embryo culture

Embryos were cultured in CCM-30 medium (Vitrolife, Lübeck, Germany) with or without labeled exosomes, 400 nM mir-30d mimic or Alexa 488-scramble miRNA (Qiagen, Valencia, CA, USA) for 72 h. Embryos were then washed 4 times in fresh CCM and used for transcriptomic assays, electron microscopy, confocal microscopy, and adhesion assays.

5.3. Adhesion assays

Epithelial endometrial cells from six donors were cultured until confluence, and 15 embryos per condition were added in 3 independent experiments (i.e., 360 embryos). Mouse blastocysts expanded with normal morphology and were cultured in the presence of 400 nM mir-30d mimic, Alexa 488-scramble miRNA, or mir-30d inhibitor (Qiagen, Valencia, CA, USA) for 72 h. The attachment of mouse blastocysts to the epithelial cell monolayer was measured at 24 and 48 h by mechanical assay. Briefly, plates were moved on a rotation shaker for 10 s, and the floating blastocysts were deemed to be unattached.

6. BIOINFORMATIC ANALYSIS

6.1. Microarrays data analysis

For miRNA microarrays, the data obtained for each probe were normalized and $\log_2$ transformed using R software and Bioconductor database libraries. The web-based Babelomics tool (Medina *et al*. 2010) was used to merge the resulting data matrix based on the mean number of replicates from each probe. Next, the data matrix was analyzed using Rank Products in MeV software with a false discovery rate (FDR) correction of less than 5%, and differentially expressed miRNAs were listed together with their fold-change number. Principal

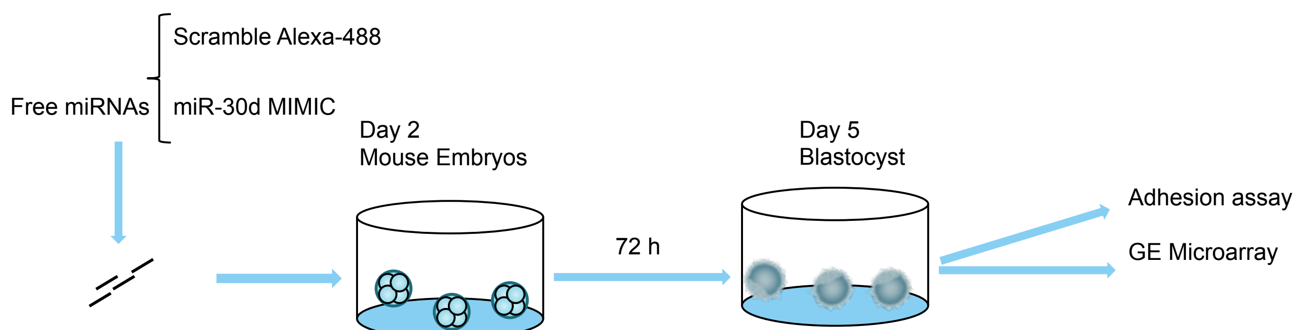


Figure 17. Experimental design for assessment of miRNA's uptake by murine embryos.

components analysis on the set of differentially expressed miRNAs was performed using Babelomics. IPA software (Ingenuity Systems, www.ingenuity.com) was used to predict the biological functions affected and any previously reported interactions between the miRNAs and the target genes.

For gene expression (GE) microarrays, the matrix containing the log-transformed, normalized, and merged-by-probe data was introduced into the Rank-Product module in MeV software for statistical analysis. Samples were assigned to different groups and were compared. A false discovery rate (FDR) correction of less than 5% was used, and mRNA fold changes between the different samples and groups were calculated. For supervised hierarchical clustering heat map representation, only the intensity values for the set of differentially expressed genes were standardized. The mRNAs identified were functionally studied *in silico* by computational analysis of the biological processes and pathways that they affected the most, using IPA software (Ingenuity® Systems). This software implements an algorithm that finds interactions and associations with processes previously reported in the literature. This tool was able to predict the 'physiological system development and functions', 'molecular and cellular functions', and 'diseases and disorders' affected; it also finds potential upstream regulators that might direct the deregulation pattern of differentially expressed genes, and that may stimulate significant inactivation (a z-score less than -2) or activation (z-score more than 2). To identify potential miRNA target genes, we used Targetscan (http://www.targetscan.org/) a publicly available target prediction algorithm.

6.2. Proteomic data analysis

For proteomic analysis, a data matrix with areas of each peptide identified was log-transformed, normalized using quantile normalization, and merged by the mean in R software. The data was subjected to Rank-product analysis with a FDR of less than 5%, and was corrected in MeV software to obtain the final list of differentially expressed proteins.

6.3. Statistics represented

The data presented in box plots represent the minimum, first quartile, median, second quartile, and maximum values. Bar graphs are expressed as means plus or minus the standard error of the mean (StEM). Pairwise comparisons between conditions were performed using the Mann–Whitney U test for continuous variables, and p-values less than or equal to 0.05 were considered as statistically significant. Mean differences among conditions in adhesion experiments were evaluated using Student's t-test (with previous testing for the equal variance assumption) in the SPSS Statistics software package (v.17). P values <0.05 were considered significant.

V. RESULTS

1. Human EF contains secreted miRNAs of maternal origin

We confirmed the presence of a large quantity of miRNAs in human EF (Figure 18) and mean of percentage of miRNAs in all samples quantified using Pico RNA LabChip (Table 5).

EFs were classified as early proliferative (EP; days 6-8; n=4), late proliferative (LP; days 9-14; n=4), early secretory (ES; days 15-18; n=4), mid secretory or window of implantation (MS or WOI; days 19-23; n=4), and late secretory (LS; days 24-28; n=4) phases.

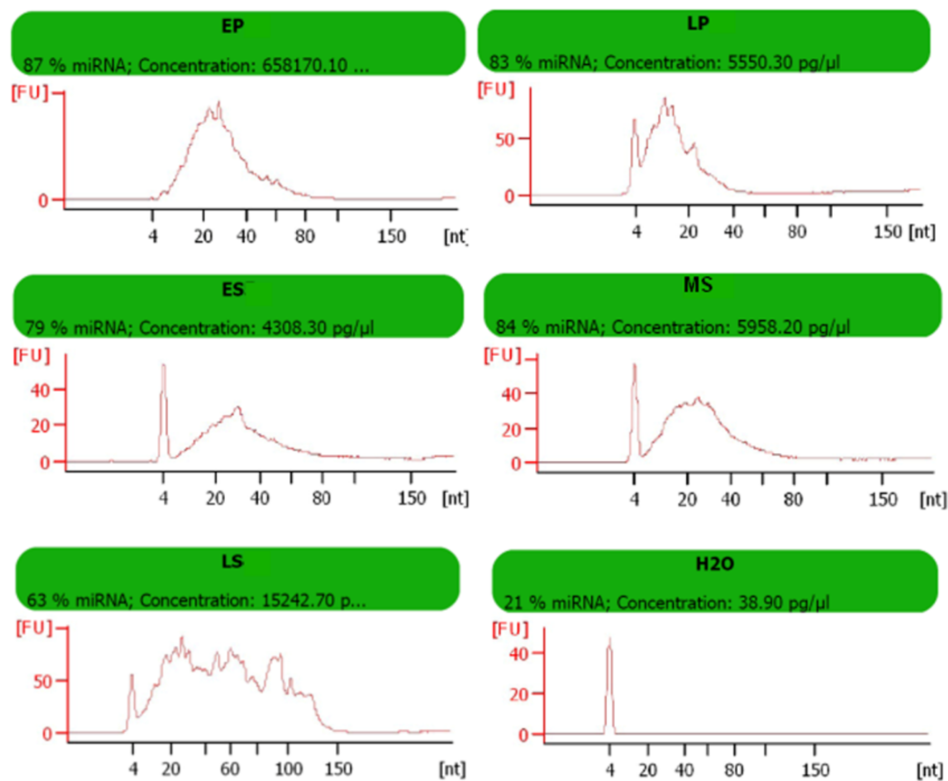


Figure 18. Small RNA content of endometrial fluid samples across the menstrual cycle using Pico RNA LabChip Bio Analyzer

Menstrual cycle stage	Day of cycle ± sd	Age ± sd	%miRNA ± sd
Early Proliferative (EP)	6.75 ± 1.25	37.25 ± 3.30	60.5% ± 12.87%
Late Proliferative (LP)	12.5 ± 1.73	37 ± 5.77	48.75% ± 7.41%
Early Secretory (ES)	16.25 ± 1.50	38.75 ± 3.09	40.5% ± 2.65%
Window Of Implantation (WOI)	20.75 ± 1.70	36 ± 3.60	64% ± 19.5%
Late Secretory (LS)	31.5 ± 4.20	40 ± 3.82	45.25% ± 12.65

Table 5. Percentage of miRNA across the menstrual cycle (n=4/group)

2. MiRNAs of maternal origin displays differential miRNAs profile during the window of implantation (WOI)

We used microarrays to compare EF miRNA expression in these sample groups with that in the WOI group. The Rank Product analysis, with a false discovery rate correction of <5%, resulted in nine differentially expressed miRNAs identified in EP, 8 in LP, 6 in ES, and 4 in LS versus the WOI (Figure 19a).

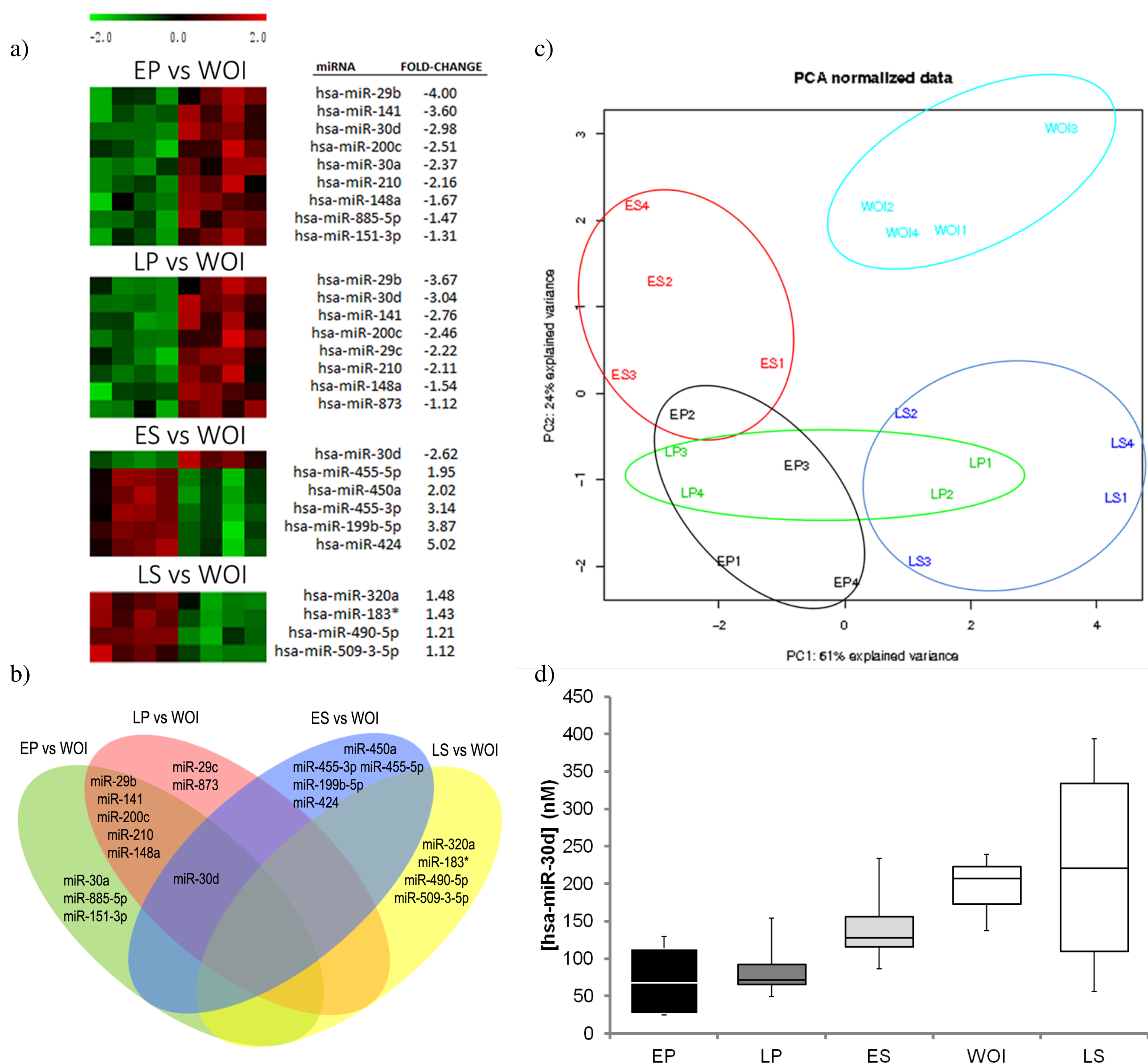


Figure 19. (**a**) Heat map of the differential expression of secreted maternal miRNAs during the WOI, relative to other phases of the menstrual cycle. (**b**) Venn diagram demonstrating the overlap of different miRNAs at different phases of the menstrual cycle (**c**) Supervised principal components analysis (PCA) to distinguish differentially expressed miRNAs in EF obtained throughout the menstrual cycle, relative to the WOI. (**d**) Hsa-miR-30d concentration in EF over the menstrual cycle.

The lists of miRNAs differentially expressed for each comparison were represented in a Venn's diagram (Figure 19b) with hsa-miR-30d being the most represented. This lists of nineteen differentially expressed miRNAs represented at Venn's diagram were used to perform a supervised principal components analysis (PCA) (Figure 19c). We determined hsa-miR-30d concentration in the EF across the menstrual cycle, reaching concentrations of 194.68 ± 29.90 nM at the WOI (Figure 19d).

Bioinformatic analysis to predict the function(s) of these differentially expressed miRNAs primarily highlighted endocrine; reproductive and cell proliferation disorders (Figure 20).

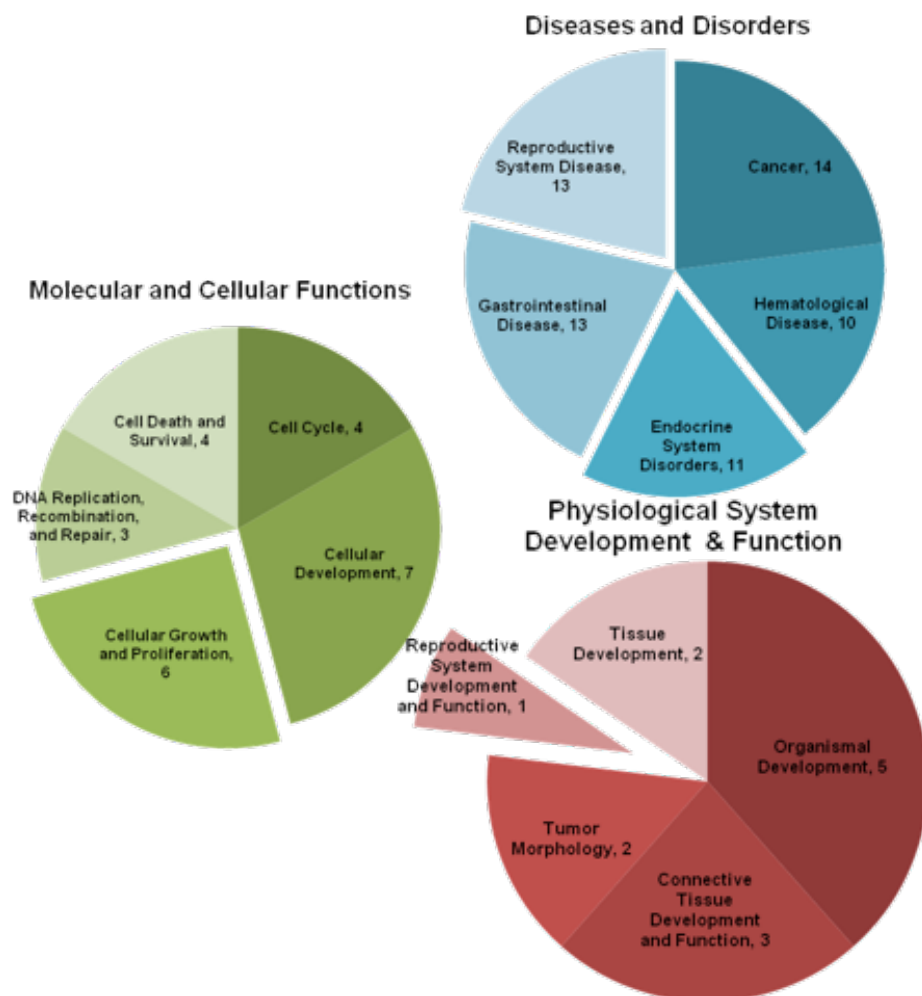


Figure 20. Functional classification of miRNAs identified on endometrial fluid.

An interaction network generated by IPA software shows a relation between genes and miRNAs based on the literature. In the graph one can also see that some of them have been associated with uterine endometroid carcinoma and diabetes mellitus diseases (Figure 21).

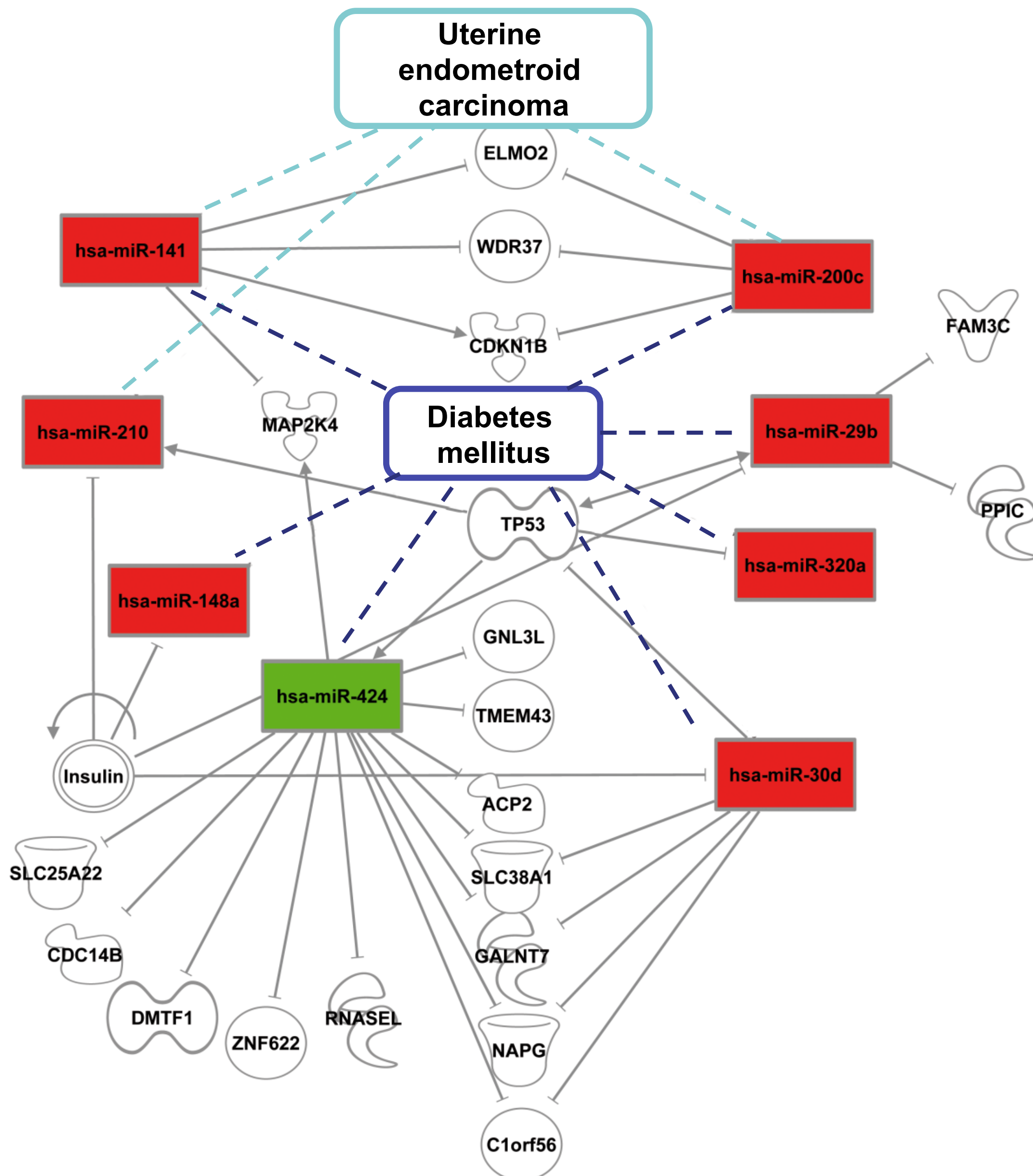


Figure 21. Interaction network between genes.

3. MiR-30d is expressed in the epithelial compartment of the receptive endometrium

Since previous studies in endometrial biopsies supported the upregulation of hsa-mir-30d during the acquisition of endometrial receptivity (Altmae *et al*. 2013, Kuokkanen *et al*. 2010, Sha *et al*. 2011), we wanted to determine which cell compartment is upregulating and actively secreting it in the endometrial lumen. Three biopsies from healthy patients during a natural cycle were obtained at the receptive phase (LH+7), and the stromal cells from epithelial cells were mechanically and enzymatically separated, followed by MiScript qPCR for hsa-miR-30d to assess the expression of miR-30d in these two cell compartments. Hsa-miR-30d expression increased by 2.65 ± 0.69 fold in the epithelial versus the stromal endometrial fraction (Figure 22).

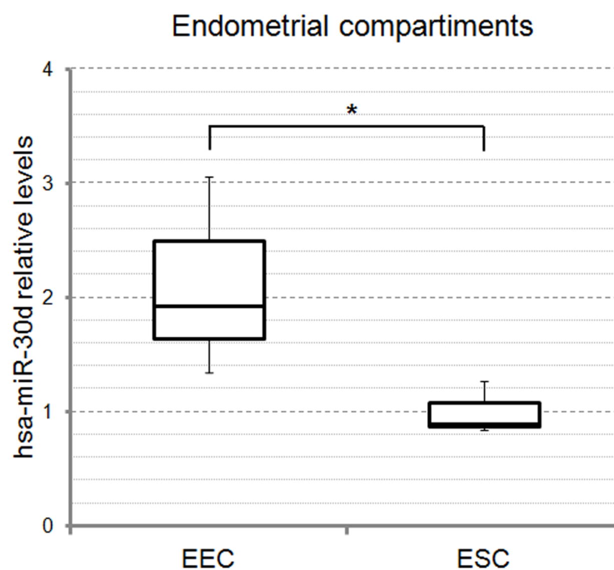


Figure 22. Real-time qPCR for hsa-miR-30d in epithelial versus the stromal endometrial cell fraction.

4. The transcriptomic effect of miR-30d transfection in primary human endometrial epithelial cells

Since the endometrium has shown to highly express hsa-miR-30d during the receptive stage, we explored the endogenous effects derived from hsa-miR-30d exogenous introduction in the endometrial epithelial cells *"in vitro"*. First, we transiently transfected and checked the efficiency in primary hEEC cultures with mimic-30d versus scramble miRNAs, showing an 89.95 ± 47.23 fold-increase in miR-30d after the intervention (Figure 23).

Once gene expression microarrays were performed, a Rank Product analysis with a FDR

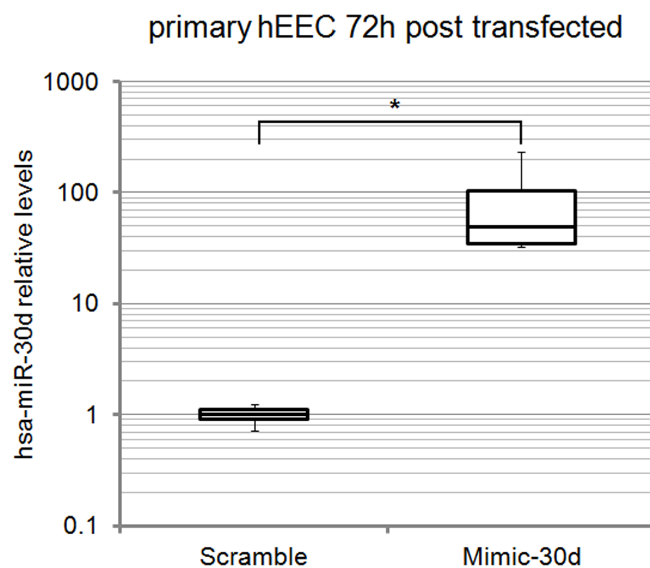


Figure 23. Transfection efficiency in primary hEEC cultures after 72 hours of transfection with mimic-30d versus scramble.

correction of less than 5% resulted in 176 genes differentially regulated after the miR-30d transfection. A total of 75 upregulated and 101 downregulated genes were registered (See CD Annexed Table 1). A standardized supervised hierarchical clustering heat map (Figure 24a) together with a 3D principal component analysis (PCA; Figure 24b) illustrate the different gene expression levels between the two conditions, and how they cluster together. These results were validated using real time qPCR for three representative genes (BMP4, H19, and FOS) that were downregulated in mimic- versus scramble-treated hEECs (Figure 24c).

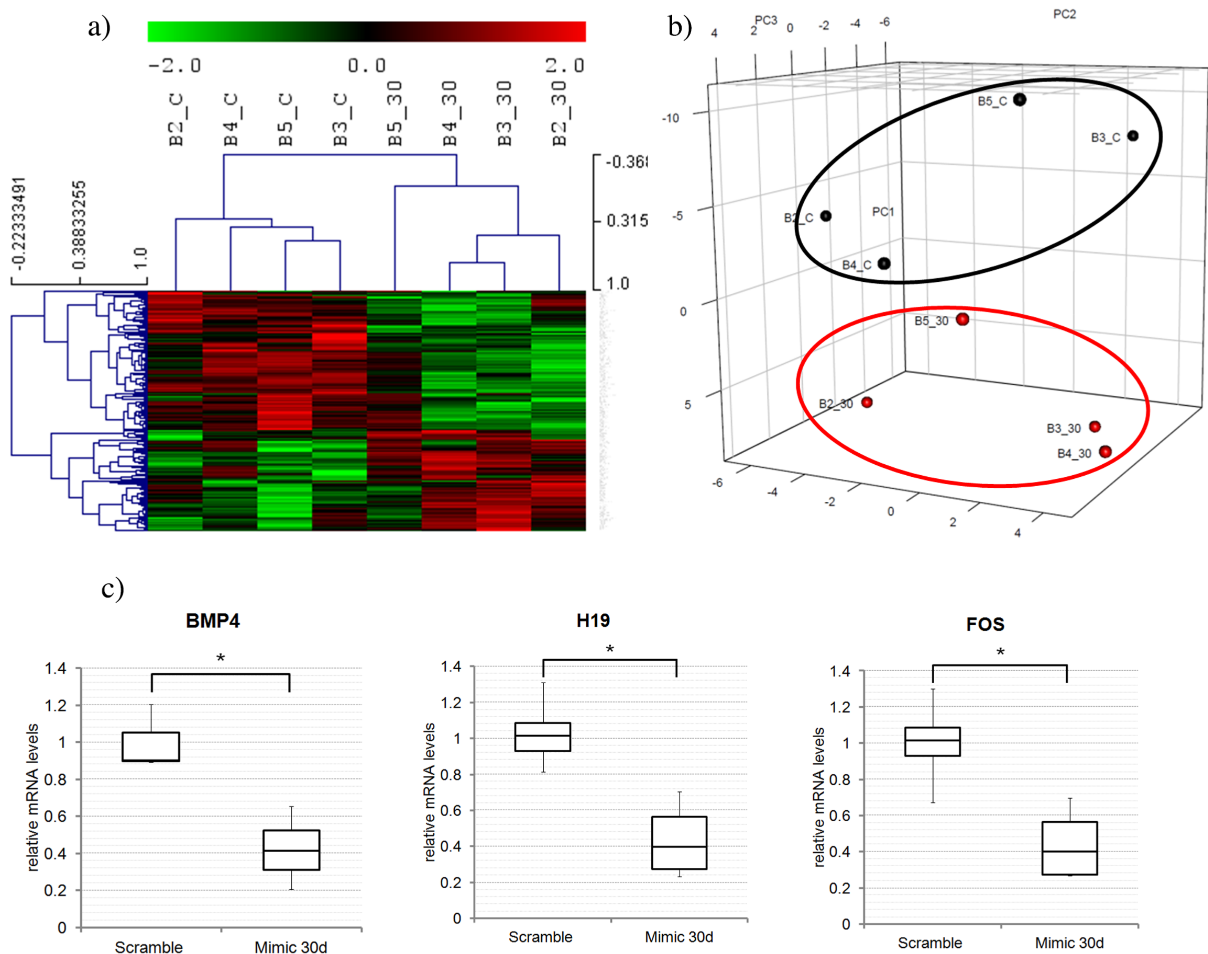


Figure 24. (a) A standardized supervised hierarchical clustering heat map and **(b)** A 3D principal components analysis (PCA) both used to visualize sample similarities between the differentially expressed genes obtained. **(c)** Real Time PCR validation for three genes down-regulated in mimic-30d vs scramble transfected cells.

5. Bioinformatic analysis of genes regulated by miR-30d in human endometrial epithelial cells

The IPA software analysis allowed us to predict the most implicated functions for the set of 251 genes that are differentially regulated by miR-30d. For "*diseases and disorders*" the most represented term was cancer, but there were also genes related to the reproductive and endocrine systems (Figure 25a). At the "*molecular and cellular functions*" level most of the

genes were involved in cell proliferation (Figure 25b). At the "*physiological system development and functions*" level, the genes appeared to be related to embryonic and tissue development (Figure 25c). Based on the literature, IPA software looks for significant interaction network nodes that are indirectly affected by fourteen differentially expressed genes. These nodes are 'all-trans retinoic acid'(Tice *et al*. 2002) and 'b-estradiol' (Symmans *et al*. 2005), which are described as essential for cell division in epithelial, stromal, and myometrial cells; these two nodes, together with 'MYC'(Koch *et al*. 2007) and 'CTNNB1'(Shimoyama *et al*. 1999), are related to the proliferation of pancreatic progenitor cells and beta islet cells (Figure

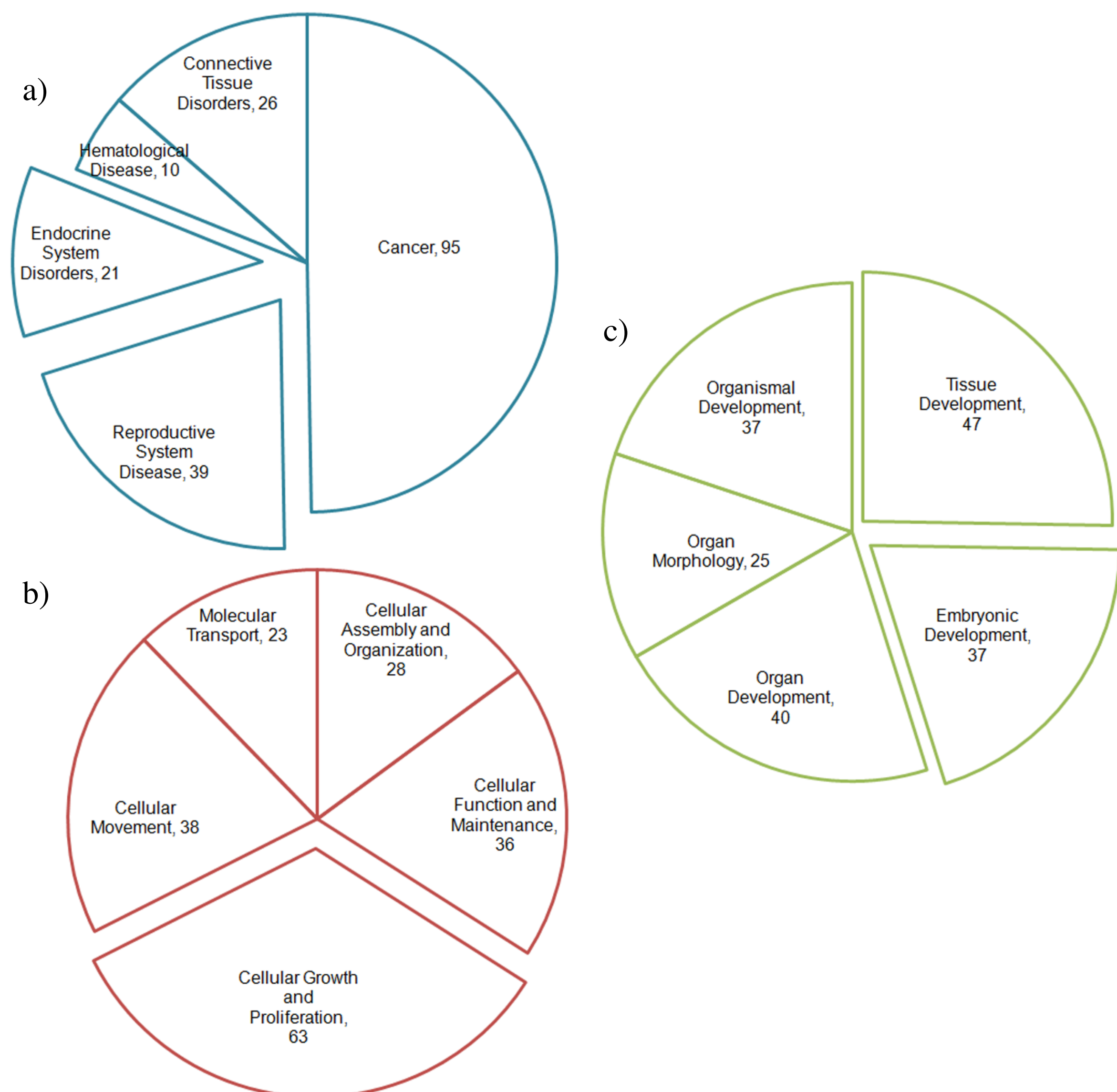
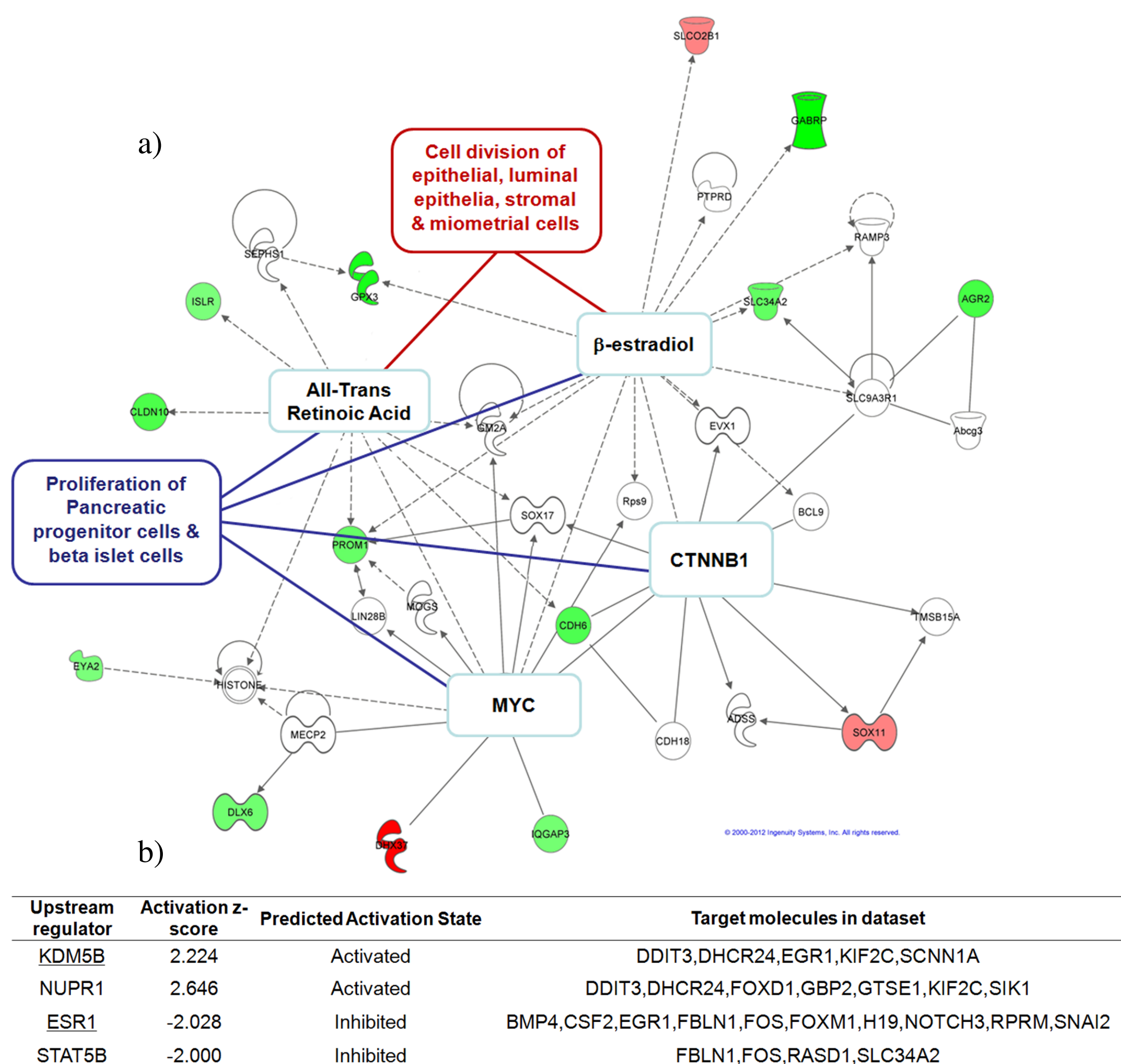


Figure 25. IPA software summarized results for **(a)** Main diseases and disorders associated with selected genes. **(b)** Main molecular and cellular functions related with the genes. **(c)** Physiological system development and functions associated with the genes.

26a).

The IPA software was also able to predict the potential upstream regulators, ESR1 and STAT5B, which reached an activation z-score of less than -2 (an inhibited state), and KDM5B and NUPR1, which achieved a z-score of more than 2 (an activated state). ESR1 (estrogen receptor alpha) was the closest to the threshold value, and the target genes for this transcription factor are BMP4, CSF2, EGR1, FBLN1, FOS, FOXM1, H19, NOTCH3, RPRM, and SNAI2 (Figure 26b).



Upstream regulator	Activation z-score	Predicted Activation State	Target molecules in dataset
KDM5B	2.224	Activated	DDIT3,DHCR24,EGR1,KIF2C,SCNN1A
NUPR1	2.646	Activated	DDIT3,DHCR24,FOXD1,GBP2,GTSE1,KIF2C,SIK1
ESR1	-2.028	Inhibited	BMP4,CSF2,EGR1,FBLN1,FOS,FOXM1,H19,NOTCH3,RPRM,SNAI2
STAT5B	-2.000	Inhibited	FBLN1,FOS,RASD1,SLC34A2

Figure 26. IPA software summarized results for (**a**) The most significant interaction network for differentially expressed genes based on the literature. **(b)** Potential upstream regulators that might control these deregulated genes.

6. The proteomic effect of miR-30d transfection in primary human endometrial epithelial cells

The proteomic analysis found 108 differentially expressed proteins (See CD Annexed Table 2) from the 2,290 originally identified (see CD Annexed Table 3). Fold-change values ranged between 3 (upregulated) and -3 (downregulated). Several upregulated (COX6C, LGALS1, DNMT1, and MIF) and downregulated (WNT1, ALDH2, PGRMC1, NOTCH4, ITGAV, and ALDH1B1) proteins are relevant for endometrial physiology. A standardized supervised hierarchical clustering heat map (Figure 27a) together with 3D PCA (Figure 27b) illustrates the different protein levels between samples and how they cluster together. Western blots for DNMT1, ALDH2, PGRMC1, and WNT1 were used to validate these iTRAQ results (Figure 27c), DNMT1 and ALDH2 were statistically significative (p-value<0.05) after Mann–Whitney U test.

The genes and proteins, which overlapped between transcriptomic and proteomic profiles, are illustrated in a Venn's diagram (Figure 27d), where only ALDH2 was identified as differentially downregulated at both the mRNA and protein levels. This gene has also been predicted to be directly be targeted by hsa-miR-30d (Figure 27e, seed region).

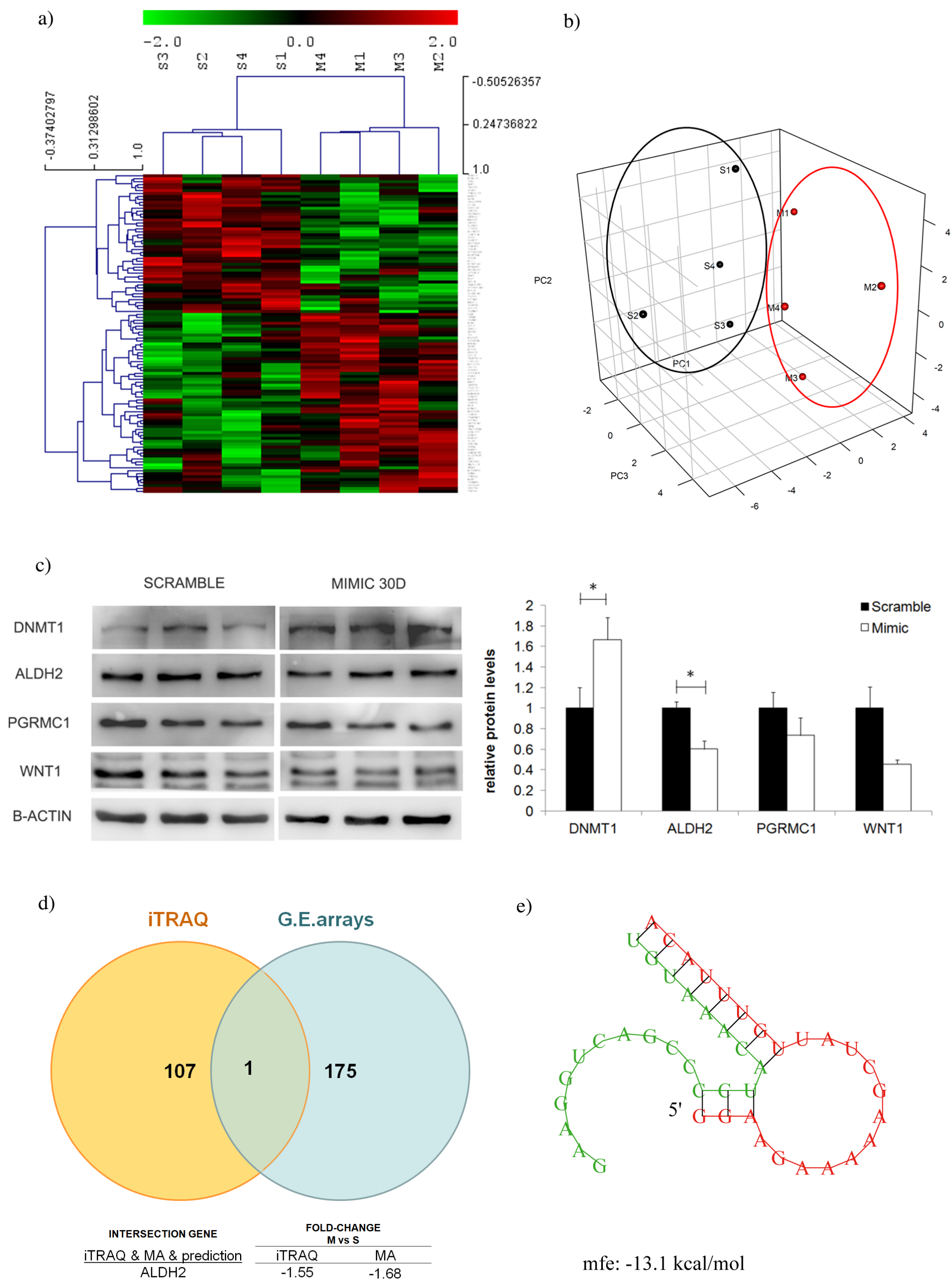


Figure 27. **(a)** A standardized supervised hierarchical clustering heat map and **(b)** a 3D PCA used to visualize the sample similarities for the differentially expressed proteins obtained. **(c)** Western blots for DNMT1, ALDH2, PGRMC1, and WNT1 for validation of iTRAQ results (*p-value<0.05 after Mann–Whitney U test). **(d)** A Venn diagram of overlapping genes and proteins between transcriptomic and proteomic studies; only ALDH2 is found in both. **(e)** The seed region of 3'-UTR complementarity with hsa-miR-30d calculated as an RNA hybrid.

7. The methylation status of the H19 gene

Focusing on the probable regulation of the DNMT1 protein and H19 gene (which is typically methylated) by miR-30d as suggested by miR-30d overexpression, we hypothesized that alteration of the H19 methylation status might lead its silencing. This gene, which has been shown to be downregulated in mimic conditions, is a well-known hemi-methylated gene in the imprinted control region (ICR; Figure 28a); hence, we performed a methylated DNA immunoprecipitation (MeDIP) analysis to assess its global methylation status. We selected UBE2B as an unmethylated housekeeping gene to validate the effectiveness of the MeDIP (Figure 28b). A statistically significant increase in the methylation status of H19 was observed for mimic- versus scramble-treated hEECs (Figure 28c) reinforcing the concept that miR-30d modifies not only the transcriptome but also the epigenome of hEECs.

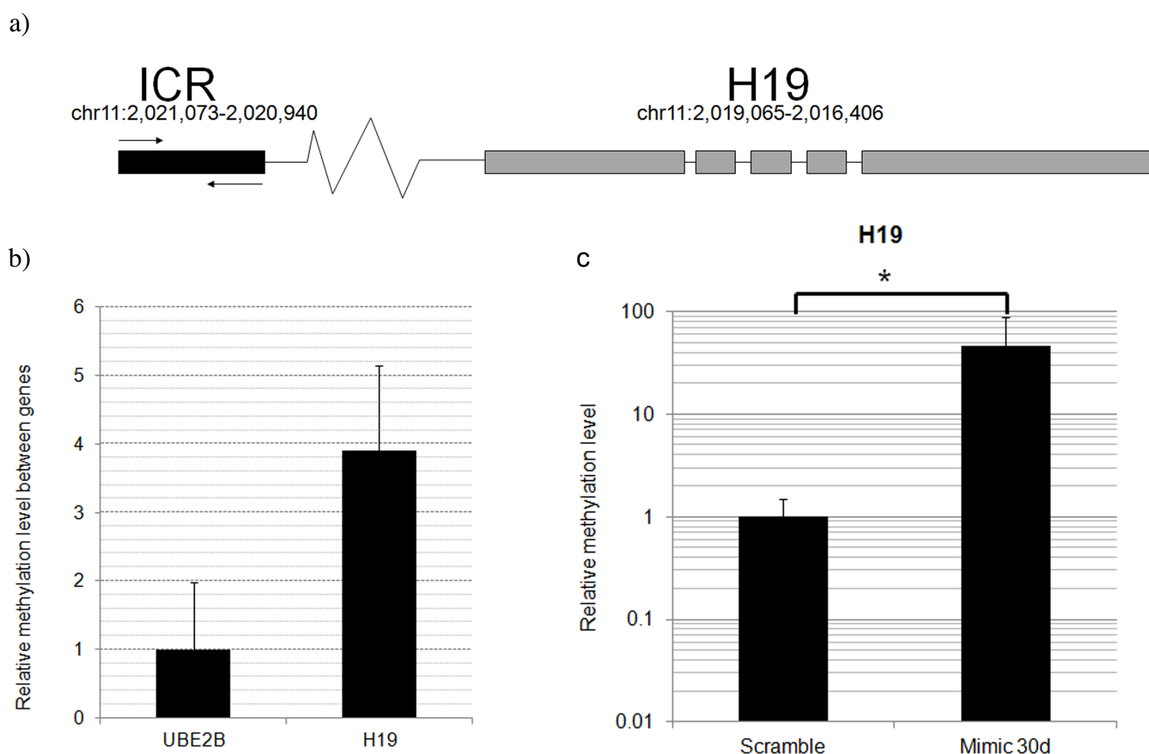


Figure 28. **(a)** A schematic of the H19 gene, indicating its ICR where methylated DNA immunoprecipitation was performed. **(b)** Real-time qPCR showing UBE2B as an unmethylated housekeeping gene commonly used to explore the effectiveness of the MeDIP, versus the H19 ICR hemi-methylated gene. **(c)** Real-time qPCR comparing the methylation status of H19 for mimic- versus scramble-treated hEECs.

8. Exosomes are secreted by the endometrial glands

Next, we investigated the cellular origin and mechanism of secretion of the endometrial miRNA into the EF. Since miRNAs are usually secreted in exosomes (Valadi *et al*. 2007), we searched for these microvesicles in the human endometrium by checking initially the expression of CD63, an established marker of exosomes (Ng *et al*. 2013). CD63 staining localized at the apical part and glycocalyx of the glandular epithelium during the WOI, and was absent in the early secretory endometrium corresponding to the pre-receptive phase (Figure 29).

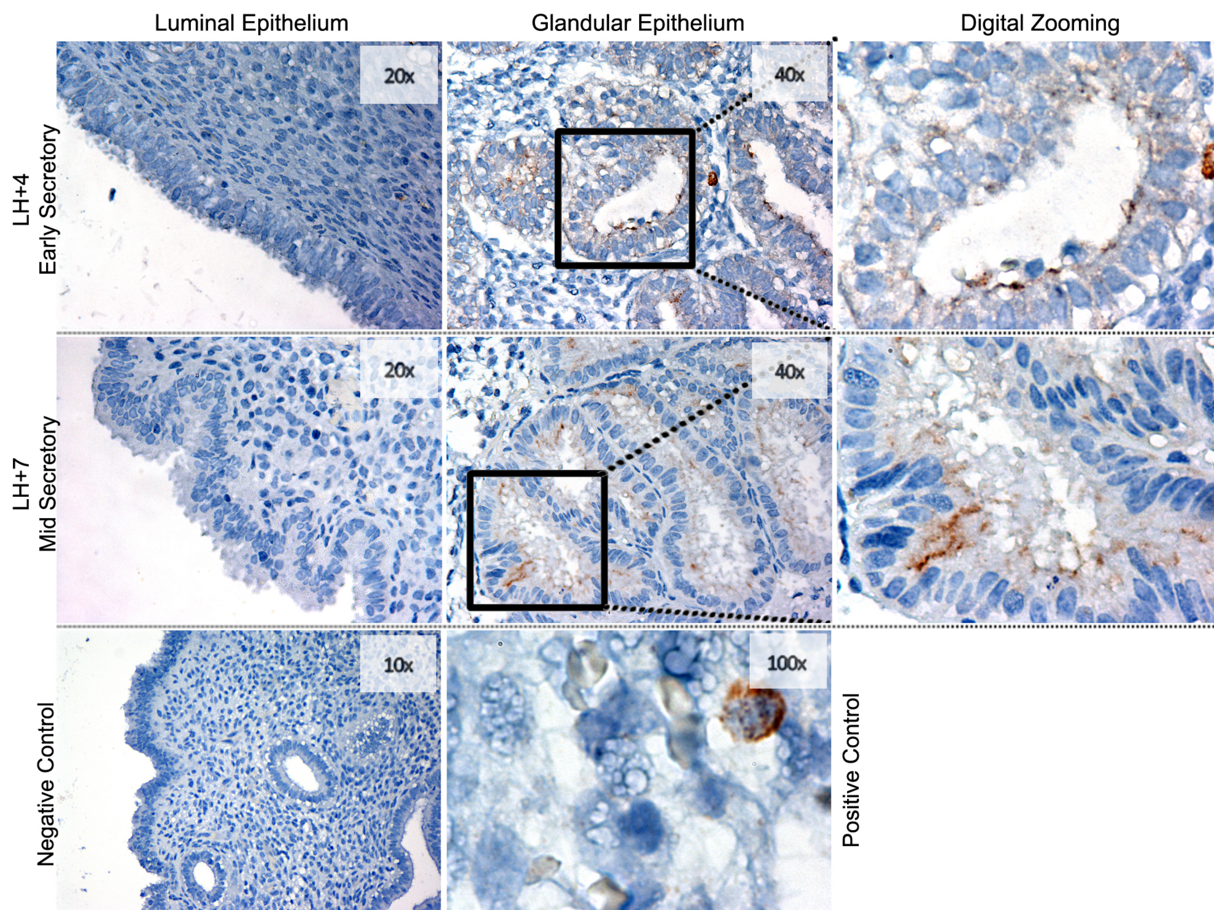


Figure 29. Immunohistochemical staining of human glandular and luminal epithelium for CD63 in the early secretory phase (4 days after luteinizing hormone peak; LH+4), window of implantation WOI (LH+7), and negative (no primary antibody) and positive controls (resident macrophage cell).

Using transmission electron microscopy (TEM), we verified that glandular epithelial cells in the secretory phase have a high endosome content, with sizes ranging from 50 to 250 nm (Figure 30a), that appear to be secreted into the endometrial cavity. In fact, negative-staining TEM after ultracentrifugation of human EF identified these small vesicles as exosomes (Figure 30b). Western blot analysis showed that the exosome fraction of primary human endometrial epithelial cells (hEEC) culture media was positive for CD63 and low content of β-actin (Mathivanan and Simpson 2009, Ng *et al.* 2013) (Figure 30c).

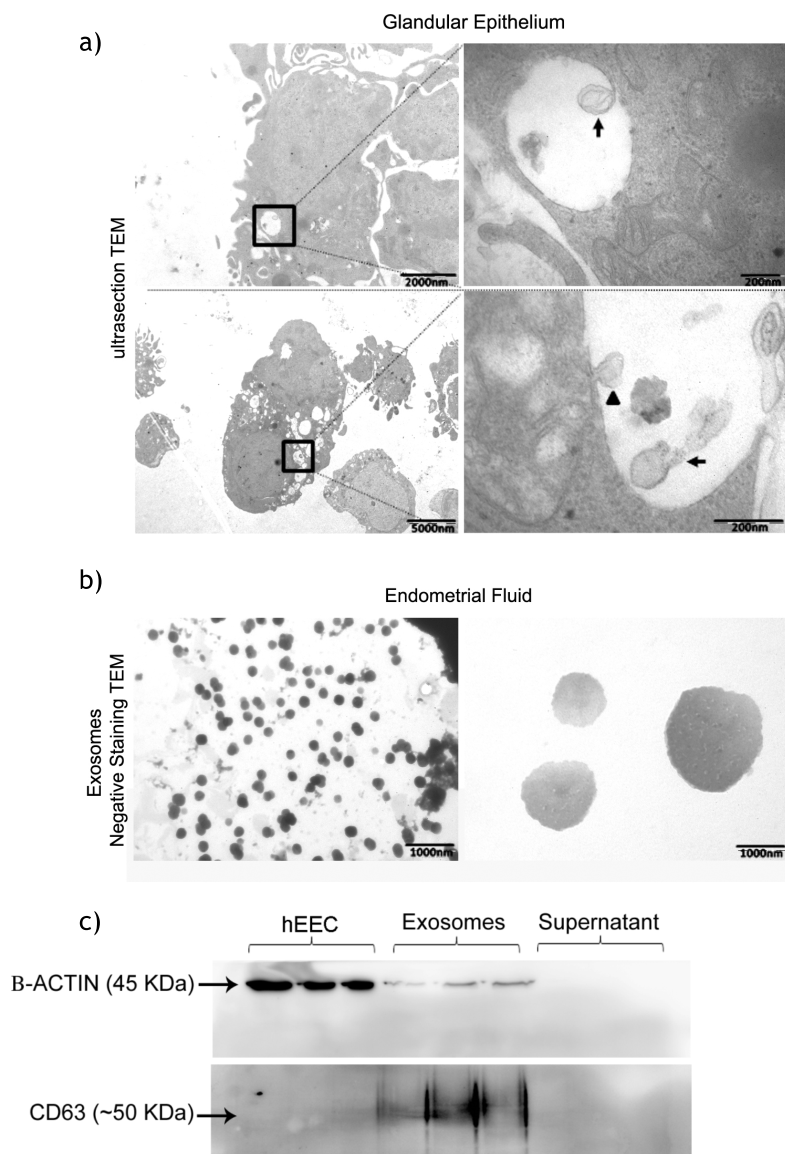


Figure 30. (**a**) Transmission electron microscopy (TEM) images from glandular hEEC show an endosome proximal to the plasma membrane with a small exosome (arrow). Another glandular hEEC shows the same type of endosome compartment in which a nascent exosome (arrowhead) and a broken exosome release their contents (arrow). (**b**) TEM images from negative staining with uranyl acetate for exosomes purified from EF. (**c**) Western blot identifying Cd63 (specific exosomes marker) and b-actin in pelleted microvesicles.

9. Maternal miRNAs are transported as free- and exosome-associated molecules

After demonstrating that the human endometrial epithelium produces and secretes exosomes, we used an *"in vitro"* model of primary hEEC to obtain large quantities of exosomes (3.5 ± 1.26 mg of total protein) from the hEEC-conditioned media and used a RNA Pico LabChip kit to identify the presence of RNAs. Using a RNase treatment, we demonstrated that exosomes protect RNAs from degradation (Figure 31a). Then, using qPCR, hsa-miR-30d was identified in the total hEEC-conditioned media, in the primary hEEC, in the exosome-depleted conditioned media (supernatant), and in the purified exosomes (Figure 31b). The data are represented as crossing points, which are inversely proportional to expression level, meaning the lower Cp value, the greater expression.

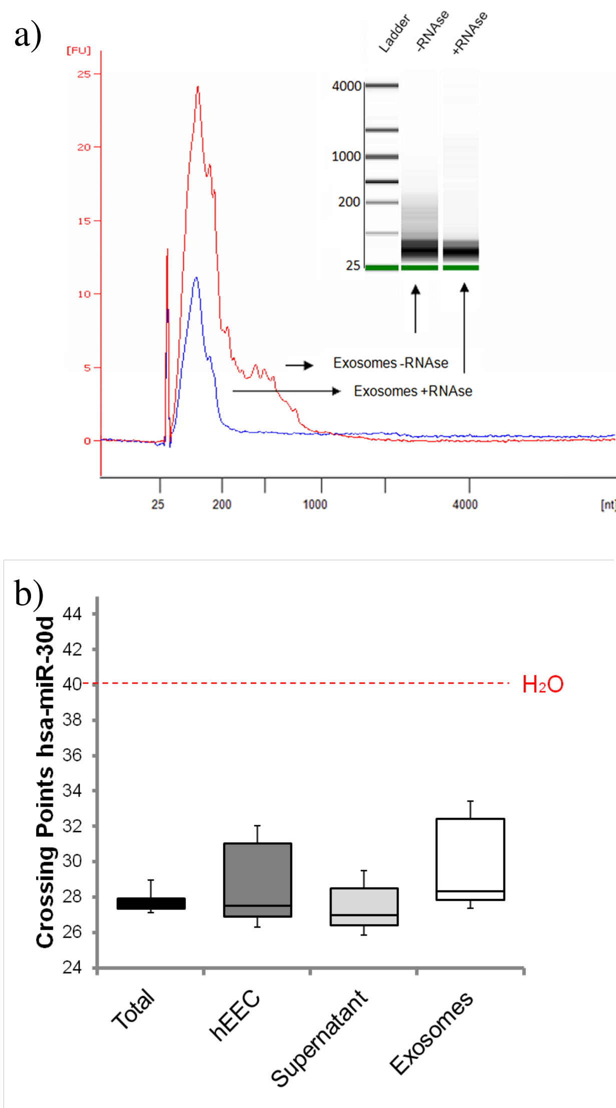


Figure 31 (**a**) Results from an RNA lab chip assay showing the RNA content from primary hEEC-derived exosomes with or without treatment with RNase A. (**b**) Crossing points (y-axis) for hsa-miR-30d in total conditioned media, cells, supernatant, and exosomes purified from primary hEEC cultures.

10. Embryo ability to uptake miRNAs

We hypothesized that the embryo is able to take up free or exosome-associated miRNAs from the EF. The uptake of free hsa-miR-30d was tested in an *"in vitro"* mouse embryo model. Day-2 mouse embryos (n=30) were cultured for 72 h with scramble Alexa 488-labeled miRNA (400 nM), and the trophectoderm uptake was confirmed by immunofluorescence, either in the hatched area or in the trophectoderm after zona pellucida removal (Figure 32a). Then, mouse embryos (n=60) were incubated with free synthetic miR-30d mimic (50 nM, 100 nM, or 400 nM) using scramble miRNA as a negative control, and the embryo uptake was detected by traditional and real-time qPCR at all concentrations and tested in the embryo and the washed medium (Figure 32b).

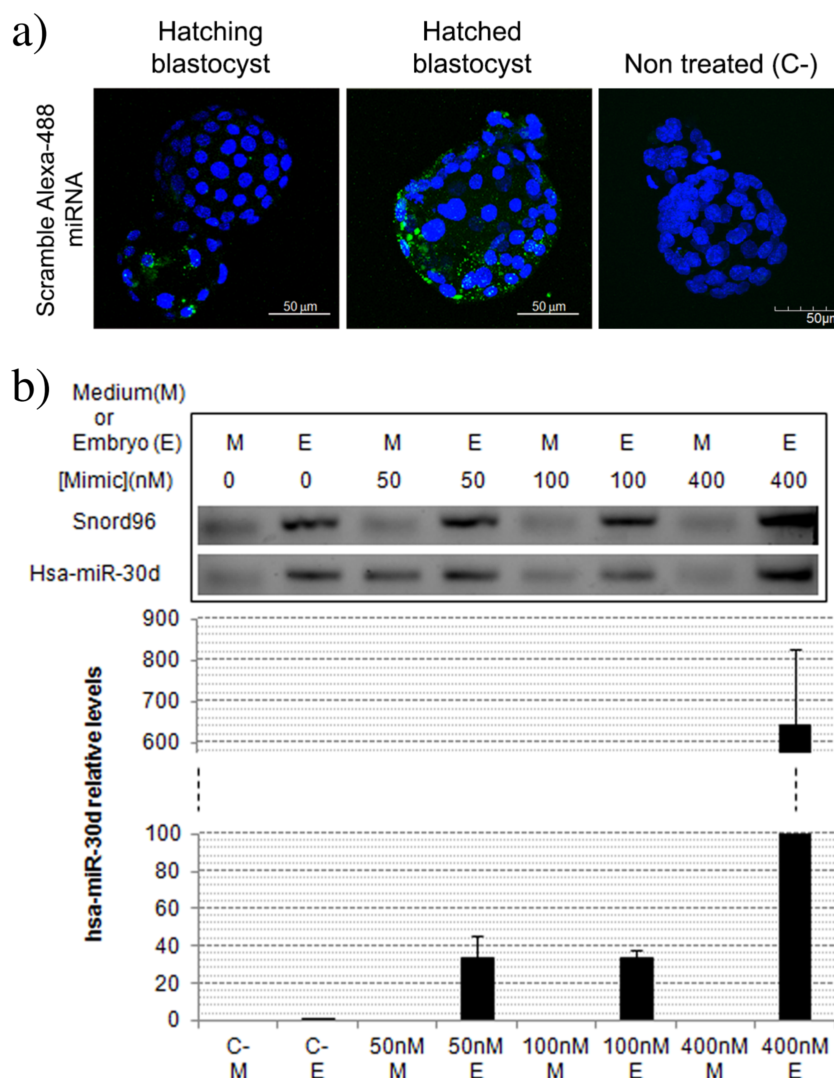


Figure 32. (**a**) Confocal fluorescence microscopy of live hatching mouse blastocysts treated with 400 nM Alexa 488-scrambled miRNA for 72 h. (**b**) qPCR of embryos (E) and wash medium (M) treated with 0, 50, 100, or 400 nM miR-30d mimic. Gel bands indicating the absence of Snord96 (housekeeping) or hsa-miR-30d in the final wash media are shown.

11. Embryo ability to uptake exosomes derived from hEEC

To test the embryo uptake of exosome-secreted miRNAs, we stained vesicles isolated from the primary hEECs with an exosome membrane marker, Vybrant DiO, and added them to culture media containing day-2 mouse embryos (n=15) for 24 h. We also verified that the trophectoderm in the hatched area (or after the zona pellucida was removed) was able to take up exosomes (Figure 33a). This was confirmed using scanning electron microscopy to visualize the trophectoderm surface of the exosome-cocultured embryos (Figure 33b). Small rounded vesicles surrounded by microvilli were found attached to the apical trophectoderm membrane close to pores or cell membrane invaginations.

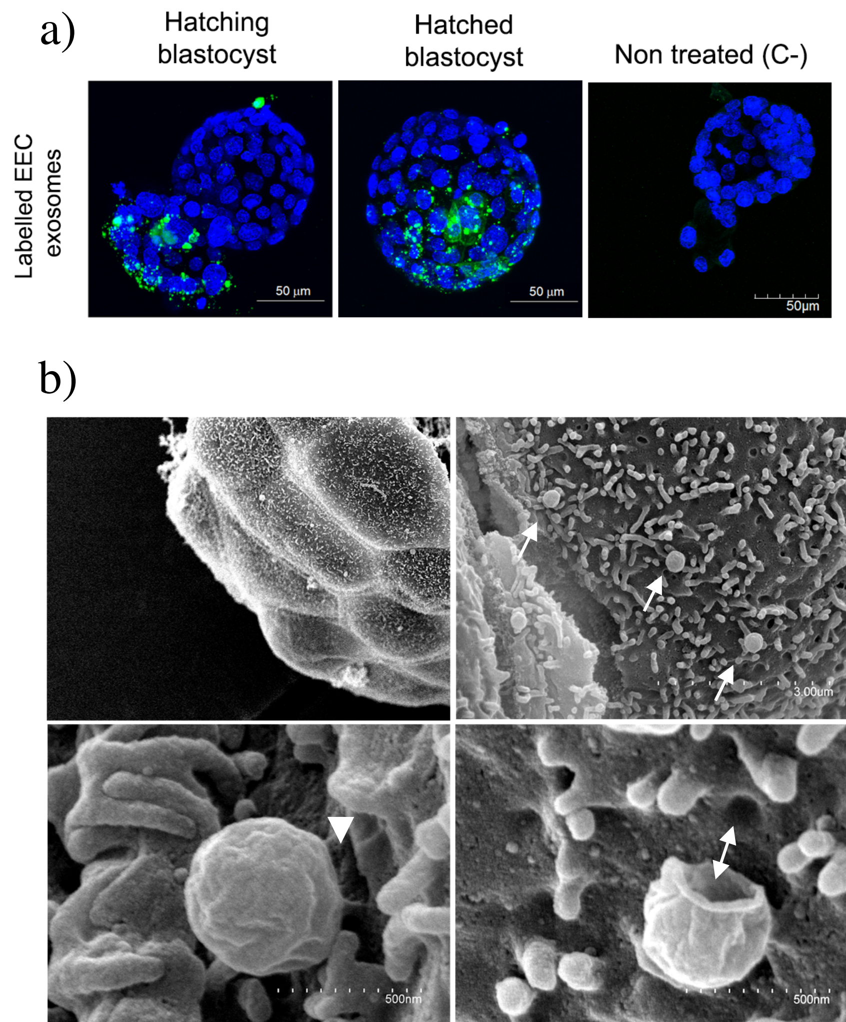
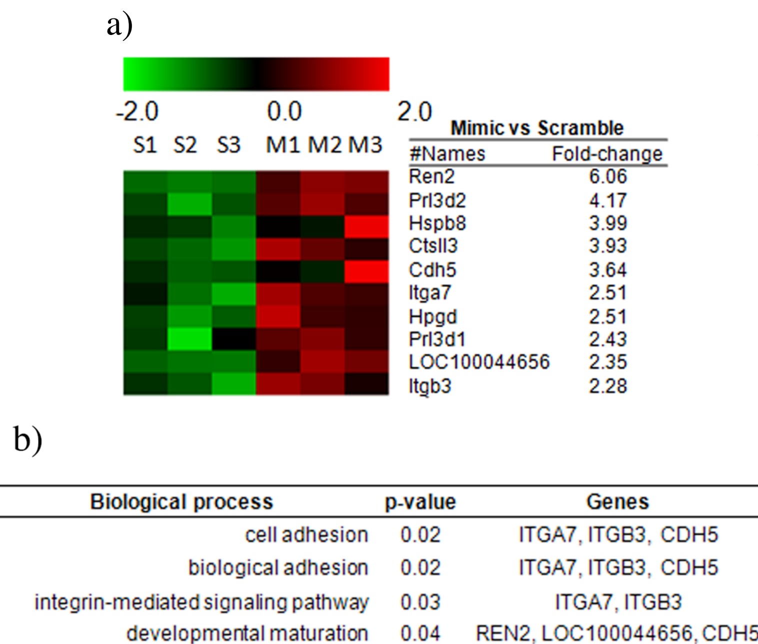


Figure 33 a) Confocal microscopy images of hatching blastocysts cultured with hEEC exosomes labeled with Vybrant DiO for 24 h. (**b**) Scanning electron microscopy images from a hatched mouse blastocyst with typical rounded exosome vesicles adhering to the trophectoderm (arrows indicates exosomes adhered to trophectoderm surface; arrowheads indicates a contact zone; and bidirectional arrow the detachment of a vesicle that seemed to be adhered).

12. Transcriptomic changes after hsa-miR-30d uptake by the murine embryo *"in vitro"*

To demonstrate the functional relevance of this maternal–embryonic communication mechanism, we investigated the effects of endometrial miRNA uptake on the embryonic transcriptome and phenotype. A gene expression microarray on day-2 mouse embryos (n=50/group in biological triplicates) cultured with hsa-miR-30d mimic or scramble at 400 nM for 72h revealed embryonic overexpression of 10 specific genes (Figure 34a). These genes were interrogated using the DAVID web-based tool (http://david.abcc.ncifcrf.gov/home.jsp) to identify the biological processes that were most likely affected. The vast majority of regulated genes were related to cell adhesion, the integrin-mediated signaling pathways, and developmental maturation (Figure 34b). Selected genes (Itgb3 and Cdh5) were validated by real time qPCR (Figure 35a)



#Names	Fold-change
Ren2	6.06
Prl3d2	4.17
Hspb8	3.99
Ctsll3	3.93
Cdh5	3.64
Itga7	2.51
Hpgd	2.51
Prl3d1	2.43
LOC100044656	2.35
Itgb3	2.28

Biological process	p-value	Genes
cell adhesion	0.02	ITGA7, ITGB3, CDH5
biological adhesion	0.02	ITGA7, ITGB3, CDH5
integrin-mediated signaling pathway	0.03	ITGA7, ITGB3
developmental maturation	0.04	REN2, LOC100044656, CDH5

Biological process	p-value
cell adhesion	0.02
biological adhesion	0.02
integrin-mediated signaling pathway	0.03
developmental maturation	0.04

Figure 34. (**a**) Supervised and standardized heat map of differentially expressed genes together with fold-changes obtained from gene expression microarrays in embryos treated with scrambled miRNA (S) or miR-30d mimic (M). (**b**) The significant biological processes affected, based on predictions made by the DAVID web-based tool.

13. Phenotypic changes after hsa-miR-30d uptake by the murine embryo *"in vitro"*

The phenotypic effect of this transcriptomic regulation was demonstrated using an *"in vitro"* model of embryo adhesion (Garrido-Gomez *et al*. 2012, Martin *et al*. 2000) in four conditions —control without miRNA, scramble miRNA, miR-30d mimic, and a miR-30d inhibitor— in six experiments (90 embryos per condition, n=360). After 32 h, miR-30d mimic increased the rate of embryonic adhesion to the endometrial epithelium relative to scramble miRNA (53.44 ± 6.40% versus 35.22 ± 7.40%, respectively), and the adhesive phenotype was impaired when a specific miR-30d inhibitor was added (53.44 ± 6.40% versus 18.55 ± 3.72%, respectively; p=0.001; Figure 35b).

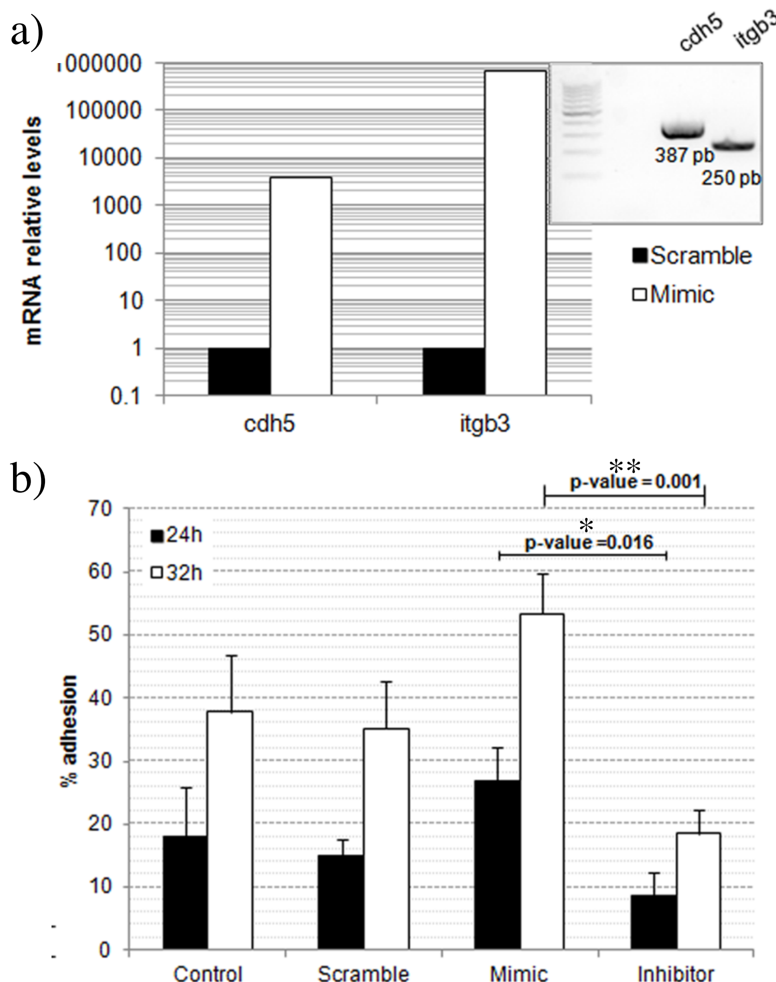


Figure 35. (**a**) Quantitative real time PCR for the validation of transcriptomic gene expression identified on the microarrays. (**b**) Mouse blastocyst adhesion assay showing a functional effect related to the miR-30d uptake.

VI. DISCUSSION

The human embryo undergoes developmental changes during its preimplantation period (Niakan *et al*. 2012) necessary to initiate the implantation process in synchrony with the receptive endometrium.

Accumulated evidences have demonstrated specific transcriptomics (Diaz-Gimeno *et al*. 2011, Diaz-Gimeno *et al*. 2012, Kao *et al*. 2002), proteomics (Dominguez *et al*. 2009), and lipidomics (Vilella *et al*. 2013) signatures of the human endometrium during the window of implantation. On top of this dynamic regulation, the blastocyst actively regulates the endometrium during the implantation process (Caballero-Campo *et al*. 2002, De los Santos *et al*. 1996). On the other hand, the endometrial fluid (EF), a viscous fluid secreted by the endometrial glands provides nutrients for blastocyst formation and constitutes a microenvironment where the embryo-endometrial dialog occurs prior to implantation. However, its impact on the embryonic implantation and future adult life are unknown.

MiRNAs are small, 19-22–nucleotide sequences of noncoding RNA that function as regulators of endogenous gene expression (Ambros and Chen 2007, Bartel 2004). MiRNAs may be secreted by cells and incorporated into microvesicles or, alternatively, may be associated with proteins that protect them from RNase degradation, endowing them with a long half-life (Turchinovich *et al*. 2011, Yoshizawa and Wong 2013). These molecules have been implicated in the regulation of the human WOI (Altmae *et al*. 2013, Kuokkanen *et al*. 2010, Ng *et al*. 2013, Sha *et al*. 2011) as well as in the decidualization process *"in vitro"* and *"in vivo"* (Estella *et al*. 2012).

In this thesis, we determined that miRNAs are secreted by the human endometrium to the endometrial fluid with specific profiles across menstrual cycle, demonstrated that these molecules are uptaken by the pre-implantation embryos acting as a previously unknown form of regulation, and discussed the potential of this new kind of biomarker for detection of endometrial receptivity.

We detected the presence of a large quantity of small RNAs in human EF that make them suitable for containing mature forms of miRNAs (19-22 nts). Moreover, after microarrays analysis we identified a differential miRNA expression pattern in EF across the menstrual

cycle. In general, the differential expression of miRNAs is narrower as we approach to the WOI. The nineteen differentially expressed miRNA were used to perform a supervised principal components analysis (PCA), which showed that the EF obtained from the WOI was different from EFs secreted at different stages of the cycle based on its miRNA composition.

More generally, some miRNAs participate in functions related to endocrine and reproductive system disorders, such as for example diabetes mellitus and endometroid carcinomas, which shows the importance of these miRNAs not only for embryonic implantation but also for the normal physiology of endometrium and pregnancy support.

Additionally, some individual observations of miRNAs correlates with previous observations associated with the endometrium physiology and the embryo implantation process, such as miR-320a that has been observed to increase during decidualization (Xia *et al*. 2010), miR-141 has been related to the increase of progesterone and to affect implantation in a murine model (Liu *et al*. 2013) and hsa-miR-30d determined to be up-regulated in receptivity in several works and also to essential processes in other tissues and cell types, such as glucose metabolism and epithelial-to-mesenchymal transitions (Altmae *et al*. 2013, Joglekar *et al*. 2009, Kuokkanen *et al*. 2010, Sha *et al*. 2011, Zhao *et al*. 2012). Since this last example also was the most differentially represented miRNA in the EF of all the comparisons, we decided to investigate its possible role at paracrine and autocrine levels, with particular focus on the receptivity stage and embryo implantation.

The miRNA “hsa-miR-30d” belongs to the “mir-30” family, which consists in six miRNAs (hsa-miR-30a,b,c,d,e,f) that posses a highly conserved seed sequence between species. Some of them are clustered in the same locus of the chromosome: hsa-miR-30c and

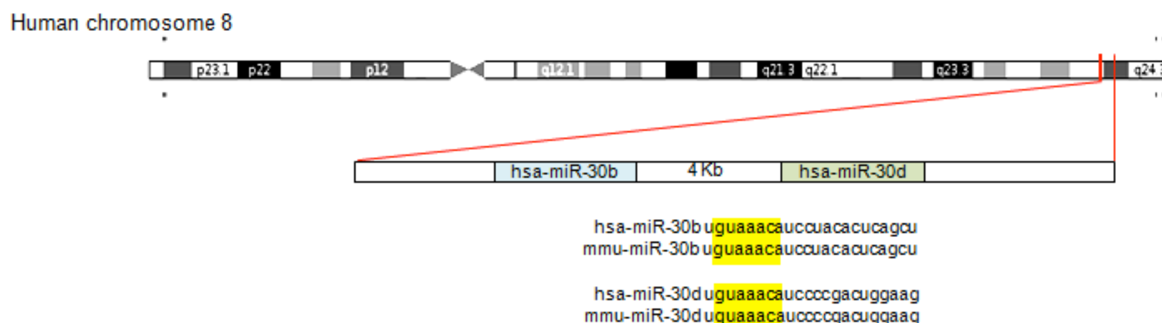


Figure 36. hsa-miR-30b and hsa-miR-30d gene cluster.

hsa-miR-30e in chromosome 1, and hsa-miR-30b and hsa-miR-30d in chromosome 8 (Figure 36).

In general, miR-30 family is ubiquitously expressed in humans and has been associated with several functions in different cell types and organisms. They have been determined to participate in epithelial-to-mesenchymal transition (Ozcan 2009), to confer epithelial phenotypes to human pancreatic cells (Joglekar *et al*. 2009), to regulate apoptosis through TP53 targeting and the mitochondrial fission machinery (Li *et al*. 2010), to be negative regulators of the BMP-2-mediated osteogenic differentiation (Wu *et al*. 2012), to participate in endothelial cell behaviour during angiogenesis (Bridge *et al*. 2012) and to play a role in cardiac functions (Pan *et al*. 2013). But their roles in endometrium were still unknown. This miRNA however, has been shown to participate in ectoderm specification during embryonic development by targeting the Embryonic Ectoderm Development (EED) protein (Song *et al*. 2011).

We were interested in the role of hsa-miR-30d progressive up-regulation in the endometrium across the menstrual cycle. We observed that epithelial cells were the main source of this miRNA, suggesting that the production in the endometrial fluid of this miRNA originates from these cells, also resulting in autocrine regulation.

To learn how miR-30d participates in the transition to a receptive phenotype, we transiently overexpressed this miRNA in hEECs *"in vitro"* by transfecting the endometrial epithelial cells, with synthetic 'mimic' or 'scramble' miRNAs. Since we decided to explore miRNA effects in primary cells instead of endometrial cell lines, the samples were derived from LH+0 (non receptive) biopsies from COS donor patients and *"in vitro"* cultured, which would potentially differ from the *"in vivo"* physiological LH+0 status.

Analyses of the genes regulated by this miRNA appear to be very interesting for endometrial physiology and were chosen for validation. These three genes were BMP4, which has been associated with decidualization, and is expressed in the endometrial epithelium during pregnancy, and is widely described as a secretion factor essential for the embryonic differentiation to trophoblast lineages (Stoikos *et al*. 2008, Xu *et al*. 2002); H19, an mRNA which is not translated into protein but whose deregulation is usually associated with

endometrial hyperplasia (Tanos *et al.* 2004), implantation failure, and DNA methylation impairment (Korucuoglu *et al.* 2010); and FOS, an essential gene associated with endometrial cell proliferation driven by b-estradiol, and which is reported to be downregulated during the WOI (Fujimoto *et al.* 1995, Kao *et al.* 2002).

The ectopic overexpression of miR-30d in hEECs affects genes that have been associated with cancer and with reproductive and endocrine system disorders. Moreover, β-estradiol receptor, which has been determined as a potential upstream regulator in our analysis, affects estrogen response that modulates endometrial proliferation (Owen 1975), suggesting that miR-30d might modulate the action of β-estradiol. All-trans retinoic acid can also target different genes to modulate local β-estradiol inactivation in the endometrium (Wang *et al.* 2011). Finally, both MYC and FOS gene transcription are rapidly activated by β-estradiol, and its presence stimulates cell cycle progression (Cheng *et al.* 2008). However, it remains to be determined if all these effects are a consequence of mir-30d levels or it causes its changes. The effect of ESR1 is likely to take place at the transcription factor activation level rather than at the production level because the amount of mRNA has not changed. Inactivation of ESR1 would lead to downregulation of BMP4, EGR1, FOS, and H19, as previously reported (Adriaenssens *et al.* 1999, Eger *et al.* 2000, Giacomini *et al.* 2009, Kim *et al.* 2011).

Proteomic analysis identified 2,290 proteins, the majority being cytoplasmic rather than transmembrane proteins, because the cell membrane precipitation fraction was not analyzed. After Rank Product analysis, 108 proteins were differentially expressed between the mimic and scramble conditions. Several upregulated proteins are relevant to endometrial physiology such as: LGALS1, also called galectin-1, a glycoprotein which has been shown to be upregulated during the late-secretory stage in human endometrium, and which seems to be implicated in immune tolerance and embryo implantation (Jeschke *et al.* 2009, Tirado-Gonzalez *et al.* 2013, von Wolff *et al.* 2005); MIF, a pro-inflammatory cytokine which has been observed in pre-decidualised stromal cells and in glandular epithelia (Arcuri *et al.* 2001); DNMT1, which has been reported not to change at the mRNA level throughout the menstrual cycle (Vincent *et al.* 2011); and COX6C, which has been reported to be involved in uterine leiomyoma (Kurose *et al.* 2000). We also found some proteins relevant for the endometrium physiology amongst the

downregulated proteins, including WNT1, which regulates the balance between estrogen-induced proliferation and progesterone-mediated differentiation in the normal endometrium (Wang *et al*. 2009); the aldehyde dehydrogenase enzymes (ALDH2 and ALDH1B1) which have been associated with endometrial cancer (Laniewska-Dunaj *et al*. 2013, Orywal *et al*. 2013), specifically ALDH2, which was the only molecule downregulated at both the protein and mRNA levels, and interestingly, is also a predicted direct target of hsa-miR-30d; PGRMC1, a type of progesterone receptor that is abundant in the endometrium during the proliferative phase and is localized in the nucleus, but whose function remains to be fully explored (Pru and Clark 2013); NOTCH4, a transmembrane receptor that has shown to be downregulated during the change from the proliferative to the secretory phase in normal human endometrium (Cobellis *et al*. 2008); and ITGAV, an essential receptor which mediates cell adhesion and embryo implantation (Erikson *et al*. 2009).

The downregulation of H19 imprinted-gene non-coding RNA and the upregulation of the DNMT1 protein (which is responsible for the maintenance of DNA methylation), may result in epigenetic regulation of the H19 methylation pattern. The global methylation status of a gene can be studied using a MeDIP approach, which enriches the gene of interest in the two conditions (mimic versus scramble). Using this type of analysis we showed that H19 methylation was elevated in mimic-treated hEECs versus scramble controls. Given this interesting result in this well-known hemi-methylated gene, it would be very interesting to analyze CpG sites, which may be enriched or differentially methylated throughout the whole genome, using microarrays or sequencing technologies to assess if miR-30d indirectly affects DNMT1, thus altering the global methylation patterns. Another interesting observation was the downregulation of the nicotinamide N-methyltransferase gene (NNMT) whose expression has been described to reduce the cellular methylation potential. Therefore, given that mimic treatment downregulates this gene, this could increase the global methylation potential (Ulanovskaya *et al*. 2013).

Previous studies has shown that miR-30 family is able to inhibit the epithelial-to-mesenchymal transition by downregulating some genes, such as SNAI1, and increasing expression of others such as e-cadherin (Zhang *et al*. 2012) – a gene that is also activated by

DNMT1 (Espada *et al*. 2011). These observations would explain the maintenance of the epithelial phenotype in primary hEECs *"in vitro"* and in part they could suggest an implication for the developing embryo that shows increased expression of epithelial markers after miR-30d incorporation. Another point that merits further research is that the postulated alterations to the methylation pattern of the ESR1 promoter would lead to a reduced response to estrogens in the endometrium (Shiozawa *et al*. 2002, Vincent *et al*. 2011), what is especially interesting because the downregulation of several genes (FOS, BMP4, H19, and EGR1) indirectly predicts ESR1 inactivation. Further investigation will be required to discover how DNMT1 is indirectly upregulated by hsa-miR-30d (Figure 37).

The miRNA profile obtained across the menstrual cycle strongly demonstrates the potential uses of this subset of miRNAs in the future development of a non-invasive tool for the detection of endometrial receptivity. But since the number of fold-change observed are relatively small, we expect that there are slight global changes in these profiles due to our sample, effecting on the statistical power, and resulting in a low number of statistically differentially expressed miRNAs.

The potential uses of miRNAs as novel biomarkers of endometrial receptivity comes from similar studies of specific profiles in some types of endometrial cancer, preeclampsia and

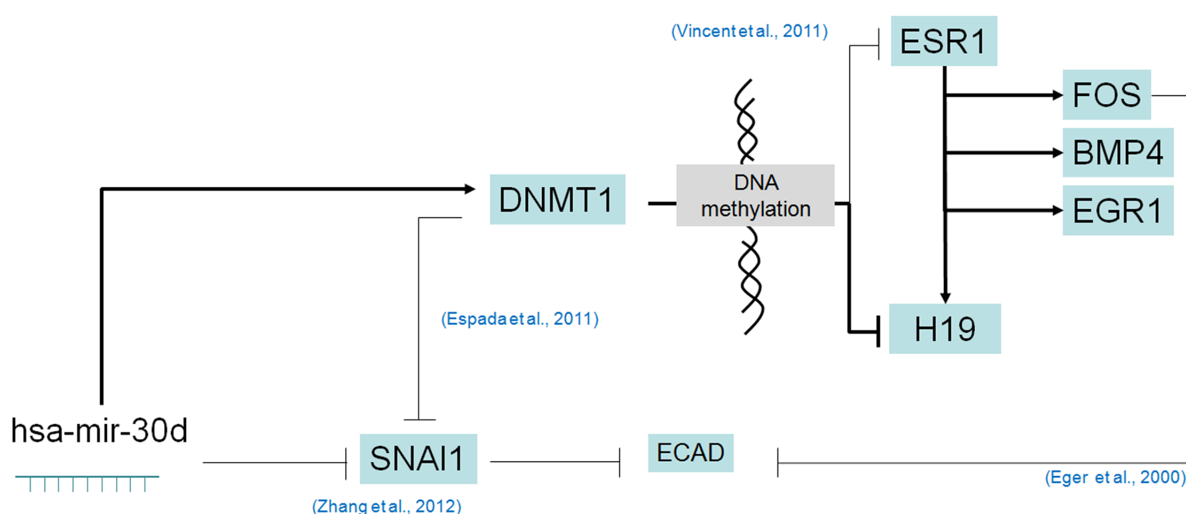


Figure 37. Schematic representation of the working hypothesis relating hsa-miR-30d effects on methylation status and epithelial phenotype of endometrial epithelial cells.

endometriosis (Cohn *et al*. 2010, Hever *et al*. 2007, Hull *et al*. 2008, Ohlsson Teague *et al*. 2009, Pan *et al*. 2007). This novel tool can be based on microarrays similar to the endometrial receptivity array tool (Diaz-Gimeno *et al*. 2011), that consists on an algorithm and a machine learning to improve detection. But alternatives, such as PCR arrays and also the development of RNA sequencing as an affordable platform, would not only determine altered expression of miRNAs and mRNAs, but also identify novel miRNAs never described before. The stability of miRNA in the endometrial fluid and their sensitivity of detection are two very important advantageous points towards considering this type of molecule for development of a non-invasive tool.

One of our goals was also to determine what mechanisms are responsible for releasing these miRNAs in the EF and for protecting them to exert their function once they reach their target cell. As we mentioned before, it has been recently proposed that miRNAs can communicate from cell to cell at very distant parts of the body by using microvesicles (exosomes), lipid carriers such as LDL or HDL, and protein complexes (Arroyo *et al*. 2011, Vickers *et al*. 2011). Since exosomes were not yet described in the endometrial fluids, we tested for the presence of a specific exosome marker (CD63) by immunohistochemistry, and obtained a positive result that was also confirmed by electron microscopy observations of small vesicles in endometrial fluid samples and in endosomes from epithelial cells. Moreover, we were able to confirm by western blots for CD63 marker that *"in vitro"* cultured primary endometrial epithelial cells are actively producing large quantities of exosomes. Not only the pelleted exosomes contained miRNAs, but also we were also able to observe miRNAs in the remaining supernatants of these endometrial fluid and conditioned media samples. These results raise the question of whether not only exosome-associated miRNAs are produced by the endometrial epithelial cells, but also free or another kind of associated soluble forms can also be present in the endometrial fluid.

Exosomes contained not only miRNAs, but also a portion of small RNAs with longer sizes, whether they also contain pre-miRNAs remain to be determined, but some works have observed that exosomes are able to transport mRNAs that cells can incorporate and translate into proteins using their own machinery (Valadi *et al*. 2007). In any case, exosomes are

protecting the RNA from degradation, as we observed after RNAse treatment for 30 minutes in extracted and non-extracted samples of exosomes, in which RNA fractions corresponding to sizes ranging from 200-1000 nts is lost.

The novel concept of intercellular communication between mother and embryo mediated by exosomes has the important caveat that at the moment it only can be studied *"in vitro"*. However, some groups are developing miRNA sensors, such as McManus' Lab at the University of California San Francisco, that it is actually developing a reporter system that would facilitate the observations in animal models by detecting the functional incorporation of exogenous miRNAs quantitatively with fluorimetric detection. However, this system will take years to be developed and will not be available in the near future to be incorporated in our studies to have the *"in vivo"* confirmation.

Fluorescent labeled exosomes derived from primary endometrial epithelial cultures showed ability to be incorporated by murine blastocysts in the hatching zones; confocal microscopy suggested that they were able to reach the trophoblastic cells and not the inner cell mass after 24 hours of co-incubation. These viable hatched embryos with exosomes attached were subjected to scanning electron microscopy, which resulted in images of embryos with small rounded exosomes in direct contact and attached to the trophoblast surface. Several mechanisms could explain the content release from exosome to target cell, although it is mainly considered to occur by endocytosis; however, other hypothesis such as fusion with plasma membrane should be considered.

Remarkably, the murine embryo presented microvilli and what seems to be small pores interspaced along the trophoblastic surface. This could explain how blastocyst also mediates a fast exchange of nutrients and molecules. In this sense, addition of free miRNAs into culture media at different concentrations resulted in incorporation by the embryos at the hatching zone of the trophoblastic cells. Transcriptomic studies of the effects of mimic miR-30d miRNAs versus Scramble miRNA in embryos only showed increase in expression of ten genes, and this slight change could be due to the fact that this miRNA plays a small role on global transcriptome at this stage of embryonic development, or to the fact that miRNA is

incorporated but it is either degraded or has not yet been incorporated in RISC complexes to be functional.

Since transcriptomic changes were found for genes classified as adhesive molecules such as Itgb3, Itga7 and Cdh5, we tested if there were any effects in the adhesive phenotype of embryos treated with Scramble, Mimics or Inhibitors for mir-30d. The results showed significant increase in adhesion in mimic versus inhibitor treated embryos: in particular, inhibitor treatment drops the adhesion significantly, suggesting that while the mir-30d might not potentiate implantation, its presence is necessary. Also, it remains to be elucidated if other family members of this miRNAs, which share the same seed sequence, have compensatory actions.

A scheme of biological mechanisms directing miRNAs communication between mother and embryo is summarized in Figure 38. Endogenous miRNAs are produced in the endometrial epithelial cells and are released into the endometrial lumen in several forms: as shedding vesicles, as protein-lipoprotein associated forms, as free miRNAs, and as exosomes. Several mechanisms of uptaking miRNAs by the trophectoderm can then take place: vesicle fusion, receptors, pores, and endocytosis can mediate their internalization. Finally, the trophectoderm cells incorporate miRNAs in the RISC complex to regulate the proper implantation.

In summary, we have demonstrated that endometrial fluid contains miRNAs, and that they show a specific profile across menstrual cycle. Hsa-miR-30d, which is the most increasing miRNA during the menstrual cycle, has functions associated to the changes in several genes that are important for the endometrial physiology, hormone response and maintenance of the epithelial phenotype through control at the epigenetic level. In addition to that, we also demonstrated that miRNAs are secreted by epithelial cells in free or as exosomes associated forms, and that they are able to reach the hatching embryo and to modify its transcriptome and adhesive phenotype *"in vitro"*.

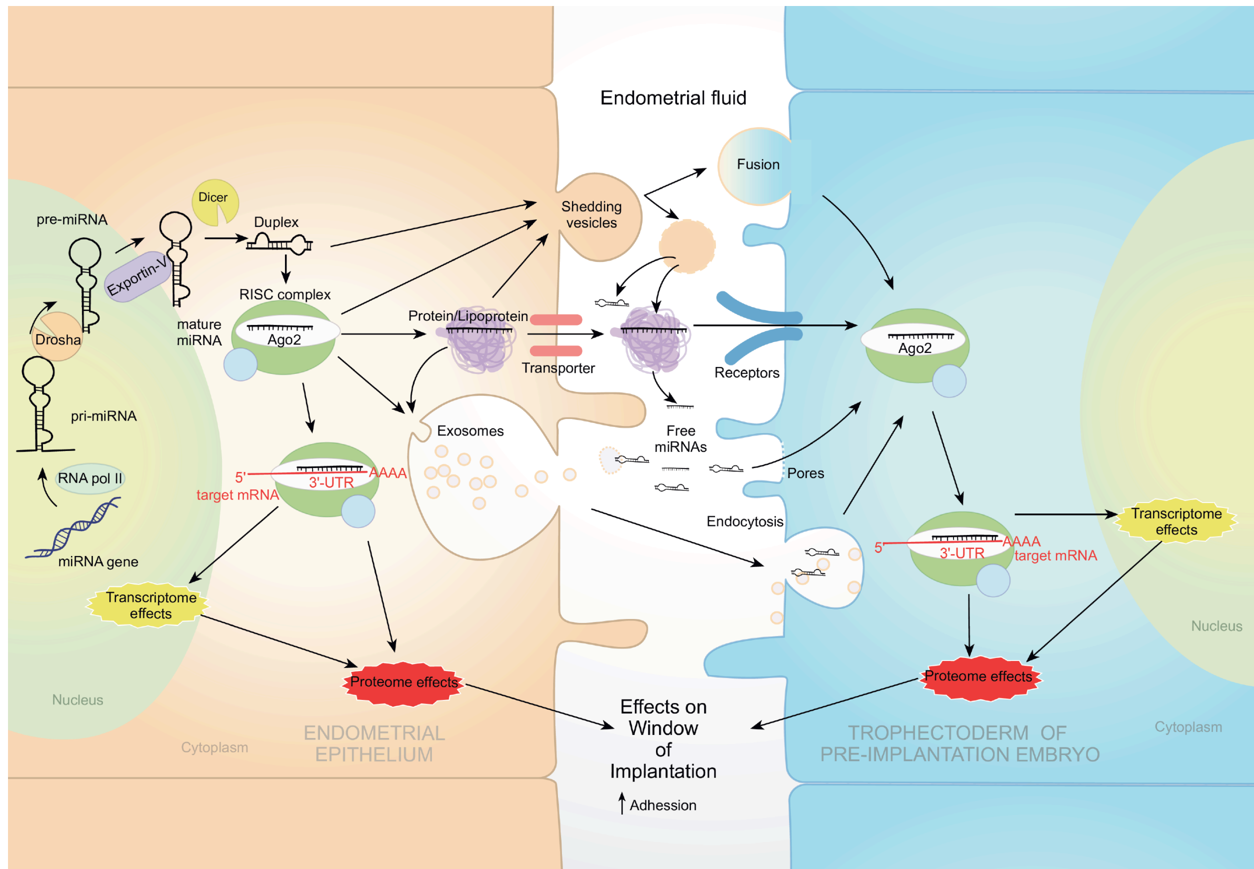


Figure 38. Schematic summary of the novel cross-talk mechanism that involves the delivery of endometrial miRNAs from the maternal endometrium to the endometrial fluid and in turn modify the embryo transcriptome, proteome and its adhesive phenotype

VII. CONCLUSIONS

VII.CONCLUSION

1. MiRNAs are present in the endometrial fluid aspiration across the menstrual cycle during natural cycles.

2. The profile of miRNAs during the window of implantation stage shows important differences compared to the rest of stages of menstrual cycle, supporting its use as a novel biomarker of endometrial receptivity.

3. The ectopic expression of hsa-miR-30d in primary hEEC induces transcriptomic modifications related to the cell cycle, proliferation and endocrine disorders that could influence endometrial receptivity.

4. The estrogen receptor seems to be an upstream regulator associated with the effects of hsa-miR-30d.

5. The ectopic expression of hsa-miR-30d in primary hEEC induces proteomic modifications relevant for the endometrial physiology and epigenetic status.

6. There is an increase in the methylation status of the DMR region of the H19 gene in the mimic mir-30d versus the scramble treated primary epithelial cells.

7. Endometrial epithelial cells secrete exosomes both in the endometrial fluid and in the culture medium *"in vitro"*.

8. Exosomes and free-form miRNAs can be internalized by the trophectoderm of murine blastocyst embryos.

9. Hsa-miR-30d is present in the secretions during endometrial receptivity stage and can modulate the gene expression and adhesiveness phenotype of the hatched, ready-to-implant murine embryos *"in vitro"*.

VIII. REFERENCES

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Adriaenssens E, Lottin S, Dugimont T, Fauquette W, Coll J, Dupouy JP, Boilly B and Curgy JJ. Steroid hormones modulate H19 gene expression in both mammary gland and uterus. *Oncogene* 1999:**18**:4460-4473.

Aghajanova L, Hamilton AE and Giudice LC. Uterine receptivity to human embryonic implantation: histology, biomarkers, and transcriptomics. *Semin Cell Dev Biol* 2008:**19**:204-211.

Altmae S, Martinez-Conejero JA, Esteban FJ, Ruiz-Alonso M, Stavreus-Evers A, Horcajadas JA and Salumets A. MicroRNAs miR-30b, miR-30d, and miR-494 Regulate Human Endometrial Receptivity. *Reprod Sci* 2013:**20**:308-317.

Ambros V. The functions of animal microRNAs. *Nature* 2004:**431**:350-355.

Ambros V and Chen X. The regulation of genes and genomes by small RNAs. *Development* 2007:**134**:1635-1641.

Aplin JD. The cell biological basis of human implantation. *Baillieres Best Pract Res Clin Obstet Gynaecol* 2000:**14**:757-764.

Arcuri F, Ricci C, Ietta F, Cintorino M, Tripodi SA, Cetin I, Garzia E, Schatz F, Klemi P, Santopietro R *et al.* Macrophage migration inhibitory factor in the human endometrium: expression and localization during the menstrual cycle and early pregnancy. *Biol Reprod* 2001:**64**:1200-1205.

Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL *et al.* Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A* 2011:**108**:5003-5008.

Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004:**116**:281-297.

Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004:**116**:281-297.

Baumann CG, Morris DG, Sreenan JM and Leese HJ. The quiet embryo hypothesis: molecular characteristics favoring viability. *Mol Reprod Dev* 2007:**74**:1345-1353.

Bazer FW, Spencer TE, Johnson GA, Burghardt RC and Wu G. Comparative aspects of implantation. *Reproduction* 2009:**138**:195-209.

Berlanga O, Bradshaw HB, Vilella-Mitjana F, Garrido-Gomez T and Simon C. How endometrial secretomics can help in predicting implantation. *Placenta* 2011:**32 Suppl 3**:S271-5.

Boomsma CM, Kavelaars A, Eijkemans MJ, Amarouchi K, Teklenburg G, Gutknecht D, Fauser BJ, Heijnen CJ and Macklon NS. Cytokine profiling in endometrial secretions: a non-invasive window on endometrial receptivity. *Reprod Biomed Online* 2009:**18**:85-94.

Borthwick JM, Charnock-Jones DS, Tom BD, Hull ML, Teirney R, Phillips SC and Smith SK. Determination of the transcript profile of human endometrium. *Mol Hum Reprod* 2003:**9**:19-33.

Braude P, Bolton V and Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 1988:**332**:459-461.

Bridge G, Monteiro R, Henderson S, Emuss V, Lagos D, Georgopoulou D, Patient R and Boshoff C. The microRNA-30 family targets DLL4 to modulate endothelial cell behavior during angiogenesis. *Blood* 2012:**120**:5063-5072.

Bulmer JN and Johnson PM. Immunohistological characterization of the decidual leucocytic infiltrate related to endometrial gland epithelium in early human pregnancy. *Immunology* 1985:**55**:35-44.

Caballero-Campo P, Dominguez F, Coloma J, Meseguer M, Remohi J, Pellicer A and Simon C. Hormonal and embryonic regulation of chemokines IL-8, MCP-1 and RANTES in the human endometrium during the window of implantation. *Mol Hum Reprod* 2002:**8**:375-384.

Carson DD, Lagow E, Thathiah A, Al-Shami R, Farach-Carson MC, Vernon M, Yuan L, Fritz MA and Lessey B. Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. *Mol Hum Reprod* 2002:**8**:871-879.

Castanotto D, Tommasi S, Li M, Li H, Yanow S, Pfeifer GP and Rossi JJ. Short hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in HeLa cells. *Mol Ther* 2005:**12**:179-183.

Castoldi M, Schmidt S, Benes V, Noerholm M, Kulozik AE, Hentze MW and Muckenthaler MU. A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). *RNA* 2006:**12**:913-920.

Cheng YH, Yin P, Xue Q, Yilmaz B, Dawson MI and Bulun SE. Retinoic acid (RA) regulates 17beta-hydroxysteroid dehydrogenase type 2 expression in endometrium: interaction of RA receptors with specificity protein (SP) 1/SP3 for estradiol metabolism. *J Clin Endocrinol Metab* 2008:**93**:1915-1923.

Chou CS, MacCalman CD and Leung PC. Differential effects of gonadotropin-releasing hormone I and II on the urokinase-type plasminogen activator/plasminogen activator inhibitor system in human decidual stromal cells in vitro. *J Clin Endocrinol Metab* 2003:**88**:3806-3815.

Cobellis L, Caprio F, Trabucco E, Mastrogiacomo A, Coppola G, Manente L, Colacurci N, De Falco M and De Luca A. The pattern of expression of Notch protein members in normal and pathological endometrium. *J Anat* 2008:**213**:464-472.

Cohen M, Meisser A and Bischof P. Metalloproteinases and human placental invasiveness. *Placenta* 2006:**27**:783-793.

Cohn DE, Fabbri M, Valeri N, Alder H, Ivanov I, Liu CG, Croce CM and Resnick KE. Comprehensive miRNA profiling of surgically staged endometrial cancer. *Am J Obstet Gynecol* 2010:**202**:656.e1-656.e8.

Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK and Calin GA. MicroRNAs in body fluids--the mix of hormones and biomarkers. *Nat Rev Clin Oncol* 2011:**8**:467-477.

Critchley HO, Robertson KA, Forster T, Henderson TA, Williams AR and Ghazal P. Gene expression profiling of mid to late secretory phase endometrial biopsies from women with menstrual complaint. *Am J Obstet Gynecol* 2006:**195**:406.e1-406.16.

Cruz M, Gadea B, Garrido N, Pedersen KS, Martinez M, Perez-Cano I, Munoz M and Meseguer M. Embryo quality, blastocyst and ongoing pregnancy rates in oocyte donation patients whose embryos were monitored by time-lapse imaging. *J Assist Reprod Genet* 2011:**28**:569-573.

D. Neill J, Plant TM, Pfaff DW, Challis JRG, M. de Kretser D, S. Richards J and M. Wassarman P. **Vascular Morphogenesis in the Female Reproductive System (3rd ed.)**. *ELSEVIER* 2006:.

de Antonellis P, Liguori L, Falanga A, Carotenuto M, Ferrucci V, Andolfo I, Marinaro F, Scognamiglio I, Virgilio A, De Rosa G *et al.* MicroRNA 199b-5p delivery through stable nucleic acid lipid particles (SNALPs) in tumorigenic cell lines. *Naunyn Schmiedebergs Arch Pharmacol* 2013:**386**:287-302.

De los Santos MJ, Mercader A, Frances A, Portoles E, Remohi J, Pellicer A and Simon C. Role of endometrial factors in regulating secretion of components of the immunoreactive human embryonic interleukin-1 system during embryonic development. *Biol Reprod* 1996:**54**:563-574.

DeSouza L, Diehl G, Yang EC, Guo J, Rodrigues MJ, Romaschin AD, Colgan TJ and Siu KW. Proteomic analysis of the proliferative and secretory phases of the human endometrium: protein identification and differential protein expression. *Proteomics* 2005:**5**:270-281.

Diaz-Gimeno P, Horcajadas JA, Martinez-Conejero JA, Esteban FJ, Alama P, Pellicer A and Simon C. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. *Fertil Steril* 2011:**95**:50-60, 60.e1-15.

Diaz-Gimeno P, Horcajadas JA, Martinez-Conejero JA, Esteban FJ, Alama P, Pellicer A and Simon C. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. *Fertil Steril* 2011:**95**:50-60, 60.e1-15.

Diaz-Gimeno P, Ruiz-Alonso M, Blesa D, Bosch N, Martinez-Conejero JA, Alama P, Garrido N, Pellicer A and Simon C. The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. *Fertil Steril* 2012:.

Dickey RP, Olar TT, Curole DN, Taylor SN and Matulich EM. Relationship of first-trimester subchorionic bleeding detected by color Doppler ultrasound to subchorionic fluid, clinical bleeding, and pregnancy outcome. *Obstet Gynecol* 1992:**80**:415-420.

Dockery P, Li TC, Rogers AW, Cooke ID and Lenton EA. The ultrastructure of the glandular epithelium in the timed endometrial biopsy. *Hum Reprod* 1988:**3**:826-834.

Dominguez F, Garrido-Gomez T, Lopez JA, Camafeita E, Quinonero A, Pellicer A and Simon C. Proteomic analysis of the human receptive versus non-receptive endometrium using differential in-gel electrophoresis and MALDI-MS unveils stathmin 1 and annexin A2 as differentially regulated. *Hum Reprod* 2009:**24**:2607-2617.

Doughty MJ, Bergmanson JP and Blocker Y. Shrinkage and distortion of the rabbit corneal endothelial cell mosaic caused by a high osmolality glutaraldehyde-formaldehyde fixative compared to glutaraldehyde. *Tissue Cell* 1997:**29**:533-547.

Durn JH, Marshall KM, Farrar D, O'Donovan P, Scally AJ, Woodward DF and Nicolaou A. Lipidomic analysis reveals prostanoid profiles in human term pregnant myometrium. *Prostaglandins Leukot Essent Fatty Acids* 2010:**82**:21-26.

Eger A, Stockinger A, Schaffhauser B, Beug H and Foisner R. Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta-catenin/lymphoid enhancer binding factor-1 transcriptional activity. *J Cell Biol* 2000:**148**:173-188.

ENCODE Project Consortium, Bernstein BE, Birney E, Dunham I, Green ED, Gunter C and Snyder M. An integrated encyclopedia of DNA elements in the human genome. *Nature* 2012:**489**:57-74.

Erikson DW, Burghardt RC, Bayless KJ and Johnson GA. Secreted phosphoprotein 1 (SPP1, osteopontin) binds to integrin alpha v beta 6 on porcine trophectoderm cells and integrin alpha v beta 3 on uterine luminal epithelial cells, and promotes trophectoderm cell adhesion and migration. *Biol Reprod* 2009:**81**:814-825.

Espada J, Peinado H, Lopez-Serra L, Setien F, Lopez-Serra P, Portela A, Renart J, Carrasco E, Calvo M, Juarranz A *et al.* Regulation of SNAIL1 and E-cadherin function by DNMT1 in a DNA methylation-independent context. *Nucleic Acids Res* 2011:**39**:9194-9205.

Estella C, Herrer I, Atkinson SP, Quinonero A, Martinez S, Pellicer A and Simon C. Inhibition of histone deacetylase activity in human endometrial stromal cells promotes extracellular matrix remodelling and limits embryo invasion. *PLoS One* 2012:**7**:e30508.

Estella C, Herrer I, Moreno-Moya JM, Quinonero A, Martinez S, Pellicer A and Simon C. miRNA signature and Dicer requirement during human endometrial stromal decidualization in vitro. *PLoS One* 2012:**7**:e41080.

Estella C, Herrer I, Moreno-Moya JM, Quinonero A, Martinez S, Pellicer A and Simon C. miRNA signature and Dicer requirement during human endometrial stromal decidualization in vitro. *PLoS One* 2012:**7**:e41080.

Ferraretti AP, Goossens V, de Mouzon J, Bhattacharya S, Castilla JA, Korsak V, Kupka M, Nygren KG, Nyboe Andersen A, European IVF-monitoring (EIM) *et al.* Assisted reproductive technology in Europe, 2008: results generated from European registers by ESHRE. *Hum Reprod* 2012:**27**:2571-2584.

Finn CA and Martin L. The control of implantation. *J Reprod Fertil* 1974:**39**:195-206.

Fujimoto J, Ilori M, Ichigo S, Morishita S and Tamaya T. Estrogen induces the expression of c-fos and c-jun genes in fibroblasts derived from human uterine endometrium. *Exp Clin Endocrinol Diabetes* 1995:**103**:378-385.

Galan A, Herrer R, Remohi J, Pellicer A and Simon C. Embryonic regulation of endometrial epithelial apoptosis during human implantation. *Hum Reprod* 2000:**15 Suppl 6**:74-80.

Galan A, Montaner D, Poo ME, Valbuena D, Ruiz V, Aguilar C, Dopazo J and Simon C. Functional genomics of 5- to 8-cell stage human embryos by blastomere single-cell cDNA analysis. *PLoS One* 2010:**5**:e13615.

Garcia-Velasco JA, Isaza V, Caligara C, Pellicer A, Remohi J and Simon C. Factors that determine discordant outcome from shared oocytes. *Fertil Steril* 2003:**80**:54-60.

Gargett CE and Rogers PA. Human endometrial angiogenesis. *Reproduction* 2001:**121**:181-186.

Garrido-Gomez T, Dominguez F, Lopez JA, Camafeita E, Quinonero A, Martinez-Conejero JA, Pellicer A, Conesa A and Simon C. Modeling human endometrial decidualization from the interaction between proteome and secretome. *J Clin Endocrinol Metab* 2011:**96**:706-716.

Garrido-Gomez T, Dominguez F, Quinonero A, Estella C, Vilella F, Pellicer A and Simon C. Annexin A2 is critical for embryo adhesiveness to the human endometrium by RhoA activation through F-actin regulation. *FASEB J* 2012:**26**:3715-3727.

Gasser R. **ATLAS OF HUMAN EMBRYOS**.

Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T *et al.* Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol* 2008:**26**:317-325.

Giacomini D, Paez-Pereda M, Stalla J, Stalla GK and Arzt E. Molecular interaction of BMP-4, TGF-beta, and estrogens in lactotrophs: impact on the PRL promoter. *Mol Endocrinol* 2009:**23**:1102-1114.

Gilabert-Estelles J, Braza-Boils A, Ramon LA, Zorio E, Medina P, Espana F and Estelles A. Role of microRNAs in gynecological pathology. *Curr Med Chem* 2012:**19**:2406-2413.

Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A and Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 2006:**34**:D140-4.

Guerau-de-Arellano M, Alder H, Ozer HG, Lovett-Racke A and Racke MK. miRNA profiling for biomarker discovery in multiple sclerosis: from microarray to deep sequencing. *J Neuroimmunol* 2012:**248**:32-39.

Guo R, Abdelmohsen K, Morin PJ and Gorospe M. Novel MicroRNA Reporter Uncovers Repression of Let-7 by GSK-3beta. *PLoS One* 2013:**8**:e66330.

Haouzi D, Dechaud H, Assou S, De Vos J and Hamamah S. Insights into human endometrial receptivity from transcriptomic and proteomic data. *Reprod Biomed Online* 2012:**24**:23-34.

Hawkins SM and Matzuk MM. The menstrual cycle: basic biology. *Ann N Y Acad Sci* 2008:**1135**:10-18.

He L and Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 2004:**5**:522-531.

Hever A, Roth RB, Hevezi P, Marin ME, Acosta JA, Acosta H, Rojas J, Herrera R, Grigoriadis D, White E *et al.* Human endometriosis is associated with plasma cells and overexpression of B lymphocyte stimulator. *Proc Natl Acad Sci U S A* 2007:**104**:12451-12456.

Horcajadas JA, Minguez P, Dopazo J, Esteban FJ, Dominguez F, Giudice LC, Pellicer A and Simon C. Controlled ovarian stimulation induces a functional genomic delay of the endometrium with potential clinical implications. *J Clin Endocrinol Metab* 2008:**93**:4500-4510.

Horcajadas JA, Riesewijk A, Martin J, Cervero A, Mosselman S, Pellicer A and Simon C. Global gene expression profiling of human endometrial receptivity. *J Reprod Immunol* 2004:**63**:41-49.

Horcajadas JA, Riesewijk A, Polman J, van Os R, Pellicer A, Mosselman S and Simon C. Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. *Mol Hum Reprod* 2005:**11**:195-205.

Hull ML, Escareno CR, Godsland JM, Doig JR, Johnson CM, Phillips SC, Smith SK, Tavare S, Print CG and Charnock-Jones DS. Endometrial-peritoneal interactions during endometriotic lesion establishment. *Am J Pathol* 2008:**173**:700-715.

Irwin JC, Suen LF, Faessen GH, Popovici RM and Giudice LC. Insulin-like growth factor (IGF)-II inhibition of endometrial stromal cell tissue inhibitor of metalloproteinase-3 and IGF-binding protein-1 suggests paracrine interactions at the decidua:trophoblast interface during human implantation. *J Clin Endocrinol Metab* 2001:**86**:2060-2064.

Jabbour HN, Kelly RW, Fraser HM and Critchley HO. Endocrine regulation of menstruation. *Endocr Rev* 2006:**27**:17-46.

Jackson RJ and Standart N. How do microRNAs regulate gene expression?. *Sci STKE* 2007:**2007**:re1.

Jarver P, Coursindel T, Andaloussi SE, Godfrey C, Wood MJ and Gait MJ. Peptide-mediated Cell and In Vivo Delivery of Antisense Oligonucleotides and siRNA. *Mol Ther Nucleic Acids* 2012:**1**:e27.

Jeschke U, Walzel H, Mylonas I, Papadopoulos P, Shabani N, Kuhn C, Schulze S, Friese K, Karsten U, Anz D *et al.* The human endometrium expresses the glycoprotein mucin-1 and shows positive correlation for Thomsen-Friedenreich epitope expression and galectin-1 binding. *J Histochem Cytochem* 2009:**57**:871-881.

Joglekar MV, Patil D, Joglekar VM, Rao GV, Reddy DN, Mitnala S, Shouche Y and Hardikar AA. The miR-30 family microRNAs confer epithelial phenotype to human pancreatic cells. *Islets* 2009:**1**:137-147.

Kabir-Salmani M, Shiokawa S, Akimoto Y, Sakai K, Sakai K and Iwashita M. Tissue transglutaminase at embryo-maternal interface. *J Clin Endocrinol Metab* 2005:**90**:4694-4702.

Kamijo T, Rajabi MR, Mizunuma H and Ibuki Y. Biochemical evidence for autocrine/paracrine regulation of apoptosis in cultured uterine epithelial cells during mouse embryo implantation in vitro. *Mol Hum Reprod* 1998:**4**:990-998.

Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA and Giudice LC. Global gene profiling in human endometrium during the window of implantation. *Endocrinology* 2002:**143**:2119-2138.

Kim JH, Jeong IY, Lim Y, Lee YH and Shin SY. Estrogen receptor beta stimulates Egr-1 transcription via MEK1/Erk/Elk-1 cascade in C6 glioma cells. *BMB Rep* 2011:**44**:452-457.

Klimenko OV and Shtilman MI. Transfection of Kasumi-1 cells with a new type of polymer carriers loaded with miR-155 and antago-miR-155. *Cancer Gene Ther* 2013:**20**:237-241.

Koch HB, Zhang R, Verdoodt B, Bailey A, Zhang CD, Yates JR,3rd, Menssen A and Hermeking H. Large-scale identification of c-MYC-associated proteins using a combined TAP/MudPIT approach. *Cell Cycle* 2007:**6**:205-217.

Koot YE, Teklenburg G, Salker MS, Brosens JJ and Macklon NS. Molecular aspects of implantation failure. *Biochim Biophys Acta* 2012:**1822**:1943-1950.

Korucuoglu U, Biri AA, Konac E, Alp E, Onen IH, Ilhan MN, Turkyilmaz E, Erdem A, Erdem M and Menevse S. Expression of the imprinted IGF2 and H19 genes in the endometrium of cases with unexplained infertility. *Eur J Obstet Gynecol Reprod Biol* 2010:**149**:77-81.

Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y and Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem* 2010:**285**:17442-17452.

Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N and Pollard JW. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. *Biol Reprod* 2010:**82**:791-801.

Kurose K, Mine N, Doi D, Ota Y, Yoneyama K, Konishi H, Araki T and Emi M. Novel gene fusion of COX6C at 8q22-23 to HMGIC at 12q15 in a uterine leiomyoma. *Genes Chromosomes Cancer* 2000:**27**:303-307.

Lala PK, Chatterjee-Hasrouni S, Kearns M, Montgomery B and Colavincenzo V. Immunobiology of the feto-maternal interface. *Immunol Rev* 1983:**75**:87-116.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W *et al.* Initial sequencing and analysis of the human genome. *Nature* 2001:**409**:860-921.

Laniewska-Dunaj M, Jelski W, Orywal K, Kochanowicz J, Rutkowski R and Szmitkowski M. The activity of class I, II, III and IV of alcohol dehydrogenase (ADH) isoenzymes and aldehyde dehydrogenase (ALDH) in brain cancer. *Neurochem Res* 2013:**38**:1517-1521.

Lee RC, Feinbaum RL and Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 1993:**75**:843-854.

Lessey BA. Endometrial responsiveness to steroid hormones: a moving target. *J Soc Gynecol Investig* 2004:**11**:61-62.

Li J, Donath S, Li Y, Qin D, Prabhakar BS and Li P. miR-30 regulates mitochondrial fission through targeting p53 and the dynamin-related protein-1 pathway. *PLoS Genet* 2010:**6**:e1000795.

Liu X, Gao R, Chen X, Zhang H, Zheng A, Yang D, Ding Y, Wang Y and He J. Possible roles of mmu-miR-141 in the endometrium of mice in early pregnancy following embryo implantation. *PLoS One* 2013:**8**:e67382.

Martin JC, Jasper MJ, Valbuena D, Meseguer M, Remohi J, Pellicer A and Simon C. Increased adhesiveness in cultured endometrial-derived cells is related to the absence of moesin expression. *Biol Reprod* 2000:**63**:1370-1376.

Mathivanan S and Simpson RJ. ExoCarta: A compendium of exosomal proteins and RNA. *Proteomics* 2009:**9**:4997-5000.

Medina I, Carbonell J, Pulido L, Madeira SC, Goetz S, Conesa A, Tarraga J, Pascual-Montano A, Nogales-Cadenas R, Santoyo J *et al.* Babelomics: an integrative platform for the analysis of transcriptomics, proteomics and genomic data with advanced functional profiling. *Nucleic Acids Res* 2010:**38**:W210-3.

Meseguer M, Aplin JD, Caballero-Campo P, O'Connor JE, Martin JC, Remohi J, Pellicer A and Simon C. Human endometrial mucin MUC1 is up-regulated by progesterone and down-regulated in vitro by the human blastocyst. *Biol Reprod* 2001:**64**:590-601.

Meseguer M, Pellicer A and Simon C. MUC1 and endometrial receptivity. *Mol Hum Reprod* 1998:**4**:1089-1098.

Mestdagh P, Feys T, Bernard N, Guenther S, Chen C, Speleman F and Vandesompele J. High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA. *Nucleic Acids Res* 2008:**36**:e143.

Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A *et al.* Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008:**105**:10513-10518.

Moreno-Moya JM, Vilella F and Simon C. MicroRNA: key gene expression regulators. *Fertil Steril* 2013:.

Movassagh M, Choy MK, Goddard M, Bennett MR, Down TA and Foo RS. Differential DNA methylation correlates with differential expression of angiogenic factors in human heart failure. *PLoS One* 2010:**5**:e8564.

Murphy CR and Shaw TJ. Plasma membrane transformation: a common response of uterine epithelial cells during the peri-implantation period. *Cell Biol Int* 1994:**18**:1115-1128.

Ng YH, Rome S, Jalabert A, Forterre A, Singh H, Hincks CL and Salamonsen LA. Endometrial exosomes/microvesicles in the uterine microenvironment: a new paradigm for embryo-endometrial cross talk at implantation. *PLoS One* 2013:**8**:e58502.

Ng YH, Rome S, Jalabert A, Forterre A, Singh H, Hincks CL and Salamonsen LA. Endometrial exosomes/microvesicles in the uterine microenvironment: a new paradigm for embryo-endometrial cross talk at implantation. *PLoS One* 2013:**8**:e58502.

Niakan KK, Han J, Pedersen RA, Simon C and Pera RA. Human pre-implantation embryo development. *Development* 2012:**139**:829-841.

Nikas G. Pinopodes as markers of endometrial receptivity in clinical practice. *Hum Reprod* 1999:**14 Suppl 2**:99-106.

Nilsen TW. Mechanisms of microRNA-mediated gene regulation in animal cells. *Trends Genet* 2007:**23**:243-249.

Noyes RW, Hertig AT and Rock J. Dating the endometrial biopsy. *Am J Obstet Gynecol* 1975:**122**:262-263.

Ohlsson Teague EM, Van der Hoek KH, Van der Hoek MB, Perry N, Wagaarachchi P, Robertson SA, Print CG and Hull LM. MicroRNA-regulated pathways associated with endometriosis. *Mol Endocrinol* 2009:**23**:265-275.

Orywal K, Jelski W, Zdrodowski M and Szmitkowski M. The diagnostic value of alcohol dehydrogenase isoenzymes and aldehyde dehydrogenase measurement in the sera of patients with endometrial cancer. *Anticancer Res* 2013:**33**:3725-3730.

Owen JA,Jr. Physiology of the menstrual cycle. *Am J Clin Nutr* 1975:**28**:333-338.

Ozcan S. MiR-30 family and EMT in human fetal pancreatic islets. *Islets* 2009:**1**:283-285.

Pan Q, Luo X, Toloubeydokhti T and Chegini N. The expression profile of micro-RNA in endometrium and endometriosis and the influence of ovarian steroids on their expression. *Mol Hum Reprod* 2007:**13**:797-806.

Pan W, Zhong Y, Cheng C, Liu B, Wang L, Li A, Xiong L and Liu S. MiR-30-regulated autophagy mediates angiotensin II-induced myocardial hypertrophy. *PLoS One* 2013:**8**:e53950.

Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P *et al.* Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 2000:**408**:86-89.

Potireddy S, Vassena R, Patel BG and Latham KE. Analysis of polysomal mRNA populations of mouse oocytes and zygotes: dynamic changes in maternal mRNA utilization and function. *Dev Biol* 2006:**298**:155-166.

Pru JK and Clark NC. PGRMC1 and PGRMC2 in uterine physiology and disease. *Front Neurosci* 2013:**7**:168.

Quinn CE and Casper RF. Pinopodes: a questionable role in endometrial receptivity. *Hum Reprod Update* 2009:**15**:229-236.

Remohi J, Ardiles G, Garcia-Velasco JA, Gaitan P, Simon C and Pellicer A. Endometrial thickness and serum oestradiol concentrations as predictors of outcome in oocyte donation. *Hum Reprod* 1997:**12**:2271-2276.

Riesewijk A, Martin J, van Os R, Horcajadas JA, Polman J, Pellicer A, Mosselman S and Simon C. Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. *Mol Hum Reprod* 2003:**9**:253-264.

Rothberg JM and Leamon JH. The development and impact of 454 sequencing. *Nat Biotechnol* 2008:**26**:1117-1124.

Ruiz-Alonso M, Blesa D and Simon C. The genomics of the human endometrium. *Biochim Biophys Acta* 2012:**1822**:1931-1942.

Ruiz-Alonso M, Galindo N, Pellicer A and Simon C. What a difference two days make: "personalized" embryo transfer (pET) paradigm: A case report and pilot study. *Hum Reprod* 2014:**29**:1244-1247.

Salamonsen LA, Edgell T, Rombauts LJ, Stephens AN, Robertson DM, Rainczuk A, Nie G and Hannan NJ. Proteomics of the human endometrium and uterine fluid: a pathway to biomarker discovery. *Fertil Steril* 2013:**99**:1086-1092.

Sha AG, Liu JL, Jiang XM, Ren JZ, Ma CH, Lei W, Su RW and Yang ZM. Genome-wide identification of micro-ribonucleic acids associated with human endometrial receptivity in natural and stimulated cycles by deep sequencing. *Fertil Steril* 2011:**96**:150-155.e5.

Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, Mortensen P, Shevchenko A, Boucherie H and Mann M. Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. *Proc Natl Acad Sci U S A* 1996:**93**:14440-14445.

Shimoyama Y, Takeda H, Yoshihara S, Kitajima M and Hirohashi S. Biochemical characterization and functional analysis of two type II classic cadherins, cadherin-6 and -14, and comparison with E-cadherin. *J Biol Chem* 1999:**274**:11987-11994.

Shiozawa T, Itoh K, Horiuchi A, Konishi I, Fujii S and Nikaido T. Down-regulation of estrogen receptor by the methylation of the estrogen receptor gene in endometrial carcinoma. *Anticancer Res* 2002:**22**:139-143.

Simon C, Gimeno MJ, Mercader A, O'Connor JE, Remohi J, Polan ML and Pellicer A. Embryonic regulation of integrins beta 3, alpha 4, and alpha 1 in human endometrial epithelial cells in vitro. *J Clin Endocrinol Metab* 1997:**82**:2607-2616.

Simon C, Mercader A, Frances A, Gimeno MJ, Polan ML, Remohi J and Pellicer A. Hormonal regulation of serum and endometrial IL-1 alpha, IL-1 beta and IL-1ra: IL-1 endometrial microenvironment of the human embryo at the apposition phase under physiological and supraphysiological steroid level conditions. *J Reprod Immunol* 1996:**31**:165-184.

Simon C, Moreno C, Remohi J and Pellicer A. Molecular interactions between embryo and uterus in the adhesion phase of human implantation. *Hum Reprod* 1998:**13 Suppl 3**:219-32; discussion 233-6.

Sledz CA, Holko M, de Veer MJ, Silverman RH and Williams BR. Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 2003:**5**:834-839.

Small EM and Olson EN. Pervasive roles of microRNAs in cardiovascular biology. *Nature* 2011:**469**:336-342.

Song PP, Hu Y, Liu CM, Yan MJ, Song G, Cui Y, Xia HF and Ma X. Embryonic ectoderm development protein is regulated by microRNAs in human neural tube defects. *Am J Obstet Gynecol* 2011:**204**:544.e9-544.17.

Steptoe PC and Edwards RG. Birth after the reimplantation of a human embryo. *Lancet* 1978:**2**:366.

Stoikos CJ, Harrison CA, Salamonsen LA and Dimitriadis E. A distinct cohort of the TGFbeta superfamily members expressed in human endometrium regulate decidualization. *Hum Reprod* 2008:**23**:1447-1456.

Strickland S, Reich E and Sherman MI. Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. *Cell* 1976:**9**:231-240.

Symmans WF, Fiterman DJ, Anderson SK, Ayers M, Rouzier R, Dunmire V, Stec J, Valero V, Sneige N, Albarracin C *et al.* A single-gene biomarker identifies breast cancers associated with immature cell type and short duration of prior breastfeeding. *Endocr Relat Cancer* 2005:**12**:1059-1069.

Tanos V, Ariel I, Prus D, De-Groot N and Hochberg A. H19 and IGF2 gene expression in human normal, hyperplastic, and malignant endometrium. *Int J Gynecol Cancer* 2004:**14**:521-525.

Tice DA, Szeto W, Soloviev I, Rubinfeld B, Fong SE, Dugger DL, Winer J, Williams PM, Wieand D, Smith V *et al.* Synergistic induction of tumor antigens by Wnt-1 signaling and retinoic acid revealed by gene expression profiling. *J Biol Chem* 2002:**277**:14329-14335.

Tirado-Gonzalez I, Freitag N, Barrientos G, Shaikly V, Nagaeva O, Strand M, Kjellberg L, Klapp BF, Mincheva-Nilsson L, Cohen M *et al.* Galectin-1 influences trophoblast immune evasion and emerges as a predictive factor for the outcome of pregnancy. *Mol Hum Reprod* 2013:**19**:43-53.

Turchinovich A, Weiz L, Langheinz A and Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res* 2011:**39**:7223-7233.

Ulanovskaya OA, Zuhl AM and Cravatt BF. NNMT promotes epigenetic remodeling in cancer by creating a metabolic methylation sink. *Nat Chem Biol* 2013:**9**:300-306.

Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ and Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007:**9**:654-659.

van der Gaast MH, Beier-Hellwig K, Fauser BC, Beier HM and Macklon NS. Endometrial secretion aspiration prior to embryo transfer does not reduce implantation rates. *Reprod Biomed Online* 2003:**7**:105-109.

Verberg MF, Macklon NS, Heijnen EM and Fauser BC. ART: iatrogenic multiple pregnancy?. *Best Pract Res Clin Obstet Gynaecol* 2007:**21**:129-143.

Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD and Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol* 2011:**13**:423-433.

Vilella F, Ramirez L, Berlanga O, Martinez S, Alama P, Meseguer M, Pellicer A and Simon C. PGE2 and PGF2alpha Concentrations in Human Endometrial Fluid as Biomarkers for Embryonic Implantation. *J Clin Endocrinol Metab* 2013:**98**:4123-4132.

Vincent ZL, Farquhar CM, Mitchell MD and Ponnampalam AP. Expression and regulation of DNA methyltransferases in human endometrium. *Fertil Steril* 2011:**95**:1522-5.e1.

von Wolff M, Wang X, Gabius HJ and Strowitzki T. Galectin fingerprinting in human endometrium and decidua during the menstrual cycle and in early gestation. *Mol Hum Reprod* 2005:**11**:189-194.

Wahlstrom T and Seppala M. Placental protein 12 (PP12) is induced in the endometrium by progesterone. *Fertil Steril* 1984:**41**:781-784.

Wang C, Mayer JA, Mazumdar A, Fertuck K, Kim H, Brown M and Brown PH. Estrogen induces c-myc gene expression via an upstream enhancer activated by the estrogen receptor and the AP-1 transcription factor. *Mol Endocrinol* 2011:**25**:1527-1538.

Wang WT, Zhao YN, Han BW, Hong SJ and Chen YQ. Circulating MicroRNAs Identified in a Genome-Wide Serum MicroRNA Expression Analysis as Noninvasive Biomarkers for Endometriosis. *J Clin Endocrinol Metab* 2013:**98**:281-289.

Wang Y, Hanifi-Moghaddam P, Hanekamp EE, Kloosterboer HJ, Franken P, Veldscholte J, van Doorn HC, Ewing PC, Kim JJ, Grootegoed JA *et al.* Progesterone inhibition of Wnt/beta-catenin signaling in normal endometrium and endometrial cancer. *Clin Cancer Res* 2009:**15**:5784-5793.

Wightman B, Ha I and Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* 1993:**75**:855-862.

Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM and Reijo Pera RA. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* 2010:**28**:1115-1121.

Wright VC, Chang J, Jeng G, Macaluso M and Centers for Disease Control and Prevention (CDC). Assisted reproductive technology surveillance--United States, 2005. *MMWR Surveill Summ* 2008:**57**:1-23.

Wu T, Zhou H, Hong Y, Li J, Jiang X and Huang H. miR-30 family members negatively regulate osteoblast differentiation. *J Biol Chem* 2012:**287**:7503-7511.

Wu WW, Wang G, Baek SJ and Shen RF. Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel- or LC-MALDI TOF/TOF. *J Proteome Res* 2006:**5**:651-658.

Xia HF, Jin XH, Song PP, Cui Y, Liu CM and Ma X. Temporal and spatial regulation of miR-320 in the uterus during embryo implantation in the rat. *Int J Mol Sci* 2010:**11**:719-730.

Xu RH, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP and Thomson JA. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 2002:**20**:1261-1264.

Yang Q, Lu J, Wang S, Li H, Ge Q and Lu Z. Application of next-generation sequencing technology to profile the circulating microRNAs in the serum of preeclampsia versus normal pregnant women. *Clin Chim Acta* 2011:**412**:2167-2173.

Yoshizawa JM and Wong DT. Salivary MicroRNAs and Oral Cancer Detection. *Methods Mol Biol* 2013:**936**:313-324.

Zhang B, Pan X, Cobb GP and Anderson TA. microRNAs as oncogenes and tumor suppressors. *Dev Biol* 2007:**302**:1-12.

Zhang J, Zhang H, Liu J, Tu X, Zang Y, Zhu J, Chen J, Dong L and Zhang J. miR-30 inhibits TGF-beta1-induced epithelial-to-mesenchymal transition in hepatocyte by targeting Snail1. *Biochem Biophys Res Commun* 2012:**417**:1100-1105.

Zhao X, Mohan R, Ozcan S and Tang X. MicroRNA-30d induces insulin transcription factor MafA and insulin production by targeting mitogen-activated protein 4 kinase 4 (MAP4K4) in pancreatic beta-cells. *J Biol Chem* 2012:**287**:31155-31164.

Zhao Z, Zhao Q, Warrick J, Lockwood CM, Woodworth A, Moley KH and Gronowski AM. Circulating microRNA miR-323-3p as a biomarker of ectopic pregnancy. *Clin Chem* 2012:**58**:896-905.

Zhu H and Fan GC. Extracellular/circulating microRNAs and their potential role in cardiovascular disease. *Am J Cardiovasc Dis* 2011:**1**:138-149.

Zygmunt M, Wienhard J, Boving B, Munstedt K, Braems G, Bohle RM and Lang U. Expression of cell adhesion molecules in the extravillous trophoblast in placentas of preterm pregnancies and in placentas at term. *Zentralbl Gynakol* 1998:**120**:488-492.

IX. ANNEXES

1. Informed written consent model

Título del Proyecto titulado: Análisis de miRNAs en fluido endometrial como diagnóstico no invasivo de receptividad endometrial.
Investigador principal: Dr. Felipe Vilella Mitjana
Servicio: xxxxxxxxxxxxxxxxxxxxxx

Yo, ____ he sido informado por el Dr. _________________, colaborador del proyecto de investigación arriba mencionado, y declaro que:

- He leído la Hoja de Información que se me ha entregado
- He podido hacer preguntas sobre el estudio
- He recibido respuestas satisfactorias a mis preguntas
- He recibido suficiente información sobre el estudio

Comprendo que mi participación es voluntaria
Comprendo que todos mis datos serán tratados confidencialmente
Comprendo que puedo retirarme del estudio:
- Cuando quiera
- Sin tener que dar explicaciones
- Sin que esto repercuta en mis cuidados médicos

Autorizo a que las muestras obtenidas durante el proyecto de investigación sean utilizadas con fines científicos en otros proyectos de investigación que tengan por objeto el estudio de mi enfermedad y que hayan sido aprobados por el Comité de Ética de Investigación Clínica del Hospital Clínico Universitario de Valencia

Sí

No

Quiero que se me pida autorización previa para utilizar mis muestras biológicas para futuros proyectos de investigación

Sí

No

Con esto doy mi conformidad para participar en este estudio,

Firma del paciente

Fecha:

Firma del Investigador:

Fecha

AUTORIZACION DEL JEFE DE SERVICIO

D.
Como Jefe del Servicio de

Declaro:

Que conozco cuanta documentación da base al trabajo de proyecto que lleva por título Análisis de miRNAs en fluido endometrial como diagnóstico no invasivo de receptividad endometrial.

Y cuyo investigador principal será el Dr. Felipe Vilella Mitjana

Que el investigador principal, así como el resto del equipo, reúne las características de competencia necesarias para realizar proyectos así como la metodología específica del proyecto de referencia.

Que autorizo la realización de este trabajo en el Servicio /Unidad que dirijo.

En Valencia a 2 de Abril de 2012

Dr.

Jefe del Servicio de