Use of Mesenchymal Stem Cells and Endometrial Co-cultures in 2D and 3D Implantation Models Constructed on Polymer-based Carriers

by

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This is to certify that I have examined this copy of a master's thesis by

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ABSTRACT

Embryo implantation is an essential and critical phenomenon in natural and assisted human reproduction. Implantation involves bi-directional series of complex cellular and molecular events that happen in a specific time interval, called as a window of implantation (WOI). The process involves cross-talking mechanisms between maternal and embryonic tissues including cytokines, chemokines, growth factors, hormones, and various matrix-degrading proteases.

Unsuccessful implantation is a major limitation to ART with likely etiologies linked to both poor embryo development and poor uterine receptivity. Understanding the molecular mechanisms of human embryo implantation helps in improving pregnancy rates, management of infertility issues and helps the regulation of fertility by novel methods. Despite new findings in Assisted Reproductive Technologies (ART), embryo implantation rate is still between 40-60%. *In vitro* studies have revealed that implantation models are not effective due to the complex mechanism of implantation process which happens in the three-dimensional tissue architecture.

As a first step in a large-scale project, it is aimed to bring a new approach for *in vitro* three-dimensional (3D) embryo culture and to create new material to be used during embryo transfer. Regarding the ultimate purpose; it was aimed to generate three-dimensional culture configurations with a polymeric film to be used for *in vitro* implantation modelling. It was hypothesized that polymeric film can greatly assist the implantation of blastocyst when coated with appropriate cell and extracellular matrix elements.

The specific aims of the thesis are to explore the role of endometrial and mesenchymal stem cells in implantation and viability of the embryo and, to compare *in vitro* three-dimensional co-culture models constructed with the biodegradable film. Related to the purpose of this thesis; a variety of two and three-dimensional culture models have been constructed to investigate the effects of these models on the implantation of the mouse embryo. The experimental settings have included routine *in vitro* fertilization cultures used worldwide, two-dimensional film cultures of the blastocyst on polymeric film, and three-dimensional cultures of blastocysts with endometrial cell co-cultures or human mesenchymal stem cells. The cultures of endometrial or mesenchymal stem cell on film structures without the blastocysts have also been constructed. The blastocysts have been cultured in all circumstances to demonstrate the cellular, molecular and morphological parameters related to implantation. Three-dimensional endometrial co-cultures on polymeric films have been found to be the most effective to support implantation related mechanisms.

Keywords:

Implantation, 3D implantation models, mesenchymal stem cell, endometrial co-culture, polymeric carrier

ÖZET

Embriyo implantasyonu, doğal siklusta ve üremeye yardımcı tedavilerde göz önünde bulundurulan kritik bir olgudur. İmplantasyon, birçok karmaşık hücresel ve moleküler etkileşimleri içerir ve implantasyon penceresi olarak adlandırılan belirli zaman aralığında (WOI) gerçekleşir. Sitokinler, kemokinler, büyüme faktörleri, hormonlar ve çeşitli matriks proteazları dahil olmak üzere maternal ve embriyonik dokular arasındaki çapraz konuşma mekanizmasını içerir.

Başarısız implantasyon, hem zayıf embriyo gelişimi hem de kötü uterus alıcılığı (reseptivitesi) ile ilişkili olmakla beraber üremeye yardımcı teknikler (ÜYTE) için önemli bir sınırlamadır. İnsan embriyo implantasyonunun moleküler mekanizmalarını anlamak, gebelik oranlarını iyileştirmeye, infertilite sorunlarının yönetimine ve yeni yöntemlerle doğurganlığın düzenlenmesinde yardımcı olur. Üremeye yardımcı teknikler deki yeni bulgulara rağmen, blastokistin transferi ile beraber embriyo implantasyon oranı hala %40 ile %60 arasındadır. Şimdiye kadar yapılan *in vitro* çalışmalarda, implantasyon sürecinin karmaşık ve üç boyutlu mimaride meydana gelmesi nedeniyle implantasyon mekanizması etkin şekilde taklit edilememiştir.

Bu çalışmada, *in vitro* üç boyutlu (3D) embriyo kültürüne yeni bir yaklaşım getirilmesi ve embriyo transferi sırasında kullanılacak yeni bir materyal yaratılması amaçlanmaktadır. Nihai amaç ile ilgili olarak; *in vitro* implantasyon modellemesi için kullanılacak polimerik film ile üç boyutlu kültür konfigürasyonlarının oluşturulması amaçlandı. Polimerik filmin, uygun hücre ve hücre dışı matris elemanları ile kaplandığında blastosistin implantasyonunu desteklediği varsayılmıştır. Bu tezin amacı, uterus reseptivitesi ve embriyo implantasyon sürecini etkileyen, endometriumu taklit etmek için kullanılan çeşitli implantasyon modellerinin kavramını genişletmektir. Tezin spesifik amaçları, insan embryo implantasyonu sırasında endometriyal ve mezenkimal kök hücrelerin rolünü araştırmak ve biyo-bozunur filmle yapılan *in vitro* üç boyutlu (3D) ko-kültür modellerini karşılaştırmaktır. Bu tezin amacı ile ilgili olarak; implantasyon üzerindeki etkilerini araştırmak için çeşitli iki ve üç boyutlu kültür modelleri oluşturulmuştur. Deney düzenekleri, dünya çapında kullanılan rutin *in vitro* fertilizasyon kültürü, film üzerinde iki boyutlu blastokist kültürü ve jel bazlı hücre dışı matris bileşenleri veya biyolojik olarak parçalanabilen polimerik hücre taşıyıcıları ile yapılan üç boyutlu kültürleri kapsamaktadır. Fare blastokistleri, implantasyonla ilgili hücresel, moleküler ve morfolojik parametreleri göstermek için kültür ortamlarında kullanılmıştır. Üç boyutlu endometriyal ko-kültürün implantasyonla ilgili mekanizmaları desteklemede daha etkili olduğu bulunmuştur.

Anahtar Kelimeler:

İmplantasyon, 3D implantasyon modeli, mezenkimal kök hücre, endometriyal (epitel /stroma) hücreler, polimerik taşıyıcı

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NOMENCLATURE

ART	Assisted Reproductive Technology
BMP2	Bone Morphogenetic Protein 2
BSA	Bovine Serum Albumin
C-AMPs	Cyclic Adenosine Monophosphates
CAMs	Cell Adhesion Molecules
COUP-TF2	Chicken Ovalbumin Upstream Promoter Transcription Factor 2
DLS	Digital Light Sheet
ECC	Endometrial Co-culture
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ESHRE	European Society of Human Reproduction
EVT	Extravillous Trophoblast
ET	Embryo Transfer
FBS	Fetal Bovine Serum
FSH	Follicular Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
HAND2	Heart and Neural Crest Derivatives-Expressed Protein 2
HE	Hematoxylin Eosin
HB-EGF	Heparin Binding-Epidermal Growth Factor
HBSS	Hanks Balanced Salt Solution
hCG	Human Chorionic Gonadotropin
Hox	Homeobox Genes
ICM	Inner Cell Mass
IGF	Insulin-Like Growth Factor
IF	Immunofluorescent Staining
IL	Interleukin
IVM	*In vitro* Maturation
IVF	*In vitro* Fertilization
KGF	Keratinocyte Growth Factor
LH	Luteinizing Hormone

LIF	Leukemia Inhibitory Factor
MAPK	Mitogen-Activated Protein Kinase
MMP	Matrix Metalloproteinases
MSC	Mesenchymal Stem/Stromal Cells
HUCMSC	Human Umbilical Cord Mesenchymal Stem Cell
MUC	Mucin
OPN	Osteopontin
PBS	Phosphate Buffered Saline
PCOS	Polycystic Ovary Syndrome
PFA	Paraformaldehyde
PGA	Polyglycolic Acid
PGD	Preimplantation Genetic Diagnosis
PGE2	Prostaglandin E2
PGs	Prostaglandins
P(L-D, L)LA	Poly(lactic-*co*-glycolic acid)
PN	Pronucleus
PR	Progesterone Receptor
RIF	Recurrent Implantation Failure
RIPA	Radio-Immunoprecipitation Assay
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
STED	Stimulated Emission Depletion Microscopy
TGF-β	Transforming Growth Factor-β
TIMP	Tissue Inhibitors of Metalloproteinases
uNK	Uterine Natural Killer Cells
WB	Western Blot
WHO	World Health Organization
WOI	Window of Implantation
2-D	Two Dimensional
3-D	Three Dimensional
i.u.	International Unit

i.p.	Intraperitoneal Injection

To my dear and loving grandmother,

Because I will never forget you.

Chapter 1

REVIEW OF LITERATURE

1. UTERUS

In female, the internal organs of the reproductive system are a pair of ovaries, a pair of uterine tubes, one vagina and one uterus. The uterus has cervix, fundus and body parts, and it has three layers from inner to outermost; endometrium, myometrium, and perimetrium. The endometrium is the layer involved in implantation (Figure 1). [1]

1.1 Endometrium

The endometrium is the inner most layer of the uterus. The endometrial epithelium is composed of simple columnar ciliated epithelium and it lines the uterine cavity. In lamina propria (connective tissue beneath the epithelial cells) there are simple branched tubular glands. Endometrium, under the effect of hypophyseal hormones, shows cyclic changes during the menstrual cycle, where functional layer is shed off and basal layer remains to regenerate functional layer. If fertilization occurs and zygote develops into a blastocyst, it would appose/adhere to the endometrium, a phenomenon that will be completed by invasion. This whole process, named as implantation, causes the formation of decidua, the endometrium of pregnant state.[2] Stromal cells, glandular and luminal epithelial cells, endothelial cells, immune cells, and stem cells are the main type of cells found in the endometrium.

1.1.1 Epithelial Cells

The endometrial cells either line the luminal surface (luminal epithelium) or compose the glandular structures (glandular epithelium). Progesterone causes an alteration in especially the glandular cells to prepare the cells for the implantation process. The blastocyst can only interact with the endometrium at a certain time interval, called as window of implantation (WOI).[3] Glycocalyx molecules determines the epithelial cells characteristics to allow these cells to be competent with the invading blastocysts. Luminal epithelial cells act in interaction with the blastocyst and prevents the presence of infectious agents.[3]

1.1.2 Immune And Stromal Cells

Immune system related cells are also found in the endometrial compartment. Mother-fetus are in a kind of interactive and symbiotic relationship. During first three months of pregnancy immune regulation is enhance the interaction of trophoblastic cells and maternal cells without any sort of rejection. In the following months immune regulation is needed for maintenance of the anti-

inflammatory conditions and changes in the cervix. The macrophages, T-lymphocytes, natural killer cells (NK) cells and neutrophils are the immune cells of the endometrium[4] Immune cells are wandering cells and their number changes related to the phase of cycle. [5] The endometrium has also been reported to have a stem cell fraction, usually referred as MSCs; epithelial stem cells and side population cells have also been reported. [6-8]

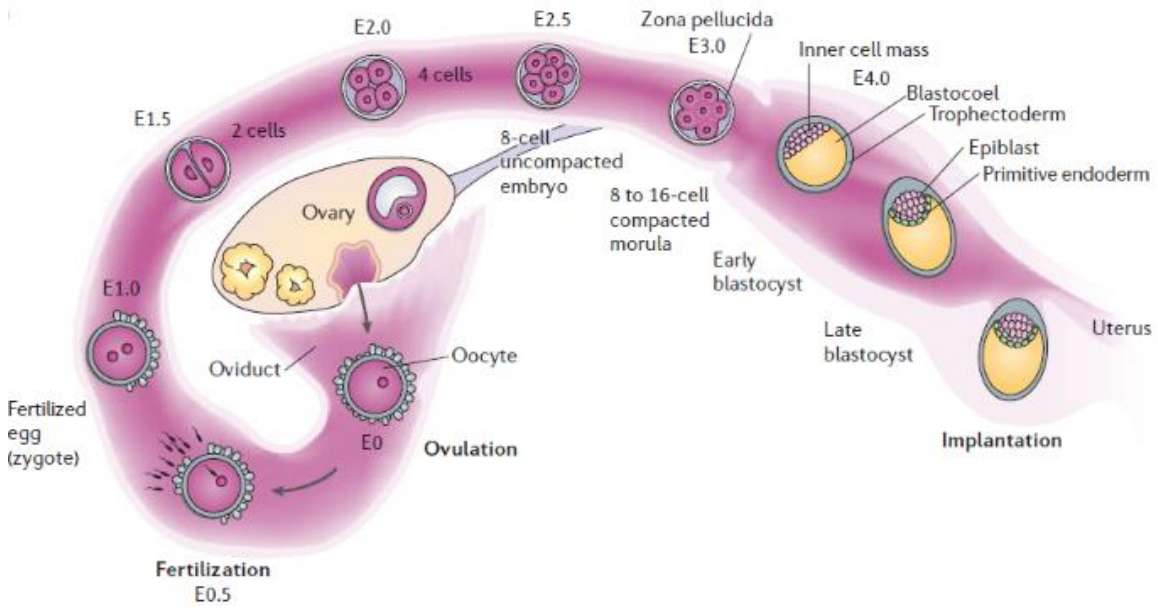


Figure 1. ***Preimplantation embryo development and implantation in mice.***[11]

2. EMBRYO IMPLANTATION

Implantation can occur when three steps are achieved successfully: apposition, adhesion, and invasion. The endometrium should be in the receptive state and the blastocyst should be competent for implantation to take place. This time interval at which implantation occurs is called as "window of implantation" period.[9] During this process, trophoblastic cells need to come in contact with the maternal luminal epithelial cells. Implantation is a very complicated process that requires intermingling interactions of extracellular matrix molecules, adhesion molecules, cytokines, etc. Endometrial cells show pinopod structures on their surface, polarity differs, cells get flat with a decreased number of apical surface microvilli.[10] The blastocysts hatch from its zona pellucida at trophoblastic cells over the inner cell mass area and attach to luminal epithelium under the effect of soluble and signaling molecules. These molecules are outlined in Figure 2.

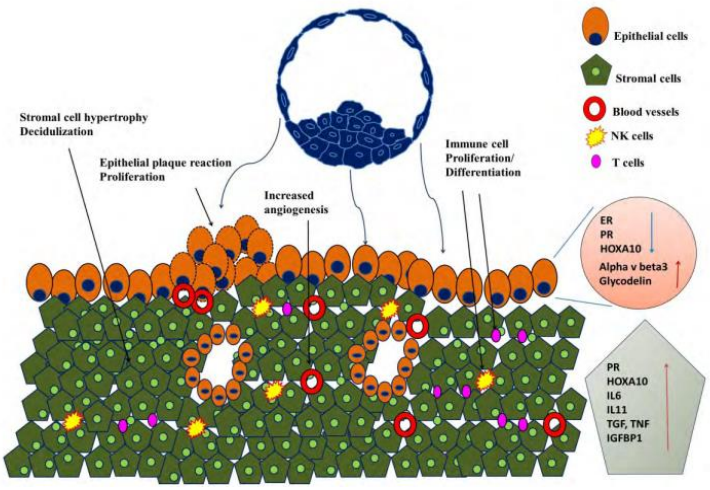


Figure 2. ***Molecular signaling during the embryo implantation.***[12]

Invasion (penetration) takes place between the surface epithelial cells and the extensions of the syncytiotrophoblasts. The trophoblasts themselves are inserted between the epithelial cells and then penetrate towards the basement membrane under the surface epithelium. During the invasion, activating and inhibiting growth factors, cytokines, and enzymes plays a crucial role.[13]

2.1 Blastocyst Activation

For successful implantation, receptive endometrium, active blastocyst and synchronous cross-talk between them are necessary. The condition of the blastocyst is important to fulfill the complicated nature of signaling events during implantation.[14] Dormant blastocysts considered to be non-active, as the mitotic divisions are decreased and less frequent, Golgi apparatus and endoplasmic reticulum are more evident compared to an active blastocyst.[14, 18] The activated blastocyst, on the other hand, has the all the energy pathways ready along with transcriptional and translational pathways, a series of events all of which are also induced by estrogen. [16 ,17]

2.2 Molecular Interactions

Competent blastocyst and receptive endometrium [19] and the interaction among them takes place only in a very distinct time interval; named as the implantation window.[20] Implantation takes place at the end of the luteal phase in the humans and diestrous phase in the mice as depicted in Figure 3.[21]

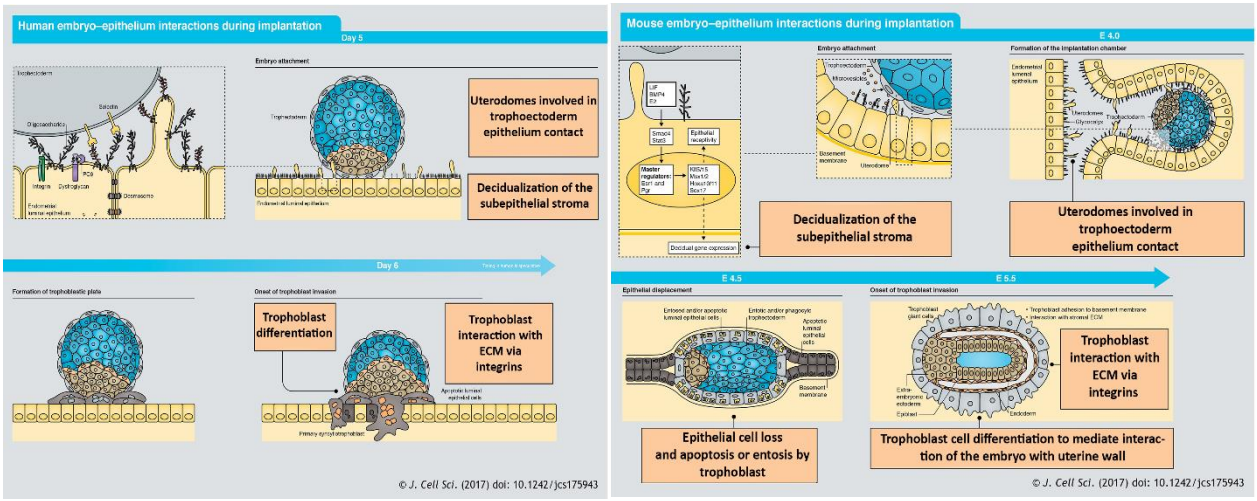


Figure 3. ***Implantation mechanism in mice and humans.***[22]

Although the main purpose is the proper invasion of the embryo to the wall of a uterus, the implantation procedure differs between the species. Different than humans, implantation chamber is formed around the attached blastocysts in rodents [23, 24] Since the blastocysts need a space for the invading trophoblast cells, apoptosis is seen under the implantation area. [25, 26]

In humans, the epithelial lining of the uterus contains microvillus and ciliated cells.[27] In rodents, the decidualization requires preexistence of the embryo and progesterone hormone in the uterine environment. On the other hand, in humans decidualization is controlled by spatiotemporal secretions of the hormones.[28]

2. 3 Decidualization

Decidualization is characterized by the growth of endometrial stromal cells in an epithelioid character. Cells accumulate large amounts of glycogen, and lipid droplets. The cells eventually become round, closely apposed and multi-faceted cells. The nucleus is active with a euchromatic nucleus that possesses prominent granular and fibrillar areas, getting ready for the necessary translational events related to nourishment of the embryo. [29] The cellular machinery related to transcription and translation of certain factors as integrin, IGF-1 increases during decidualization. Decidual cells also show definitive changes of intermediate filaments with increased amount of desmin and vimentin, along with a rise in total protein concentration. In the rodents, the primary decidual zone, which is devoid of vascular structure, leads to formation of secondary decidual zone by differentiation.[30-32]

3. MOLECULAR DETERMINANTS OF EMBRYO IMPLANTATION

Several genes are responsible for the establishment of the uterine receptivity.[34] The molecular and cellular determinants of the uterine receptivity are depicted in Figure 4. The molecular interactions are controlled by several growth factors, adhesion molecules, hormone receptors, developmental genes and cytokines.

3.1 Cytokines

LIF, IL-1 and IL-6 are the cytokines that have been studied for implantation.[35] IL-1 and IL-6 induce the expression of laminin and collagen receptors synthesized by the trophoblast cells. These two cytokines are synthesized by the trophoblast cells and act in an autocrine / paracrine manner to induce their expression. The Leukemia Inhibitor Factor (LIF) is produced in the mouse endometrium to prepare the glandular epithelial cells for the receptivity and establishment of cross-talk between the embryo and uterus. The secretion of LIF is a prerequisite for implantation in these species. It is estimated that, endometrial LIF controls trophoblast invasion by inhibiting the activity of cytotrophoblasts which express the laminin and integrin receptors. [36]

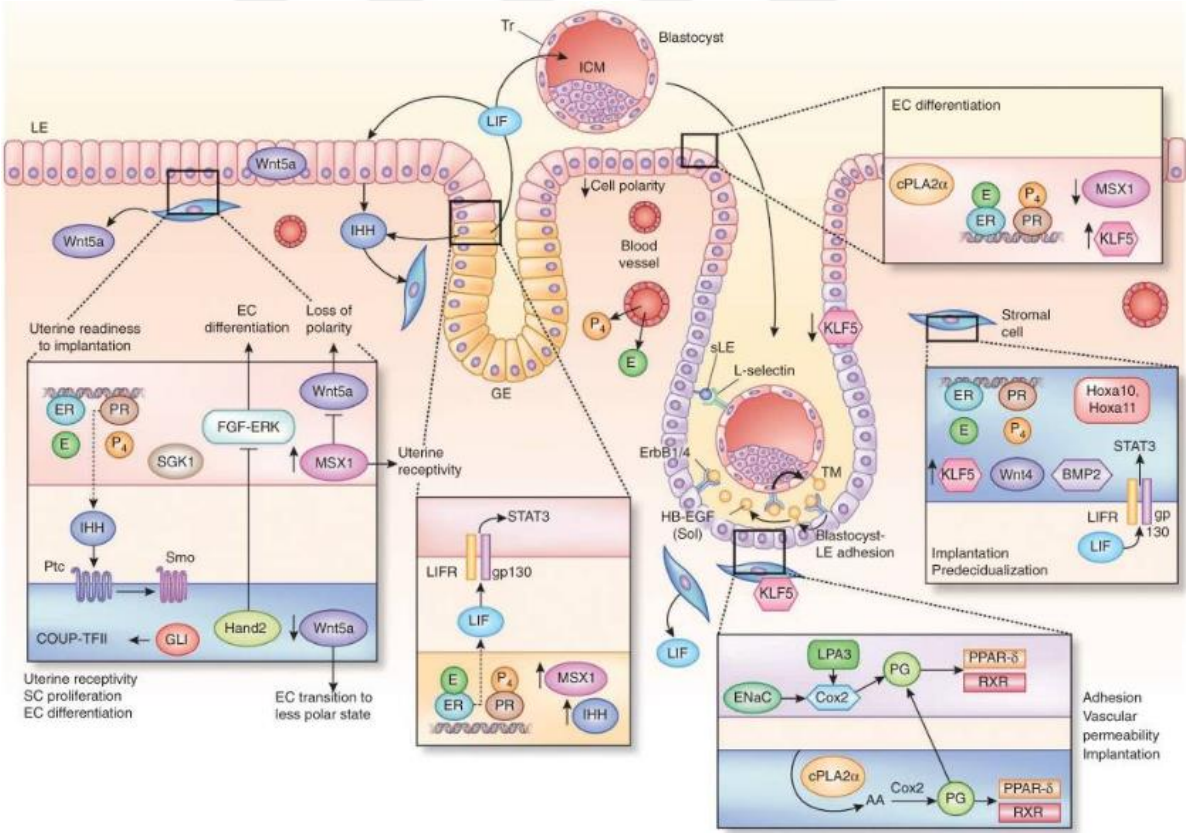


Figure 4. ***Secreted molecules during the implantation.***[33]

3.2 Developmental Genes

The developmental genes that have a role during implantation are generally transcription factors of Homeobox transcription factors, Wnts, IHH and several other genes. The transcription factors of Homeobox genes regulate the embryo morphology and cellular differentiation during the development. In rodents, the expression of homeobox transcription factors is mainly controlled by the secretions of the estrogen and progesterone. [37] It has been proved that silencing of Hox genes causes failure of the embryo implantation.[38] Indian Hedgehog (IHH), and Wnt have been proved to have a role in the conversation of the evolutionary process of epithelial to mesenchymal interactions for the proper development of the embryo. [39] Formation of glandular structure and reproductive tract are controlled mainly by the Wnt secretions which are necessary for the formation of the uterus.[40, 41]

3.3 Cell Adhesion Molecules

3.3.1 Integrins

Integrins are multi adhesive glycoproteins involved in intercellular and cell-matrix adhesions. They are found in endometrial cells, decidua cells and extravillous trophoblasts. The apposition and attachment of the blastocyst to endometrial surface is maintained by integrins, as well as some other adhesion molecules. [42] Subunit V is important to start the intercellular events related to adherence of trophoectodermal cells to endometrial area. [43]

3.3.2 Trophinins

Membrane protein trophinin makes up a complex with a cytoplasmic protein, bystin to mediate intercellular adhesion between is a and mediates hemophilic cell adhesion between the trophoectodermal cells to endometrial area. During early secretory phase trophinin is seen on endometrial epithelium and it aids in adherence step of implantation. [44]

3.3.3 Selectins

The initial contact between the endometrium and blastocyst is contributed by L-selectins, which are transmembrane proteins located in cell membranes of white blood cells. The travel of the blastocyst through tuba uterine is also retarded by this molecule.[45-47]

3.3.4 Cadherins

Cadherins are Calcium-dependent adhesion molecules. Among three subtypes (E, N, P-Cadherins), E-cadherin has been reported to be related to infertility in mice, when mutated. Cadherins are important for differentiation of cells, recognition, morphogenesis and cellular migration, as well. The cadherin expression needs to decline to let the newly adhered blastocyst to invade. [48-51]

3.4 Extracellular Matrix Proteins

3.4.1 Fibronectins

Being a multi adhesive glycoprotein, fibronectin has the ability to adhere glycosaminoglycans as heparin, large molecules as gelatin and fibrin, and other glycoproteins as integrins to help attachment phase of implantation.[52, 53] As implantation takes place, fibronectin decreases for endometrial remodeling. During embryological development, fibronectin is also important for new vessel formation and hemostasis.

3.4.2 Laminins

Laminin is a glycoprotein that constitutes the major component of the basal lamina. Uterine tissue, as most of the tissues, has laminin, including peri and myometrial areas. The role of laminin at the early development is to maintain the integrity of basal membrane and to guide the separation of primitive germ layers.

3.4.3 Entactins

Cell-extracellular matrix interactions can be maintained by, entactins, which are strongly depend on calcium for their functions. At first phases of implantation, entactins contribute integrity of basal lamina under the trophoblastic cells. As cell invade to the underlying endometrial tissue entactin expression decreases and usually not seen in trophoblasts. Not only trophoblasts but also endometrial and mesenchymal cells can express entactin. [57]

3.4.4 Collagens

Collagens are the major fibers of extracellular area. Most important collagen types related to implantation are Collagen I, III, IV, all of which have been shown to decrease in the implantation area in some rodent species. Collagen I and reticular fibers (col III) are seen as fine fibrils in the uterine stroma, while collagen IV is the major component of the basement membrane. Among all collagen IV has a variety of functions related to migration of cells, viability of cells, secretion of growth factors. The expression of collagen IV varies, as it increases in the decidual zone, but decrease in implantation areas as collagen I rises here[58-63].

3.5 Enzymes

The invasion process of the blastocyst is regulated by two important tissue modeling enzyme families; matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMPs). While TIMPs are important for the regulation of the activity during invasion, MMPs break down the

extracellular matrix and the basement membrane components. MMPs/TIMPs ratio is not static and the equilibrium between them is important for pathological conditions.[64] MMP2 and MMP9 have been reported to be most effective for remodeling of endometrium. [65]

3.6 Growth Factors

Some growth factors are intensely related to implantation event[67, 68, 69, 70, 71]. These are: i. Transforming growth factor-β: stem cell characteristics of the stromal cells, ii. Heparin-binding epidermal growth factor: vital for blastocyst-endometrium interaction, iii. Keratinocyte growth factor: important for vasculogenesis in implantation area, iv. Colony-stimulating factor and insulin-like growth factor: aid in function of trophoblasts.

3.7 Hormone Receptors

The key steroid for hormones of physiological events during implantation are estrogen and progesterone. Estrogen induces proliferation and differentiation of the endometrial cells. Erα is the main subunit of the ER that has both alpha and beta subunits. The failure in Erα has been shown to cause desensitization of estrogen treatment.[72, 73] Disruption of the ER by the proteolysis mechanism leads induction of the blastocyst to implant.[74] Progesterone, on the other hand, controls the uterine receptivity via its nuclear isoform receptors of PRA and PRB. [75] In humans, estrogen hormone induces both PR isoforms to maintain the uterus receptivity. The PR expression increases in epithelial and stromal cells notably in the proliferative phase of the menstrual cycle. With the increment of the progesterone in the uterine environment, the PR expression induces the downstream genes to regulate the invasion and development of the blastocyst. [76]

HB-EGF is expressed by the endometrial epithelial cells and by the endometrial stromal cells under the control of estrogen and progesterone, respectively. Located on apical and luminal cells, HB-EGF mediates the effects of estrogen on cell proliferation, and via its receptor ErbB4 it can induce trophoblast cell development (Figure 5).[77, 78] Dimerization of the receptors (*Erbb1-4*) is required for its functions.[79, 80]

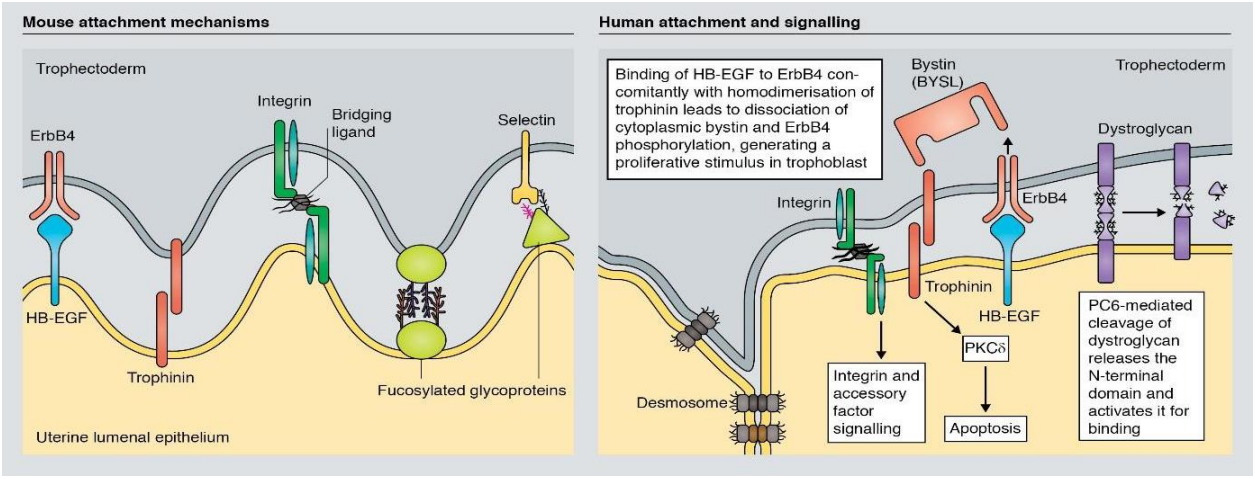


Figure 5. ***Signaling molecules during implantation in mice and humans.***[22]

4. MESENCHYMAL STEM CELLS (MSCs)

MSCs are adult stem cells found in connective tissues of cells. They make up the basis of the stromal cells that are known as the supportive cells in the tissues. The need for intercellular tissue compatibility caused the formation of open use donor pools by biotechnology companies. They provide stromal support in order to contribute to the development and function of related tissue cells. Their ability to differentiate makes them ideal for clinical therapies. Ability to fuse with damaged cells, contribute the damaged tissue, migrate to damaged tissue, be immunosuppressive / non-immunogenic make MSCs important for clinical use. [81] The criteria for MSC identity are based on their plastic adherence, expression of certain CD markers (CD105, CD90, CD73, etc.) and *in vitro* differentiation to cells of different lineages as adipocytes, osteocytes, and chondrocytes.[82] A variety of cell sources have been used to enhance the repair of tissue injuries.[83]

4.1 Secretions of MSCs

Immunoregulation maintained by MSC occurs via the soluble secretions and their diffusion to neighboring cells. These secretions are composed of mainly anti-inflammatory cytokines such as; TNF-stimulated gene 6 protein (TSG-6), Prostaglandin E2 (PGE2), Interleukin 10 (IL-10) and Interleukin 6 (IL-6). Allogenic cell-based therapies are constructed on the basis of lacking HLA-DR expression in MSCs, which gives them potential not to induce rejection mechanisms.[84, 85] MSCs are found mainly in the bone marrow, placenta, amniotic fluid, and fetal tissues; the source may affect MSC identity. Human umbilical cord-derived MSCs do not express the vascular endothelial growth factor A (VEGF-A) and can transfer their mitochondrial DNA to the other cells.[86, 87]

4.2 Immune Regulation During Pregnancy

MSCs have immunosuppressive and anti-inflammatory characteristics therefore; they have been used in several culture setups *in vitro*.[88] They provide immunomodulatory effects by dendritic cells, NK cells, B lymphocytes, especially T cells. During the pregnancy, the decidual immunity system is repressed for the replacement by the trophoblast cells of the embryo. The NK cells communicate with the extra villous cytotrophoblastic cells and their ligands (HLA-I) to prevent the disruption of the cells.[89] Also, these cells become nontoxic to the endometrial cells during the implantation and placentation. NK cells trigger the embryo invasion by inducing the stromal cells to produce chemokines. [9] Proliferation of the T cells under the implantation area is inhibited by hepatocyte growth factor (HGF) and Heme oxygenase I (HO1).[91] IL-6 and PGE2 secretions from the MSCs are also important for immune regulation during the pregnancy.[91]

4.3 Applications of MSCs For Infertility

The cryopreservation of the MSCs do not affect the characteristics of the cells therefore, they can be used as an alternative treatment method in the treatment of gynecological solid tumors, prenatal transplantation and gene treatment in uterus. In infertility and *in vitro* fertilization research and treatment, it has been reported that intrauterine treatment of uterine metabolic diseases with stem cells is possible.[92] Up to now; the MSCs from the different sources have been used for infertility treatments to induce cell proliferation, migration and the uterus regeneration.[93, 94]

5. *IN VITRO* CULTURE MODELS FOR IMPLANTATION

Due to complexity and 3D microenvironment of the implantation process, the *in vitro* studies have been constructed with several combinations and techniques to obtain two-dimensional (2D) and three-dimensional (3D) culture models. These models are outlined in the Figure 6. Gel-based co-culture systems with the endometrial cells and transwell systems have been constructed to study molecular regulation during the implantation. The monolayer culture models have been used by endometrial cells or their surrogates *in vitro*. However, due to lack of extracellular matrix components and intercellular interactions in 3D environment, these models were unable to provide evidences for *in vivo* implantation process.[95] The adherence and invasion process of the implantation have been investigated by constructing the multilayer invasion and transwell migration assays. Matrigel has been used for production of 3D environment. The embryo surrogates of trophoblast spheroids and human and mouse embryos have been also used in *in vitro*. [97]

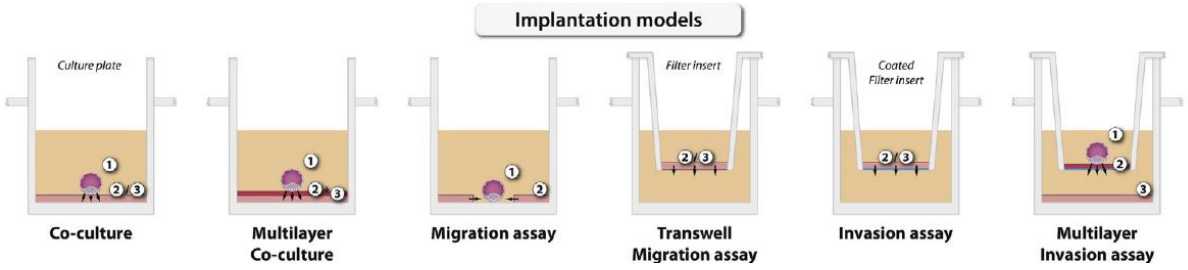


Figure 6. ***Schematic representation of the in-vitro models of human embryo implantation***.[96] *Cross-section of the culture systems was depicted. The last two culture systems with the blue line represent the Matrigel coating. The corresponding numbers are; 1: human embryo, or its surrogate; 2: human endometrial epithelial cells; 3: human endometrial stromal cells, or their surrogates. The direction of the invasion or migration is represented with the arrows.*

5.1. Challenges Of *In Vitro* Implantation Models

Duration of implantation process varies between the species. Although the WOI takes only 24 hours in mice; it takes about 5 days in humans (Figure 7).[9] Ethical and technical difficulties make *in vivo* study of implantation harder than *in vitro* studies. Due to limited intercellular interactions, cell to extracellular matrix interactions and cell types, the *in vitro* studies were insufficient to understand and mimic implantation mechanisms.

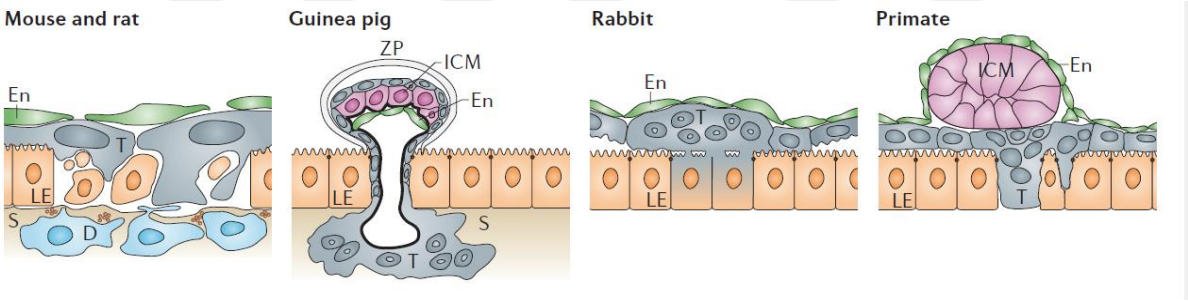


Figure 7. ***Interspecies differences of implantation.***[98] *En= embryonic endoderm, LE= luminal epithelium S=stroma, ZP=zona pellucida.*[98]

Although the main idea of the implantation is the proper interaction of the trophoblast cells of the embryo and stromal cells of the uterus, the process differs between the species. In mice and rats, the syncytiotrophoblast cells make some protrusions to the apoptotic luminal epithelial cells. In rabbit, syncytium of trophoblast and luminal epithelium cells occurs and in primates, trophoblast cells invade the underlying epithelial cells during the implantation. [99]

6. POLYMERIC CELL CARRIERS

Due to low risk of side effects and high efficiency, the polymeric carriers have been used in pharmaceutical industry and clinical studies. [100] Adhesive, degradable biomaterials and hydrogels have been selected for construction of pharmaceutical formulations.[101] Cellular attachment and proliferation can be modified by changing the composition of the natural and synthetic cell carriers. In most cases, PLA, PLGA, and PGA have been used for the construction of biodegradable polymeric structures. [102]

6.1 Biomaterials Used For 3-D Culture

6.1.1 Scaffolds In Cell Culture

The scaffolds used in cell cultures provide a 3D environment for the cell to cell and cell to extracellular matrix interactions. These constructions provide the tissue growth and development. The scaffolds used in cell culture environment need to be biocompatible and recognizable by the cells. [103] Depending on the scaffold used in cell culture; the cell type for the culturing varies to induce cellular differentiation and proliferation. In most cases, the scaffolds are used for culturing the stem cells. It also has been showed that embryonic stem cells (ESCs) can also be cultured on scaffolds with Matrigel to provide 3D environment.[104]

6.1.2 Natural Materials

Natural materials can be derived from collagen, gelatin, chitin, cellulose and the decellularized tissue fragments such as liver or blood vessels. They are biocompatible due to the presence of extracellular matrix proteins in their native tissues. They are mostly used in regeneration or replacement of the organs or the tissues.[105]

6.1.3 Synthetic Materials

The ceramics, metals, degradable and non-degradable biomaterials compose the synthetic materials. The chemicals of polyglycolic acid (PGA), polylactic acid (PLA) and the copolymer polylactide-co-glycolide (PLGA) have been used for the construction of synthetic materials. [106] The composition and amount of the chemicals can be adjusted in synthetic and biodegradable polymeric cell carriers to manipulate the degradation rate and time within the body.[107]

Chapter 2

MATERIALS & METHODS

1. CHEMICALS AND REAGENTS

HBSS - Hank's Balanced Salt Solution (HBSS), Phosphate-Buffered Saline (PBS), DMEM-F12 Ham's culture media, charcoal stripped fetal bovine serum (12676029), fetal bovine serum (FBS) (Biochrom AG, Berlin) Penicillin-Streptomycin-Amphotericin B Solution, L-Glutamine, RIPA Lysis and Extraction Buffer were purchased from Life Technologies (Thermo Fisher Scientific Inc., MA, USA). *β*-Oestradiol (E2758), progesterone (P8783), EGF (E9644), Collagenase/Dispase (10269638001), Trypsin/EDTA 0.05%, Collagen (11179179001), Glutaraldehyde solution (G5882), Paraformaldehyde powder (158127) were provided from Sigma (St.Louis, MA, USA). Quinn's SAGE sequential embryo culture medium was from ORIGIO (Denmark). Corning Matrigel Basement Membrane Matrix (354234) was obtained from Corning (NY 14831, USA). Quick-RNA Kit (Macharey-Nagel) was from Zymo Research (USA). Dexamethasone (D4902), IBMX (I5879), Indomethacin (7378), Insulin (I6634), Ascorbic Acid (A4403), β-Glycerophosphate (G9422) were from Sigma (St.Louis, MA, USA). Oil Red O (19056), Alizarin Red S (26206) were purchased from EMS (England). Anti-CD34, anti-CD45, anti-CD73, anti-CD105 antibodies purchased from BD Biosciences (USA). Anti-tubulin (T5201) was purchased from Sigma, USA. Anti-H3K4me3 (ab8580), Anti-E Cadherin (ab76055), Anti-LIF (ab113262), Anti-MMP9 (ab38898), Anti-pancytokeratin (ab6401), Anti-Vimentin (ab92547), Anti-Fibronectin (ab2413), Anti-Integrin alpha 5 (ab150361), Anti-Laminin (ab11575), Anti-Entactin (ab14511), Anti-Collagen I (ab34710), Anti-Collagen IV (ab6586), Anti-Trophinin (ab78117), Anti-EGFR (ab2430) antibodies were purchased from Abcam (UK). Anti-Mitochondria (MTCO2) antibody was purchased from Thermo Scientific, USA. Anti-ERα (sc-8002) anti-PR antibodies (sc-398898) and Anti- HBEGF (sc28908) were purchased from Santa Cruz Biotechnology (USA). Anti- CD62L (bs1036R) was obtained from bioss, USA. TRITC-phalloidin (R415), Dylight 649, Oregon 488, Alexa Fluor 488, 514,532,568,594 dyes, Hoechst 33342 and Live/Dead Viability/Cytotoxicity kit (L3224) were obtained from Life Technologies (Thermo Fisher Scientific Inc., MA, USA). 7-amino actinomycin (A9400) was obtained from Sigma, USA. P (L-D, L) LA was purchased from Purac company (Corbion Inc., Netherlands). Pregnant mare serum gonadotropin (C8554, Sigma, St.Louis, MA, USA), BSA (A-3311, Sigma, St.Louis, MA, USA), hyaluronidase (H1136, Sigma, St.Louis, MA, USA) were purchased from Sigma. 75-cm2 and 10 cm diameter tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) were used in cell culture experiments. Hematoxylin (105174), Eosin (109844) and entellan (107961) were purchased from Merck, Germany. Ovitrelle® (rHCG) 250 micrograms/0.5 ml was from Merck Millipore (Germany).

2. PREPARATION OF BLASTOCYSTS

2.1. Animal Preparation

The animals used in this experiment were obtained from the Animal Facility Centre of Koç University. Animals survived at 12 hours of light and 12 hours of dark and are fed ad-libitum. The ethical approval number: 2015/15.

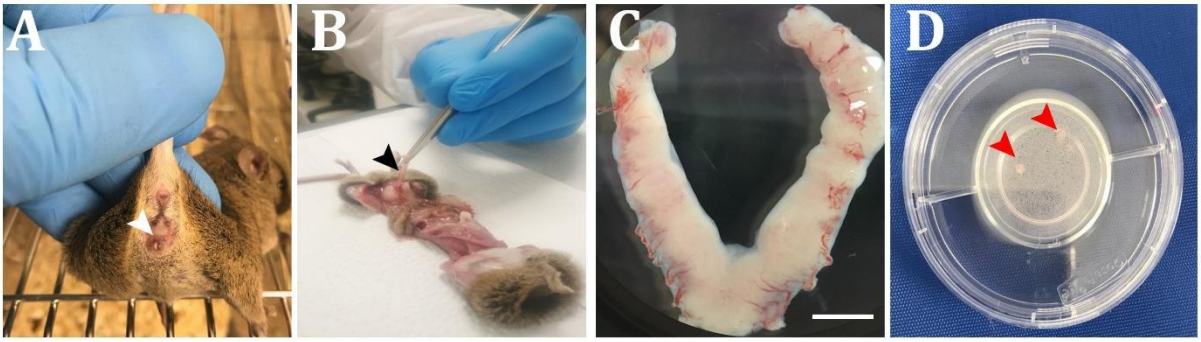


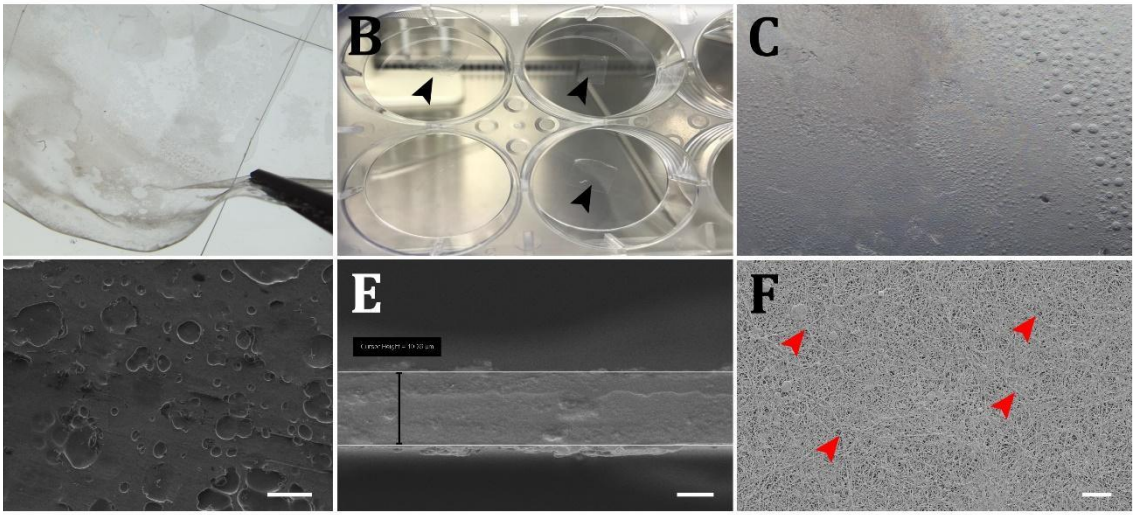
Figure 8. ***Preparation of blastocyst culture****. Mouse with a vaginal plug (A, white arrowhead) was obtained. The uterus and ampullary regions were removed (B, black arrowhead) and taken under stereomicroscope for the cell isolation and blastocyst culture (C). Ampullary regions (D, red arrowhead) were ruptured and cultured in HEPES -buffered solution. Bar=1,5 cm.*

2.2. Culture And Selection Of Blastocysts

Approximately a total of 500 blastocysts were used in all experimental groups. For superovulation CB6F1 (C57BL⁄6 X BALB⁄C) female 6-8 weeks mice were stimulated by 10 iu pregnant mare serum gonadotropin i.p., followed by 10 iu injection of hCG after 48 hours. Superovulated female mice mated with CB6F1 (C57BL⁄6 X BALB⁄C) male mice. The preparation procedure was outlined in Figure 8. Vaginal plaque formation of mated females was determined. These females were then sacrificed and ampullary regions were ruptured in BSA including HEPES supplemented with hyaluronidase. Pronuclear (PN) embryos and zygotes obtained from the fallopian tubes were cultured in Quinn's SAGE sequential embryo culture medium in 10 µl culture drops under oil (see section 4) in a 37 ° C incubator supplemented with 5% CO_2. After 24 hours of culture in cleavage medium, embryos were placed in blastocyst medium until the development of blastocysts (4.5 days of fertilization in mice).

For the experimental groups, embryos (day 4,5) with the highest quality based on Gardner's blastocyst scoring criteria were selected.[108] Briefly blastocysts with expanded blastocoele or the hatching ones that have tightly packed inner cell mass and a cohesive layer of many trophoectoderm cells were selected.

2. PREPARATION OF POLYMERIC FILMS



9. *Polymeric film.* *Film structure has a transparent view that can be seen in the stereomicroscope image (A). Film fragments were placed in culture dishes for culturing (B). The film seemed to have a uniform surface as seen in closer look under a stereo microscope (C). Surface (D) and thickness (C) of the film can be observed in the SEM images (vertical line, E=10 µm). Film fragments were covered with the collagen (F, red arrowheads) before cell seeding. Bars= 10 µm (D), 5 µm (E), 2 µm (F).*

In this study, films were used as polymeric cell carriers. Polymers of poly (L-lactic acid-co-D, L-lactic acid) (P (LD, L) LA) and poly (D, L- lactic acid- co- glycolic acid) (PLGA) solutions were prepared. The solution was poured into a bowl and spread to a homogeneous sieve. The solution was obtained by blowing the solvent of the solution and then removing the polymeric film from the petri dish after evaporation.

Before the establishment of the experimental groups, for the groups cultured with the film, film was coated with collagen (Figure 9, F). The collagen from rat tail tendon was diluted in so that the final concentration should be 5 µg/cm^2 in PBS and 100 µl collagen solution dropped on the film fragments. The film fragments incubated at 37 °C for 1h. After aspiration of the remaining solution, fragments were let to dry for overnight.

4. PREPARATION OF CULTURE SYSTEMS

4.1. Isolation And Culture Of Epithelial And Stromal Cells Of Mouse Uterine Endometrium

To prepare epithelial cells, uteri were removed, cut open lengthwise, pooled, and incubated with 0.25% trypsin /0.1% collagenase-dispase for 2 hours at 4 °C and 60 minutes at room temperature. Following transfer to ice-cold (3 °C) HBSS, digested uteri were vortexed to release sheets of epithelial cells. Uterine tissues were rinsed and vortexed for an additional three times and resulting cell suspensions were pooled.

Epithelial sheets recovered by passing the cell suspension through a 70 µm nylon mesh were collected, the second filtration of the filtered solution was carried out through a number 40 µm wire mesh sieve. The epithelial cells were trapped on the 70 µm sieve and backwashed from the sieve with a medium, while stromal cells passed through to a 60 mm sterile dish (Figure 10). Epithelial and stromal cells were resuspended in a complete medium consisting of DMEM/Ham's F-12 1:1 serum supplemented with 10% FBS, 100 U/ml penicillin, and 2 mM L-glutamine.

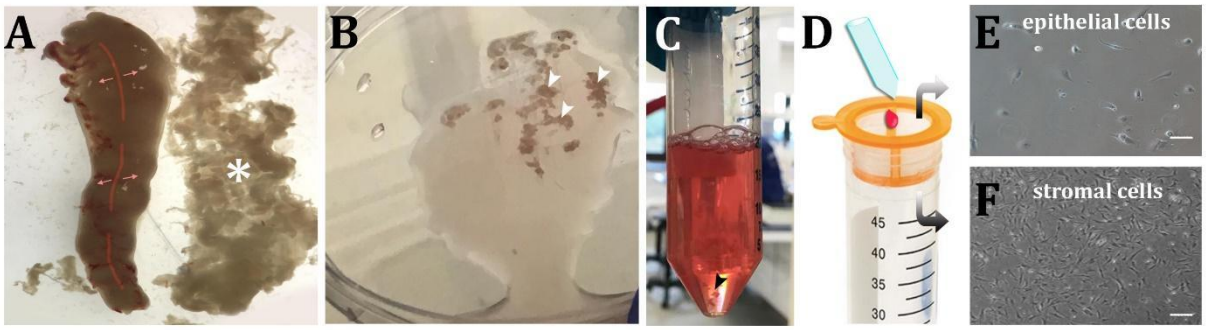


Figure 10. ***Isolation of epithelial and stromal cells of mouse endometrium****. Uterus was obtained from the mouse and opened horizontally under a stereomicroscope (A, red arrowheads). The horizontally opened uterus can be seen (A, asterisk). After epithelial layer was shed, the stromal compartment of the uterus was chopped (B, white arrowheads). Enzymatic digestion(C) was performed in a falcon tube and epithelial layers were obtained (E, black arrowhead). After flushing with the sieve (D), endometrial (E) and stromal cells (F) were cultured separately. Bars= 50 µm.*

4.2 Isolation And Culture Of Human Umbilical Cord Derived Mesenchymal Stem Cells

Umbilical cords were obtained from consenting patients delivering full-term infants by Caesarian section (*n= 6*), who faced no complications throughout pregnancy. Fifteen-centimeter-long cords were immersed in sterile Hanks' balanced salt solution supplemented with penicillin (200 units/ml), streptomycin (200 µg/ml), and amphotericin-B (5 µg/ml) and immediately transferred to the laboratory. Ethical approval was obtained from the Ethical Review Board (approval no:2016.055.IRB2.019)

4.2.1 Hematoxylin And Eosin Staining Of The Umbilical Cord

For the Hematoxylin and Eosin staining; cord biopsies were taken and incubated at %4 PFA overnight. After immersing the cord biopsies in increasing concentrations of the sucrose (20%,30%) for overnight, the 10 µm sections of the cord was obtained with the cryosectioning. The cryosections were stained with Haematoxylin and Eosin. For dissolution of the cryomatrix, the sections were immersed in water solution. After 45 seconds of Haematoxylin incubation and rinsing in water, the sections were immersed solutions with increasing alcohol concentrations for five seconds. Before closing with the entellan, sections were immersed in xylene for 2 minutes.

4.2.2 Isolation Of Human Umbilical Cord Derived Mesenchymal Stem Cells

The isolation protocol was outlined in figure 11. Arteries and vein were removed by blunt dissection, and the remaining tissue was chopped with scissors and scalpels. Tissue pieces were placed in a Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 (1:1 vol/vol) culture medium supplemented with 10% (vol/vol) fetal bovine serum (FBS), collagenase type B (1 μg/ml) ,penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin-B (2.5 μg/ml) and digested at 37 ° C in a gentle orbital shaker until a tissue homogenate was obtained in approximately 4 hours. The homogenate was centrifuged at 500*g* for 20 minutes. The cell pellet was resuspended in the culture medium, and viable cells were counted using a trypan blue dye exclusion assay. The cells were seeded to a single 75-cm^2 and 10 cm diameter tissue culture flask at a density of 5.000 cells per square centimeter.

For the explant culture of HUCSCs, tissue fragments are placed on 60 mm diameter tissue flasks with the interval of 50 cm. Droplet of Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 (1:1 vol/vol) culture medium supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 U/ml penicillin, and 2 mM L-glutamine. Plates were not moved for at least 5 days for not to inhibit the tissue attachment on a plate and cell isolation.

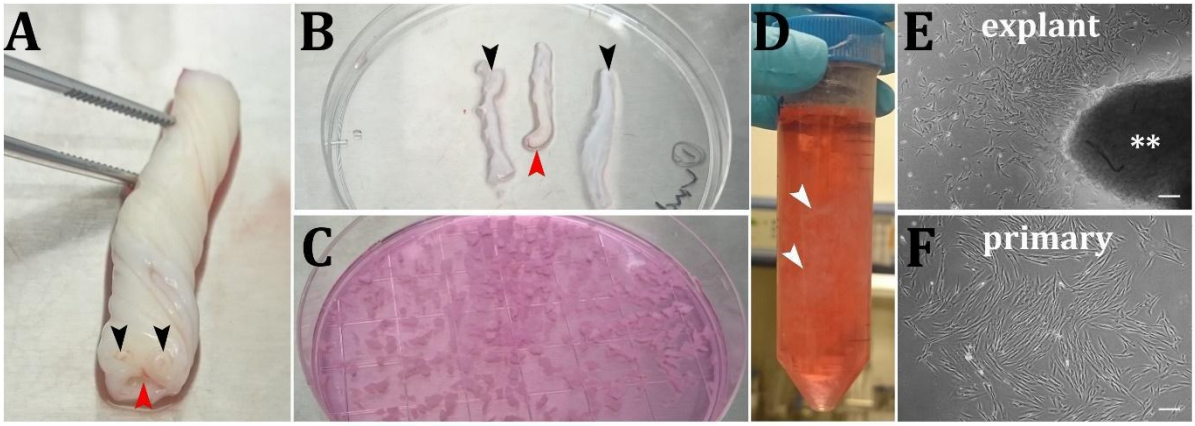


Figure 11. ***Isolation of mesenchymal stem cells of the human umbilical cord.*** *The umbilical cord was obtained and divided into 5 cm fragments for cell isolation (A). Two-arteries (A, black arrowheads) and one vein (A, red arrowhead) structures were removed from the Wharton's Jelly (B). Remained tissue was chopped (C) and incubated in enzyme solution (D). Explant (E) and primary cultures (F) were maintained separately. Bars= 50 μm.*

4.2.3. Characterization Of HUCSCs

4.2.3.1 Adipogenic Differentiation

Subconfluent (80%–90%) HUCSCs from passages 3– 6 grown on 24- culture dishes and glass coverslips were induced for adipogenic differentiation by an administration of 1 M dexamethasone, 500 M IBMX, 60 M indomethacin, and 5 g/ml insulin in Dulbecco's modified Eagle's medium (DMEM)-low glucose (low glucose, 1 g/l) supplemented with 10% fetal bovine serum (FBS). Noninduced cells

were cultured in DMEM-Ham's F-12 (1:1 [vol/vol]) culture medium supplemented with 10% (vol/vol) FBS.

4.2.3.2 Oil Red O Staining

Adipogenically induced cells were fixed with 10% formalin for thirty minutes at room temperature. Oil Red O powder was dissolved in 60% isopropanol and filtered. After rinsing in 60 % isopropanol for thirty seconds, the cells on the coverslips were incubated in Oil Red O solution for twenty minutes at room temperature. After rinsing with the 60% isopropanol, IF staining protocol was applied for multiple staining of the induced cells.[118]

4.2.3.3 Osteogenic Differentiation

P2-P3 cells growing on glass coverslips in 24-well plates were subjected to osteogenic medium composed of DMEM-LG with 10% (vol/vol) FBS, 100 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM glycerophosphate. Induced monolayers were fixed with 4% (wt/vol) paraformaldehyde at the end of each week during the following 4 weeks. Induced cells were labeled with rabbit polyclonal anti-osteopontin (Abcam, UK) antibody diluted 1:50 in PBS. Alexa 488 conjugated secondary antibodies were used as secondary antibody diluted 1:100 in PBS. For the visualization of calcium deposits, fixed cultures were stained with Alizarin red S after being fixed in 10% formalin for 20 minutes at room temperature.[118]

4.2.3.4 Alizarin Red S Staining

Osteogenically induced cells were fixed with 10% formalin for twenty minutes at room temperature. Alizarin Red S was prepared from dissolving it in distilled water and adjusting the pH to 4.2. The fixed cells are hydrated to 70% alcohol and rinsed rapidly with the distilled water. Alizarin Red S was applied to fixed samples for five minutes to stain the calcium deposits. The samples were cleared in xylene and mounted with entellan.

4.2.3.5 Flow Cytometry Analysis

Flow cytometry analysis was performed for surface markers important for MSCs. Briefly, cells were isolated in the umbilical cord and a portion of the cells passaged. After passaging, cells were trypsinized and washed 2 times with PBS to remove phenol red in the medium. Cells were stained with PerCP conjugated CD105 and FITC conjugated CD73 antibodies and hematopoietic cells were excluded by PE-conjugated CD34 and APC conjugated CD45. After incubating the cells with antibodies at RT for 15 minutes, the suspension is centrifuged 20000 rpm for 5 min and the pellet is resuspended in 500 µl PBS before BD AccuriTM C6 Plus Flow Cytometer.

5. ESTABLISHMENT OF THE EXPERIMENTAL GROUPS

The experimental groups were constructed as mentioned below (Figure 12).

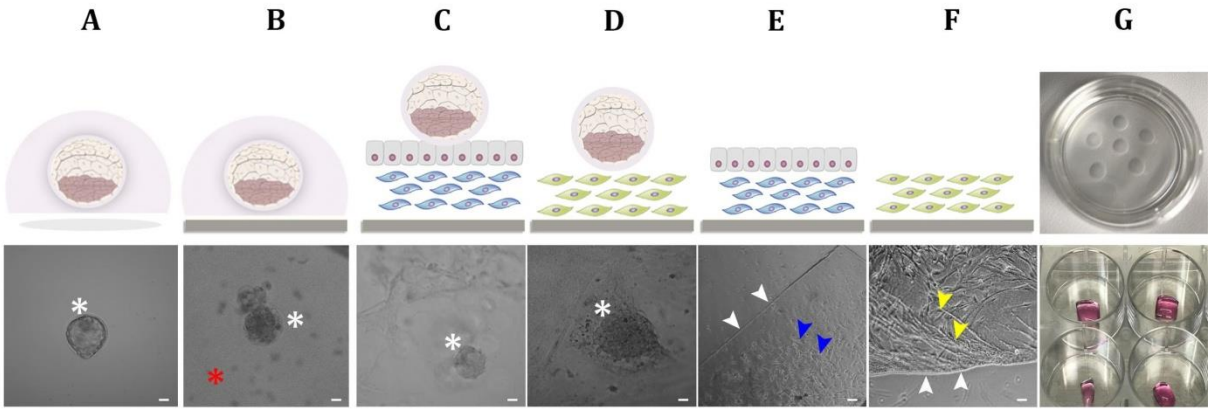


Figure 12. ***The experimental groups.*** *The experimental groups were schematically represented in the figure. For the routine IVF culture, embryos were cultured in 10 µl culture drops under light paraffin oil (A). Blastocyst (B, red asterisk) was placed on the film fragments (B, white asterisk). ECCs were constructed in/on Matrigel for the culture of blastocyst (C, asterisk). Blastocyst on the MSC covered film (D, asterisk). Cultures without the blastocyst were constructed (E, F). Epithelial and stromal cells can be seen in two different foci (E, blue arrowhead) with the edge of the film (E, white arrowhead). The MSCs (F, yellow arrowhead) were allowed to spread on the film structures (F, white arrowhead). Routine and film based culture set up can be seen in G. Asterisk= blastocyst. Bars=50 µm (A, C), 25 µm (B, D, E, F).*

5.1 Routine IVF Group

Blastocysts were cultured in Quinn's Advantage Medium under light paraffin oil at 37 °C for 48 and 96 hours under 5% carbon dioxide in a complete medium consisting of DMEM/Ham's F-12 1:1 serum supplemented with 10% charcoal-stripped FBS, 63.5 nmol /L progesterone, 7.14 nmol/L *β*-oestradiol, 20 ng/ml EGF, 100 mg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine.

5.2 2D And 3D Culture Models

The 2D models were based on the culture of blastocysts on the adherent collagen coated film. 3D culture models were constructed in two ways, by either separate culture of epithelial and stromal cells on and inside Matrigel®, which is a commercial extracellular matrix-based gel or culture of human mesenchymal stem cells on a film carrier.

5.2.1 Construction Of 2D Film Culture

After the sterilization process with 70% ethanol was completed, and films were dried overnight, the films were covered with collagen. The coated surface was washed with the medium. The blastocyst was placed on film fragments placed on wells with a mouth pipette in a complete medium of DMEM/Ham's F-12 1:1 serum supplemented with 10% charcoal-stripped FBS, 63.5 nmol/L progesterone, 7.14 nmol/L *β*-oestradiol, 20 ng/ml EGF, 100 mg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine.

5.2.2 Construction Of 3D ECC

Endometrial epithelial cells and stromal cells were prepared from freshly isolated primary or early passage epithelial and stromal cells (passages 1–3). Matrigel® was diluted with the serum-free medium and overlaid on a film (100 µl/film). The gelation process was completed after 30 min at 37 °C in an incubator. After gel formation, the epithelial cells, stromal cells (105 cells/each case) in single cell suspensions were plated separately on the Matrigel-coated chamber slides in Ham's F-12 medium supplemented with 10% charcoal-stripped FBS, 63.5 nmol /L progesterone, 7.14 nmol/L *β*-oestradiol, 20 ng/ml EGF, 100 mg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine. Typically, stromal cells were grown within the Matrigel, and epithelial cells were plated onto the Matrigel after gel formation. The blastocysts were placed on film fragments placed on wells with a mouth pipette.

5.2.3 Construction Of 3D hMSC Culture

Human mesenchymal stem cells (P3-P8) were cultured in complete DMEMF12/Ham's (1:1) medium supplemented with 10% charcoal-stripped FBS, 63.5 nmol /L progesterone, 7.14 nmol/L *β*-oestradiol, 20 ng/ml EGF, 100 mg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine. After the film was covered with collagen, coated surface was washed with the medium and cells were seeded. (50000 cells/film). The blastocysts were placed on film fragments placed on wells with a mouth pipette.

5.2.4 Construction Of ECC On Film (Film+ ECC)

Early passage of endometrial epithelial and stromal cells (passages 1–3) were used for the experimental group. Matrigel® was diluted with the serum-free medium and overlaid on a film (100 µl/film). For the gelation process, samples were incubated at 37 °C in an incubator for 30 minutes. The epithelial cells (105 cells/each case) in single cell suspensions were plated separately on the Matrigel-coated chamber slides in Ham's F-12 medium supplemented with 10% charcoal-stripped FBS, 63.5 nmol /L progesterone, 7.14 nmol/L *β*-oestradiol, 20 ng/ml EGF, 100 mg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine. Typically, stromal cells were grown within the Matrigel, and epithelial cells were plated onto the Matrigel after gel formation on the film fragments. There were no blastocysts in this group in order to see the effects without blastocysts.

5.2.5 Construction Of MSC On Film (Film+ MSC)

Human mesenchymal stem cells (P3-P8) were cultured in complete DMEMF12/Ham's (1:1) medium supplemented with 10% charcoal-stripped FBS, 63.5 nmol /L progesterone, 7.14 nmol/L *β*-oestradiol, 20 ng/ml EGF, 100 mg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine. After the film was covered with collagen, coated surface was washed with the medium for preparation of the cell seeding (5000 cells/film). With a 100 µl of a cell suspension, cells were seeded on film

fragments and incubated at 37 °C, %5 CO_2 in the incubator. There were no blastocysts in this group in order to see the effects without blastocysts.

6. EXPERIMENTAL GROUPS

6.1. Routine IVF Group

Blastocysts were cultured in Quinn's Advantage Medium under light paraffin oil at 37 °C for 48 and 96 hours under 5% carbon dioxide. Washing droplets of pre-warmed PBS to 37 °C was prepared in 10 µl droplets on the 35 mm culture plate to purify from light paraffin oil. Oil-free blastocysts were taken into lysis buffer required for culture purposes. For PCR and Western Blot, 2 blastocysts were used for each marker. Cultured blastocysts for qPCR were harvested in RNA lysis buffer and stored at -80 °C. Cultured blastocysts for Western Blot were harvested in RIPA buffer and stored at -80 °C. One blastocyst was used for each marker in immunofluorescent staining. Blastocysts were fixed in 4% PFA and then stored at +4 °C.

6.2 2D Film Culture Onto Film

Blastocysts cultured at 37 °C under oil in droplets of 10 µl for 48 and 96 hours were washed 3 times in preheated PBS droplets. The washed blastocysts were placed on collagen-coated film with a mouth-pipette. At the end of the culture period, 100 µl of RNA lysis buffer was added to each culture dish for qPCR. The blastocyst was taken in lysis buffer and stored at -80 °C. RIPA buffer (150 µl) and protease inhibitors (10 µl) were added to the cultured group for Western blot. Cells in lysis buffer were stored at -80 °C. 2D culture of blastocyst was fixed with 4% PFA and stored at +4 °C.

6.3 Endometrial Co-Cultures With Matrigel Onto Film

A 3D Matrigel structure was constructed on film to mimic the 3D structure of the endometrium. Blastocysts cultured at 37 °C under oil in droplets of 10 µl for 48 and 96 hours were washed 3 times in preheated PBS droplets. The washed blastocysts were placed on the 3D endometrial co-culture on film with a mouth-pipette. At the end of the culture period, 100 µl of RNA lysis buffer was added to each culture dish for qPCR. Epithelial and stromal cells and blastocyst were taken in lysis buffer and stored at -80 °C. RIPA buffer (150 µl) and protease inhibitors (10 µl) were added to the cultured group for Western Blot. Cells in lysis buffer were stored at -80 °C. 3D endometrial co-cultures with gel onto film were fixed with 4%PFA and stored at +4 °C.

6.4 3D hMSC Cultures Onto Film

Blastocysts were placed on collagen and hMSC coated polymeric films. Blastocysts cultured at 37 °C under oil in droplets of 10 µl for 48 and 96 hours were washed 3 times in preheated PBS droplets. The washed blastocysts were placed on the hMSC culture with a mouth-pipette. At the end of the culture period, 100 µl of RNA lysis buffer was added to each culture dish for qPCR. Mesenchymal stem cells and blastocyst were taken in lysis buffer and stored at -80 ° C. RIPA buffer (150 µl) and

protease inhibitors (10 µl) were added to the cultured group for Western Blot. Cells in lysis buffer were stored at -80 °C. 3D hMSC cultures with the film were fixed with 4%PFA and stored at +4 °C.

6.5 Film Culture Of ECC (Film+ECC)

After the seeding of the MSCs, film fragments were incubated as the experimental groups to 48th hour and 96th hour. At the end of the culture period, 100 µl of RNA lysis buffer was added to each culture dish for qPCR. Mesenchymal stem cells were taken in lysis buffer and stored at -80 °C. RIPA buffer (150 µl) and protease inhibitors (10 µl) were added to the cultured group for Western blot. Cells in lysis buffer were stored at -80 °C. hMSC cultures on the film were fixed with 4% PFA and stored at +4 °C.

6.6 Film Culture Of MSC (Film+MSC)

After construction of ECC, film fragments were incubated as the experimental groups to 48th hour and 96th hour. At the end of the culture period, 100 µl of RNA lysis buffer was added to each culture dish for qPCR. Endometrial epithelial and stromal cells were taken in lysis buffer and stored at -80 °C. RIPA buffer (150 µl) and protease inhibitors (10 µl) were added to the cultured group for Western blot. Cells in lysis buffer were stored at -80 °C. ECCs on the film were fixed with 4%PFA and stored at +4 °C. The total number of blastocysts used in PCR, WB, IF, SEM and live-dead analysis was outlined in Figure 13.

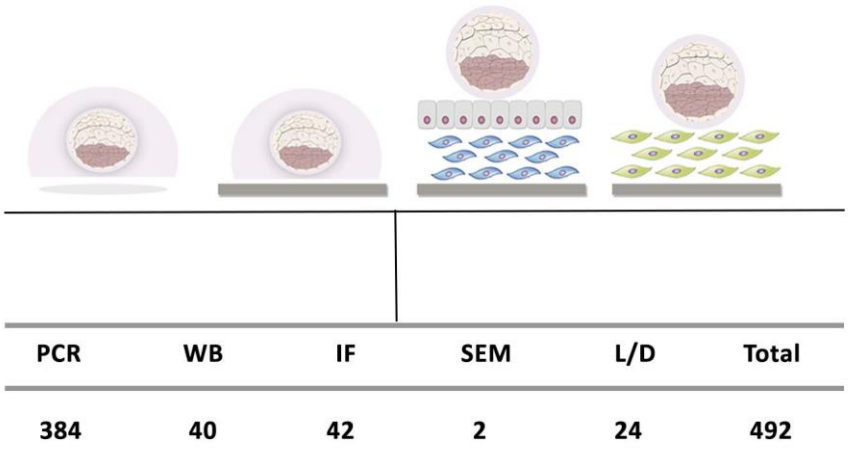


Figure 13. ***The number of blastocysts used in all experiments.*** *A total of almost 500 blastocysts were used.*

7. EVALUATION OF IMPLANTATION

Of all the markers listed in Chapter 1 (review of literature), implantation markers were divided into four main groups; functional, adhesion, development and invasion-related genes. LIF, ErbB1, ErbB2, HBEGF, ER, and PR were selected as functional; HOXA10, HOXA11, Wnt4, and IHH were selected as developmental; MMP9 and TIMP3 were selected as invasion; E-Cadherin, Fibronectin, L-Selectin, Laminin, Entactin, Integrin, Trophinin, Collagen (I, III, IV, V) were selected as adhesion genes. SEM, IF/Confocal imaging, qPCR and Western Blot methods were used to evaluate the implantation. The basic methodology was outlined in figure 14.

7.1 Immunofluorescence Staining

In the IVF group, blastocysts were fixed in room temperature for 30 minutes in a 4% PFA solution. After fixation, the blastocysts were washed in PBS drops. Blastocysts were kept at room temperature for 30 minutes in blocking buffer (1X PBS / 5% normal goat serum / 0.3% Triton™ X-100). After the blastocysts were washed in PBS drops, they were taken into the primary antibody droplets. Primary antibodies were diluted in antibody dilution buffer (1X PBS / 1% BSA / 0.3% Triton™ X-100) at 1:100 respectively. Blastocysts were incubated in primary antibody drops for overnight at 4 °C. After rinsing with PBS, blastocysts were incubated with fluorochrome-conjugated secondary antibody (Alexa 488, Alexa 594, Alexa 568, Alexa 532, Alexa 514) diluted in PBS and incubated for 90 minutes at 37 °C. The blastocysts were washed in a PBS drop and placed in a 10 µl drop of 1:2000 diluted Hoechst in PBS: Glycerol (1:1) solution on glass bottom Petri dishes. Light paraffin oil was used to prevent evaporation during imaging.

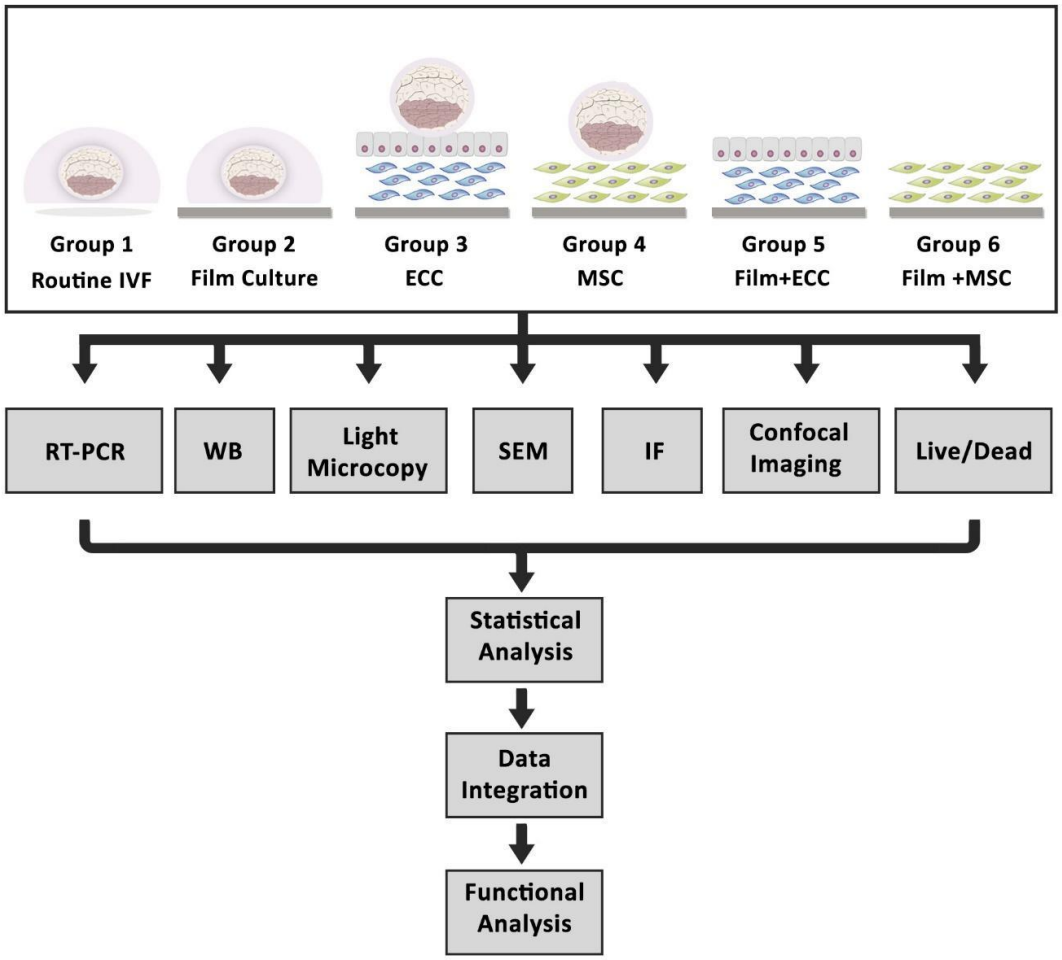


Figure 14. ***The outline of methodology related to the effects of culture systems on implantation.***

For immunofluorescence staining, film fragments with two dimensional and three dimensional experimental groups were fixed with 4% PFA for 30 minutes at room temperature, followed by washing twice with PBS and incubated at 37 °C for 90 minutes with primary antibodies. After washing again with PBS, they were incubated with a fluorochrome-conjugated secondary antibody (Alexa 488, Alexa 594, Alexa 568, Alexa 532, Alexa 514, Molecular Probes, USA) diluted in PBS and incubated for 90 minutes at 37 °C. This step was followed by rinsing the film fragments and adding Hoechst 33342 (1µg/mL) for DNA staining.

The imaging of the blastocysts in routine IVF group was achieved in 10 µl PBS glycerol solution on glass bottom Petri dishes covered with oil. The blasts in film cultures were mounted by high closure to not compress the film under the coverslip. For this purpose, the pieces of a broken coverslip were glued to the plate, the film was positioned, and the covering coverslip was placed on the pieces of broken glass to provide a higher distance.

Imaging was performed under a Leica DMİ8/SP8 TCS (True Confocal Scanning)-DLS microscope and Leica DMi8/ SP8 TCS-STED microscope. Embryos and cultures were imaged in the z axis to show the 3D structure. The 3D structure of the embryo was revealed by maximum projection of optical z-stacks to show invasive structure.

7.2 Live Cell/Death Cell Analysis

LIVE/DEATH ® Viability/Cytotoxicity kit was used to measure viability rates at 48h and 96h. 20 µl of 2mM EthD-1 were added into 10 ml of DPBS and mixed by pipetting then, 5 µl of 4mM calcein-AM was added and vortexed briefly. The cells were incubated for 30-45 minutes at room temperature and in the dark. After the incubation, 10 µl of LIVE/DEATH ® cell reagent solution was added to glass bottom Petri dishes. Using fine-tipped forceps, film fragments were carefully inverted and placed on cell viability reagent. To prevent evaporation, glass bottom Petri dishes were covered with oil. Imaging was performed with Leica DMi8/ SP8 TCS-STED microscope. The living cells appeared green while the dead cells appeared red.

7.3 Scanning Electron Microscopy (SEM) Analysis

The cells were washed 3 times with 1 ml of 0.2M Sorensen's buffer. Later they were fixed with 1 ml of 2.5% glutaraldehyde in a 0.1M buffer solution at room temperature for 2 hours. After washing 3 times with Sorensen's buffer, cells were dried at RT overnight. Before imaging samples were vacuumed and coated with 5nm Au. SEM images were obtained after covering, and surface analysis regarding the cellular interaction of cells with the film was performed. SEM images were taken at KUYTAM by a Zeiss EVO LS 15 equipped with secondary electron detector at high vacuum conditions. The electrons acceleration voltage was set to 4keV and working distance was set about 8-12 mm. The procedure was outlined in figure 15.

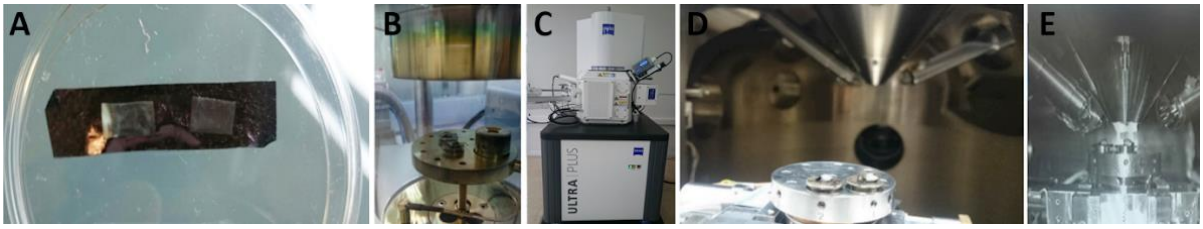


Figure 15*. SEM analysis.* *The samples were dried and coated with 5nm Au particles (A, B) and placed on the holder (D) of Zeiss EVO SEM (C), where the focus was maintained (E).*

7.4 Western Blot Analysis

Endometrial and mesenchymal stem cells were seeded separately on film fragments. Cells were detached by trypsinization and the cell pellet was obtained. After 48 and 96 hours in 3D and 2D culture, cells were detached by trypsinization and cell pellet was obtained with centrifugation at 500 g for 5 minutes. Pellets were then washed with ice-cold PBS and then re-suspended in an appropriate

volume of RIPA cell lysis buffer containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, 1X phosphatase inhibitor cocktail and 1X protease inhibitor cocktail (Sigma, MA, USA). Following 20 minutes of incubation on ice, centrifugation at 14,000g for 20 min at 4 °C was performed to obtain supernatants containing total cell extract. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane by Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit. The membranes were blocked with 5% non-fat dry milk in TBS-T (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.1%, v/v Tween-20) at room temperature for 1 hour. Then the primary antibodies were added and incubated rocking overnight at 4 °C.

Anti-pan Cytokeratin and Anti-Vimentin antibodies were used at 1:1000 and 1:500 dilutions to evaluate the origins of epithelial and stromal cells. Stromal cells were expected to be vimentin positive and pan-cytokeratin negative and epithelial cells as the opposite. Western antibodies were used at 1:1000 dilution. Anti-actin or anti-vinculin at a dilution of 1:10000 were used as a loading control. Secondary antibody conjugated to HRP, anti-rabbit, and anti-mouse was used in 1:2000. Quantification of protein within membranes was done by using Clarity™ Western ECL Substrate. Chemiluminescence detections were performed by ChemiDoc XRS+ Imaging System (Biorad, USA).

7.5 Real-Time Reverse Transcription PCR (RT-PCR)

In the routine IVF group, the blastocysts were incubated for 48 and 96 hours, after which the blastocysts were transferred into RNA lysis buffer. In 2D and 3D culture groups, cells and blastocyst were detached by trypsin and the collected pellet was taken into RNA lysis buffer. RNA isolation was performed by Quick-RNA® Kit (Macharey-Nagel) following the manufacturer's instructions. RNA quantification was performed by spectrophotometric read at 260 nm by Nanodrop (Thermo Scientific). Reverse transcription of RNA using M-MLV Reverse Transcriptase was performed to obtain 250 ng cDNA. Relative mRNA expression levels of indicated anti-apoptotic genes were detected by using Light Cycler® 480 SYBR Green I Master. List of primers are shown in Table 1.

7.6 Statistical Analysis

The experimental groups were compared according to the expression levels of the implantation markers from 48th hour to 96th. Experiments were repeated three times independently of each other. Wilcoxon's Signed Rank Test was performed to compare hour and experimental group effect on the expression of genes for statistical significance. GAPDH gene was selected as an internal control of gene expression to normalize the gene expressions. The normalization was performed by subtracting Ct of a reference gene from target gene Ct (a target gene) − Ct (GAPDH). For the expression difference from 48th hour to 96thhour; Δ*ΔCT* values were measured which is $_{[}Ct_{48}$(target gene) $-Ct_{48}$(GAPDH)]- $_{[}Ct_{96}$(target gene) $-Ct_{96}$(GAPDH)]. The results were presented as -Δ*ΔCt to* observe the gene expression change relative to the 48th-hour expression, normalized to GAPDH. The -Δ*ΔCt* values

of the experimental groups between the 48 and 96 hours were represented as boxplot for each gene. The biological and technical replicates were shown with various graphical shapes. Significance level was set at 5% ($p < 0.05$). P values lower than 0.05 were indicated with (*), and those lower than 0.01 were indicated with (**).

GENE (m/h)		MOUSE	HUMAN
Cdh1 /CDH1	**F**	ATCCTCGCCCTGCTGATT	CGAGAGCTACACGTTCACGG
	R	ACCACCGTTCTCCTCCGTA	GGGTGTCGAGGGAAAAATAGG
Mmp9 /MMP9	**F**	TCCCCAAAGACCTGAAAACC	TGTACCGCTATGGTTACACTCG
	R	CTGCTTCTCTCCCATCATCTG	GGCAGGGACAGTTGCTTCT
Lıf/LIF	**F**	AAACGGCCTGCATCTAAGG	CCAACGTGACGGACTTCCC
	R	AGCAGCAGTAAGGGCACAAT	TACACGACTATGCGGTACAGC
Esr1/ESR1	**F**	TCCAGCAGTAACGAGAAAGGA	GAAAGGTGGGATACGAAAAGACC
	R	AGCCAGAGGCATAGTCATTGC	GCTGTTCTTCTTAGAGCGTTTGA
ıtgaV/ITGAV	**F**	AGCCAGAGGCATAGTCATTGC	ATCTGTGAGGTCGAAACAGGA
	R	CATCTCCATTGGTATCAGTGGC	TGGAGCATACTCAACAGTCTTTG
Hbegf/HBEGF	**F**	CTTAGTGGAACCTCGCTGTC	ATCGTGGGGCTTCTCATGTTT
	R	AGAAAGAGCTTCAGCATCACC	TTAGTCATGCCCAACTTCACTTT
ErbB1/ERBB1	**F**	ATGAAAACACCTATGCCTTAGCC	AGGCACGAGTAACAAGCTCAC
	R	TAAGTTCCGCATGGGCAGTTC	ATGAGGACATAACCAGCCACC
ErbB2/ERBB2	**F**	ACCGACATGAAGTTGCGACTC	TGTGACTGCCTGTCCCTACAA
	R	AGGTAAGCTCCAAATTGCCCT	CCAGACCATAGCACACTCGG
Wnt 4/WNT4	**F**	AGACGTGCGAGAAACTCAAAG	AGGAGGAGACGTGCGAGAAA
	R	GGAACTGGTATTGGCACTCCT	CGAGTCCATGACTTCCAGGT
Hoxa10/HOXA10	**F**	GGCAGTTCCAAAGGCGAAAAT	CTCGCCCATAGACCTGTGG
	R	GTCTGGTGCTTCGTGTAAGGG	GTTCTGCGCGAAAGAGCAC
Hoxa11/HOXA11	**F**	ATTGAGCCCGCCACTAAATGG	CCCGCAGTCTCGTCCAATTT
	R	TCGAAAAACTGGTCGAAAGCC	AGGCTGTCTCGAAAAACTGGT
Ihh/IHH	**F**	CTCTTGCCTACAAGCAGTTCA	AACTCGCTGGCTATCTCGGT
	R	CCGTGTTCTCCTCGTCCTT	GCCCTCATAATGCAGGGACT
Fn1/FN1	**F**	GATGTCCGAACAGCTATTTACCA	AGGAAGCCGAGGTTTTAACTG
	R	CCTTGCGACTTCAGCCACT	AGGACGCTCATAAGTGTCACC
Lama1/LAMA1	**F**	ACTATGCCGTCAGCGATACAG	TTAGCCACCGGGAACCTAAAG
	R	GGCACCAGCTTTGAATAATACGA	GCCATAGCAGATACACATGCCT
Nid1/NID1	**F**	CCCAGCTTCGGCTCAGTAG	TCTACGTCACCACAAATGGCA
	R	AACGGGGATAAGTCTTCTCGAT	GCGACTGCACCGAATGTTG
Pgr/PGR	**F**	GCCAGCCAGAGCCCACAATA	GAGCACTGGATGCTGTTGCT
	R	TGTGCTGCCCTTCCATTGCC	GGCTTAGGGCTTGGCTTTC
Sell/SELL	**F**	TGCAGAGAGACCCAGCAAG	ACCCAGAGGGACTTATGGAAC
	R	CAGACCCACAGCTTCAGGAT	GCAGAATCTTCTAGCCCTTTGC
Tro/TRO	**F**	AACTGCCTAACAAGGGAAGAGT	CACCCTTGCAGGTCCTAAAGC
	R	CCACCAAAGCTAATACCAGCA	GGGAACTGGCTGACTCATTGG
Col1/COL1	**F**	GCTCCTCTTAGGGGCCAC T	GAGGGCCAAGACGAAGACATC
	R	ATTGGGGACCCTAGGCCAT	CAGATCACGTCATCGCACAAC

Col4/COL4	**F**	CTGGCACAAAAGGGACGAG	GGGATGCTGTTGAAAGGTGAA
	R	ACGTGGCCGAGAATTTCACC	GGTGGTCCGGTAAATCCTGG
Col3/COL3	**F**	ACGTAGATGAATTGGGATGCAG	TTGAAGGAGGATGTTCCCATCT
	R	GGGTTGGGGCAGTCTAGTG	ACAGACACATATTTGGCATGGTT
Col5/COL5	**F**	TTGGAAACCTTCTCCATGTCAGA	TACAACGAGCAGGGTATCCAG
	R	TCCCCAGTGGGTGTTATAGGA	ACTTGCCATCTGACAGGTTGA
Tımp3/TIMP3	**F**	ACACGGAAGCCTCTGAAAGTC	CATGTGCAGTACATCCATACGG
	R	ACTTTGTGGAGAGGTGGGAC	CATCATAGACGCGACCTGTCA
Gapdh/GAPDH	**F**	AGGTCGGTGTGAACGGATTTG	ACAACTTTGGTATCGTGGAAGG
	R	TGTAGACCATGTAGTTGAGGTCA	GCCATCACGCCACAGTTTC

Table 1. ***List of primers and their sequences used in qPCR.***

Chapter 3

RESULTS

1. CHARACTERIZATION OF MOUSE ENDOMETRIAL CELLS

Separately cultured epithelial and stromal cells were characterized by protein expression level with Western Blot and IF analysis of epithelial and mesenchymal markers as pancytokeratin (ecto/endodermal marker) and vimentin (a mesenchymal marker) for confirmation of expression of intermediate filament proteins to show their origin. As expected, epithelial cells reacted negatively with vimentin and positively with pancytokeratin, on the other hand, stromal cells were negative for pancytokeratin and positive for vimentin. Immunofluorescence staining and Western Blot of the cells for the corresponding markers was also performed for characterization of the cells. The results were as depicted in Figure 16.

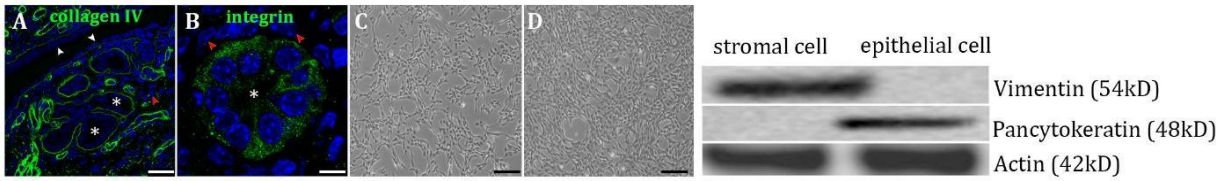


Figure 16. ***Characterization of endometrial cells.*** *The antibodies were tested to be used as implantation markers in positive controls. Collagen IV (A) and integrin (B) showed positive staining with a uterus. Endometrial glandular epithelium (A, B, asterisks), luminal epithelium (A, white arrowhead) and stromal cells (A, B, red arrowheads) can be seen. After isolation epithelial (C) and stromal cells (D) are cultured separately. Epithelial cells were positive for cytokeratin and stromal cells were positive for vimentin as shown by WB results. Blue (Hoechst): Nuclei. Bars=20 µm (A), 5 µm (B), 50 µm (C).*

2. CHARACTERIZATION OF HUMAN UMBILICAL CORD DERIVED MESENCHYMAL STEM CELLS

Since the umbilical cord is one of the sources of mesenchymal stem cells, the cord sections were stained with HE and immunofluorescent markers for phenotyping. Cells were characterized by their adherence of the plastic surface in both explant and primary cultures. Dual-labeled three-dimensional images with anti-pancytokeratin (red) and vimentin (green) showed that vimentin was exclusively expressed in MSCs as expected due to its mesenchymal origin. Also, MSCs were negative for the cytokeratin (Figure 17).

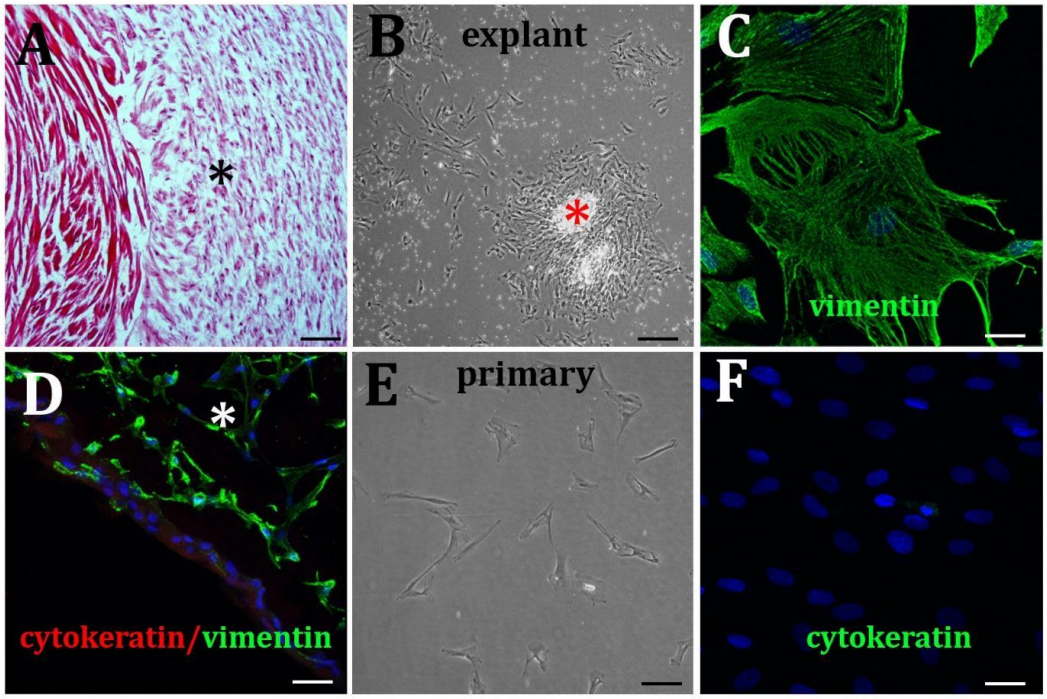


Figure 17. ***The culture of hMSCs.*** *Umbilical cord stained with HE to show the structure (A). Perivascular MSCs can be seen (A, asterisk). Explant of the cells was obtained. (B, asterisk). Cultured MSCs were positive for vimentin (C). Cord sections were stained for vimentin and cytokeratin (D). Peri-amniotic MSCs can be seen (D, asterisk). Primary cultured MSCs were also obtained (E). MSCs were negative for cytokeratin (F). Blue (Hoechst): Nuclei. Bars=25 µm (C, F), 50 µm (B, E, D), 100 µm (A).*

To determine the cell morphology of undifferentiated MSCs, the cellular skeleton, mitochondria and histone protein in the nuclei were marked by IF (Figure 17). Beta-tubulin, vimentin and actin staining of the cells gave the general idea of the MSCs morphology. The fibroblast type cells are further stained for histone and mitochondria to obtain details of the MSCs. Histone proteins were found in the nuclear portion and mitochondria were found especially in peri-nuclear portions of the cells as expected. Super-resolution images of the tubulin structures were also obtained in the MSCs (Figure 18, E, G).

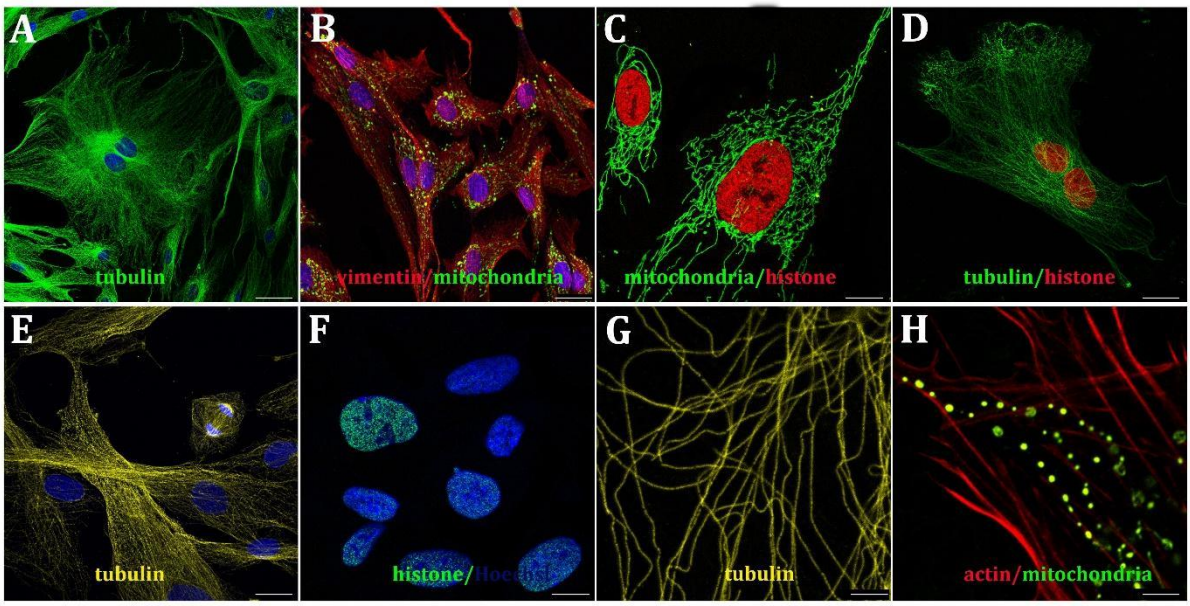


Figure 18. ***Cytoplasmic and nuclear structure of the undifferentiated hMSCs.*** *Cellular structures were validated with tubulin staining (A, B, D, E, G). Mitochondrial distribution of the cells was also obtained (B, C, H). Histone protein was detected in the nucleus (C, F). Super-resolution images were taken for a more resolved investigation (E, G). Actin was also used for validation of cell structure (H). Blue (Hoechst): Nuclei . Bars=20 µm (A), 25 µm (B, D), 10 µm (C, F), 5 µm (G, H).*

The golden standard of characterization of the mesenchymal cells is to induction of the cells to adipocytes and osteocytes. Immunofluorescence staining of the induced cells showed formation of oil droplets after adipogenic induction. Adipogenic differentiation of HUCSCs was achieved in 21 days. Adipocytic phenotypes in induced HUCSCs were first noticed by the appearance of tiny intracytoplasmic lipid droplets in fusiform shaped cells. Induced cells transformed into a rounder or cuboid shape and retracted their cellular extensions, although a few retained their fusiform shape. MSCs produced rounder cells having numerous, homogeneous lipid droplets. In the nondifferentiated control groups, lipid droplets were not detected (Figure 19, B). Osteogenic induced cells were characterized by their distended cell bodies. Alizarin red S staining for calcium mineralization showed direct evidence of calcium deposits as accumulations between cells. Noninduced cultures did not exhibit any calcium deposits. Osteopontin expression was detected as a fine punctate pattern on the cell surface after induction (Figure 19, H-J).

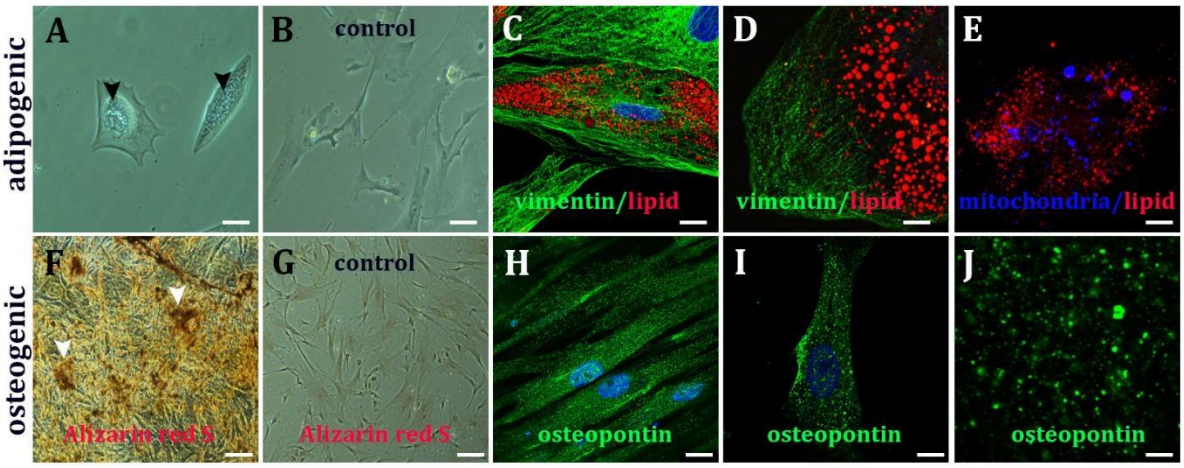


Figure 19. ***Induction characteristics of hMSCs.*** *As a result of adipogenic induction, rounder morphology was observed with the oil droplets in the cytoplasm (A, black arrowhead) when compared to the control (uninduced) cells (B). Adipogenic induction showed increased fat granules stained with oil red O (C, D, E, red). Osteogenic induction was shown by Alizarin Red S to stain Ca accumulations (F, white arrowheads) compared to control (uninduced) cells (G). Punctuate appearance of the osteopontin was observed in the osteogenic induced cells (H, I) also with the super resolution microsopy (J). Blue (Hoechst): Nuclei. Bars= 50 µm (A, B, F, G), 20 µm (H), 10 µm (C, E, I), 5 µm (D), 3 µm (J).*

Lastly, the surface markers of the cells were detected with the multiple coloring immunophenotyping. Surface markers of the CD34, CD45 negative and CD73, and CD105 positive cells were labeled and phenotyped. The results were outlined in the figure 20.

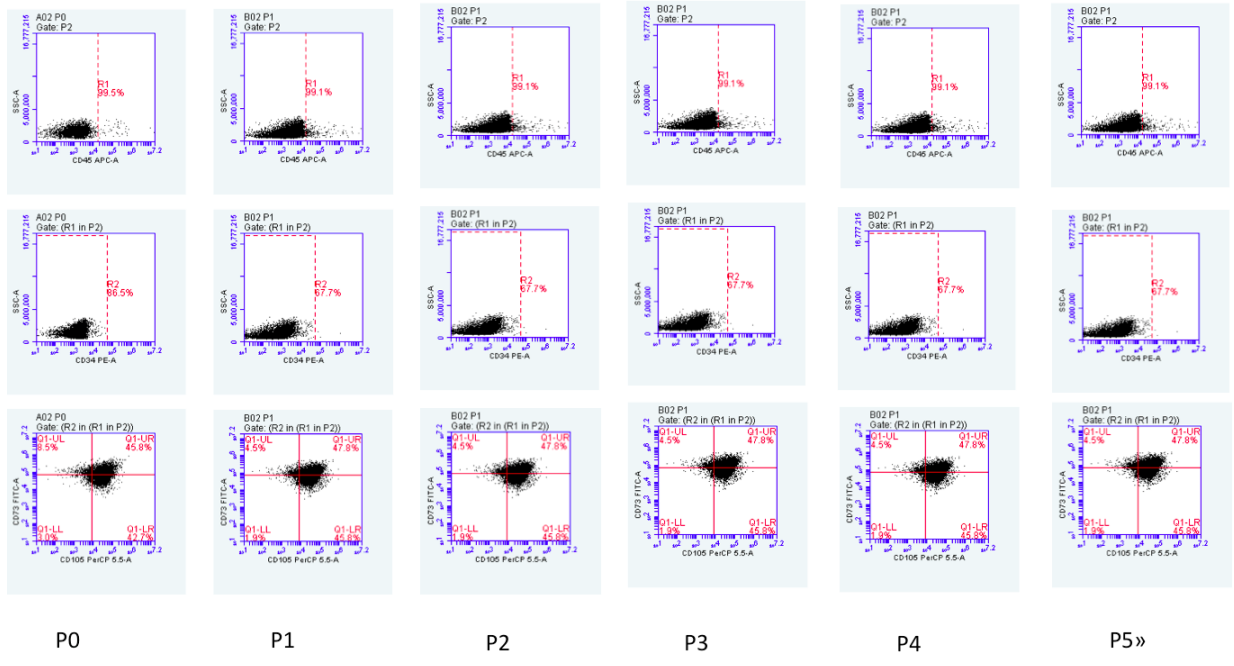


Figure 20. ***hMSCs immunotyping.*** *CD34 and CD 45 negative cells were selected. From these cells, CD73 and CD105 positive cells were gated.*

The common positive and negative CD markers of passages 4 and 5 were more prominent as shown in figure 21. Therefore, these passages were generally used in induction experiments. This was also the case for implantation experiments.

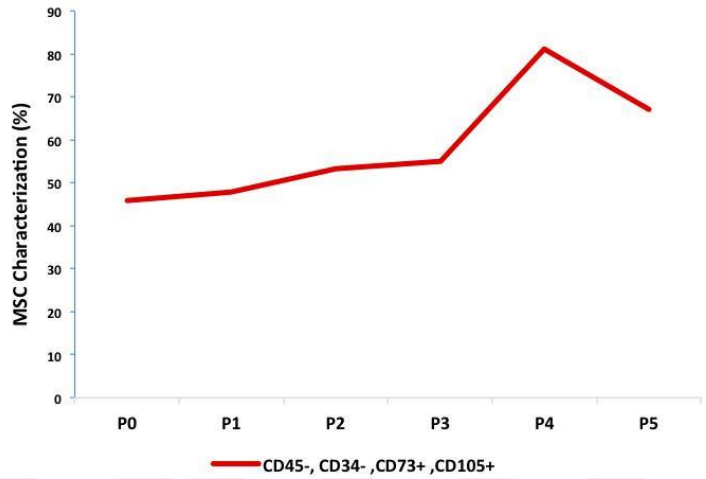


Figure 21. ***Comparison of the surface marker profiles of the cells in P 0-5 between passages.***

3. EVALUATION OF EXPERIMENTAL GROUPS

Experimental groups were constructed as; routine IVF (R), and blastocyst cultured on film (F), blastocyst on MSC cultured film (MSC), blastocyst on film covered with endometrial co-culture (ECC), MSC cultured on film (FM), and endometrial coculture on film (FE) were evaluated at 48h and 96h. SEM, IF/Confocal imaging, qPCR and Western Blot methods were used to compare the implantation markers. Comparative analysis of qPCR results of the experimental groups was investigated in section 4.

3.1 Immunofluorescent Analysis

The blastocysts with the compact ICM and trophectoderm layer and smooth blastocoel were selected for the experimental groups. Blastocysts morphology was observed as the typical appearance of compact ICM and trophectoderm cells. The selected blastocysts were cultured as routine IVF group. Blastocysts have had 3D structures; therefore, imaging was performed in drop culture not to damage their composition (Figure 22).

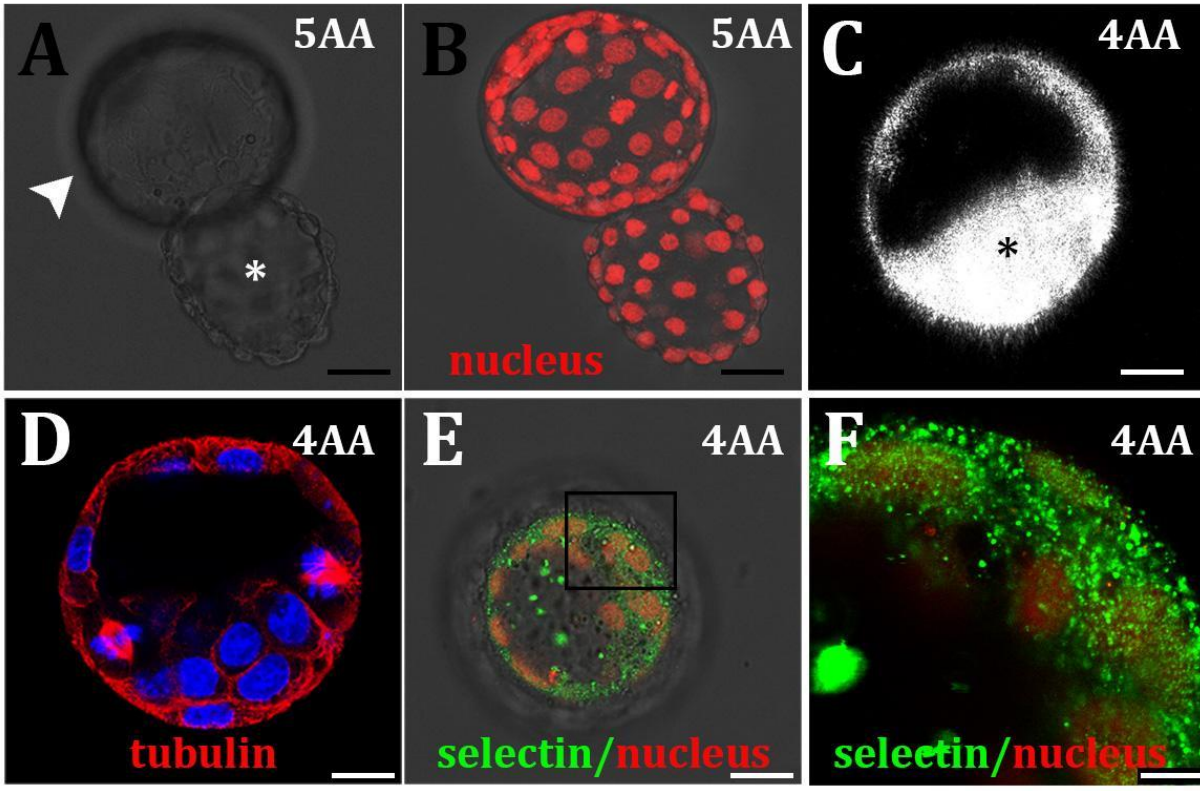


Figure 22. ***The morphology of the selected blastocysts****. DIC image of blastocyst with the hatching ZP (A, arrowhead) and trophoectodermal cells (A, asterisk) can be seen. DIC and 7AAD staining of the hatching blastocyst (B). DLS image of the blastocyst with compact ICM (C, asterisk). Morphology of the blastocyst observed with tubulin staining (D). L-selectin expression on the selected blastocyst was also validated (E, rectangle) especially in the trophectodermal area (F). Blue (Hoechst): Nuclei. Bars= 25 µm (A, B, E), 20 µm (C, D), 8 µm (E).*

According to the statistical analysis (see section 4), IF staining was performed for some of the genes with a significant difference. For this purpose, E-cadherin, MMP9, ER, PR, LIF, fibronectin and entactin were selected to observe the changes of selected markers in the experimental groups.

In the routine IVF group, some nuclei showed of ER and PR positivity along with mild level at the cytoplasm of the cells at 48h (Figure 23). MMP9 expression was observed as punctuated formation which is monitored by E-cadherin fibrils in the routine IVF culture. Entactin was particularly prominent in the basal membrane of the cells (Figure 23, E). Decrease in E-cadherin expression at hour 96 was also prominent in routine IVF blastocysts (Figure 23, D).

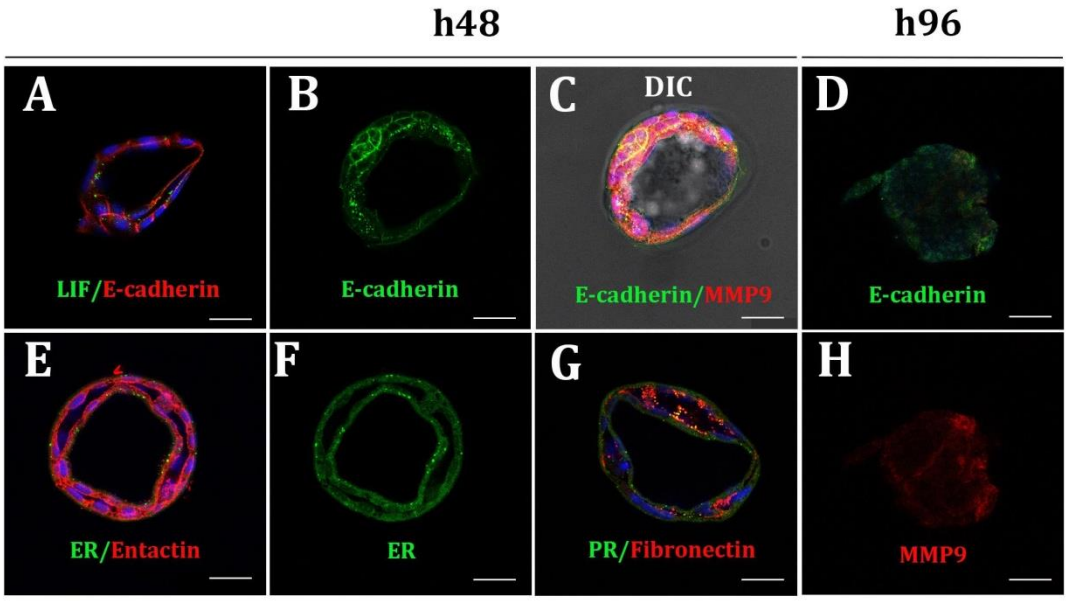


Figure 23. ***Immunofluorescent stainings in routine culture group****. The decrease in e-cadherin is prominent at hour 96. MM9 increase was not reflected in this staining. LIF and MMP9 are seen as punctate depositions (A,C). E-cadherin contructs a filamentous in between the cells (A, B). ER and PR were seen as faint staining in nuclear and cytoplasmic areas. Blue (Hoechst): Nuclei. Bars=25 µm*

In the group of blastocysts on film, ER, MMP9 and fibronectin stainings were found to be milder than the routine group at hour 48. LIF and entactin were evident in the film cultured blastocyst at 48h. (Figure 24, A, C). E-cadherin had decreased and LIF had increased at hour 96 which was also validated with the qPCR.

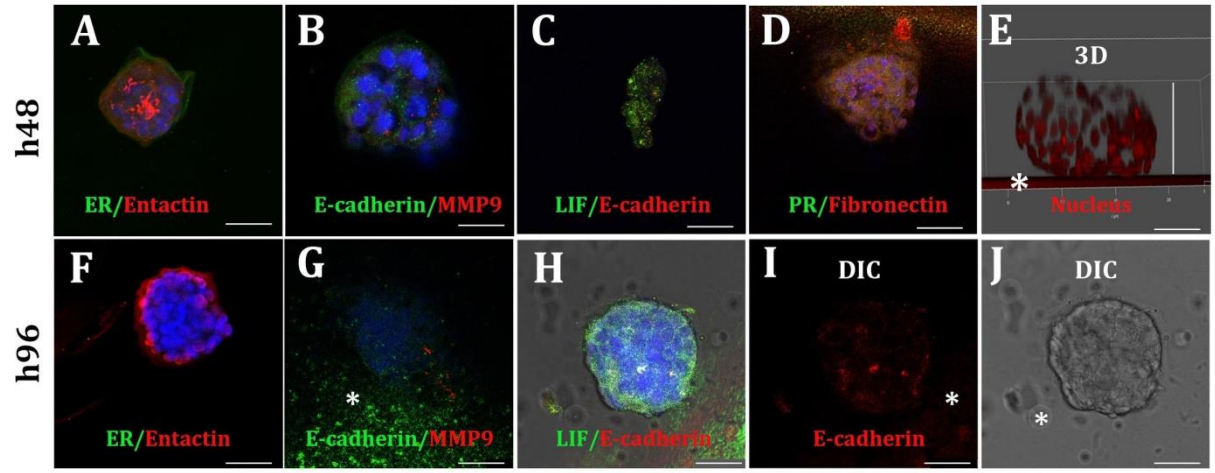


Figure 24. ***Immunofluorescent stainings in film group.*** *The decrease in e-cadherin (B, G, E) can be seen. LIF had increased (C, H). ER had a faint staining (A, F). The height of the blast of the blast on the film (asterisk) was 70 µm (E, vertical line). Polymeric film gave autofluorescence (G, I; asterisk). Blastocyst with the DIC image of the film can be seen (J, asterisk). Blue (Hoechst): Nuclei. Nucleus: 7AAD. Bars= 25 µm (A-D, F-J); 10 µm (E).*

In the ECC culture, co-culture areas formed in Matrigel were indicated by fluorescent / DIC images. In general, Matrigel is rich in entactin and fibronectin which are the natural component of the Matrigel. The positivity of entactin and fibronectin in ECC was shown in Figure 25. The increase in entactin expression was not prominent. Decrease in E-cadherin and increase in PR and LIF were validated with the IF results. Both the blastocyst and the endometrial cells were positive for the LIF and entactin expression. The MMP9 staining were positive at the cytoplasm at hour 48 however, nuclear positivity at hour 96 observed in ECC culture. PR was positive especially in the endometrial cells at hour 96 of ECC (Figure 25).

In the MSC culture of the blastocysts, E-cadherin expression was evident especially on the blastocyst at hour 48. Increase in LIF, MMP9, Entactin and decrease in E-cadherin and fibronectin expression from hour 48 to 96 were also depicted in the figure 26. Fibronectin positivity observed in the MSCs under the blastocyst (Figure 26, C). PR stainings were faint at hour 96 of ECC group. Increase in ER expression observed, and ER was positive in both nucleus and cytoplasm of the cells at hour 96 (Figure 26, K).

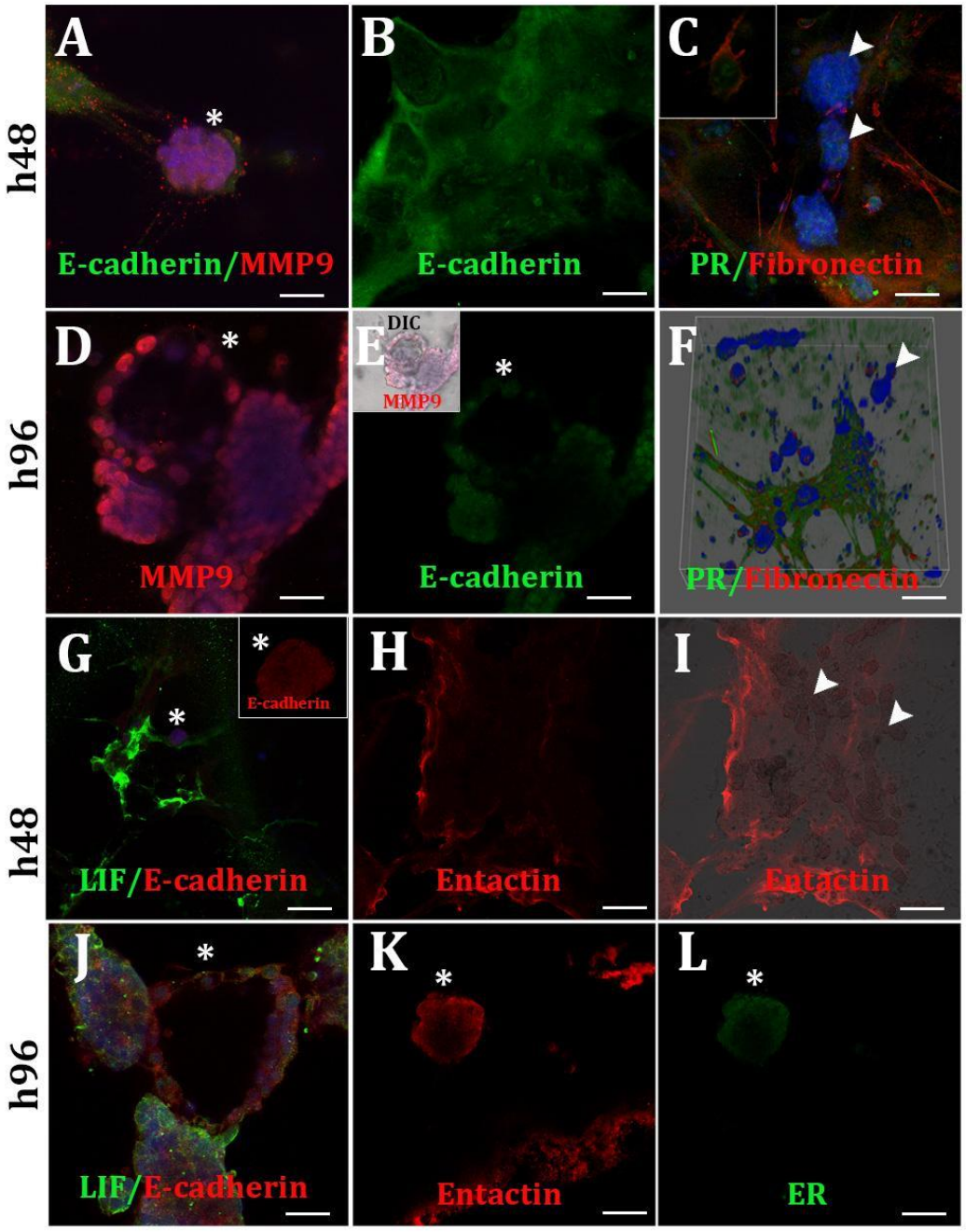


Figure 25. ***Immunofluorescent stainings in ECC group.*** *E-cadherin and fibronectin had decreased during the culture period (A, B, E, C, F). LIF (G, J), PR (C, F), entactin (H, I, K) and ER (L) had increased during the culture period. Some of these changes were recorded as significant (see text). Since Matrigel is an extracellular matrix it also had some amount of entactin (H). Blastocysts (asterisk) resembled the conglomerates of ECC cells (arrows), so previous markings were needed. The inlets show images of a blastocyst in the Matrigel (E). Blue (Hoechst): Nuclei. Bars= 25 µm, exceptions: 50 µm (F), 75 µm (B), 150 µm (I, J).*

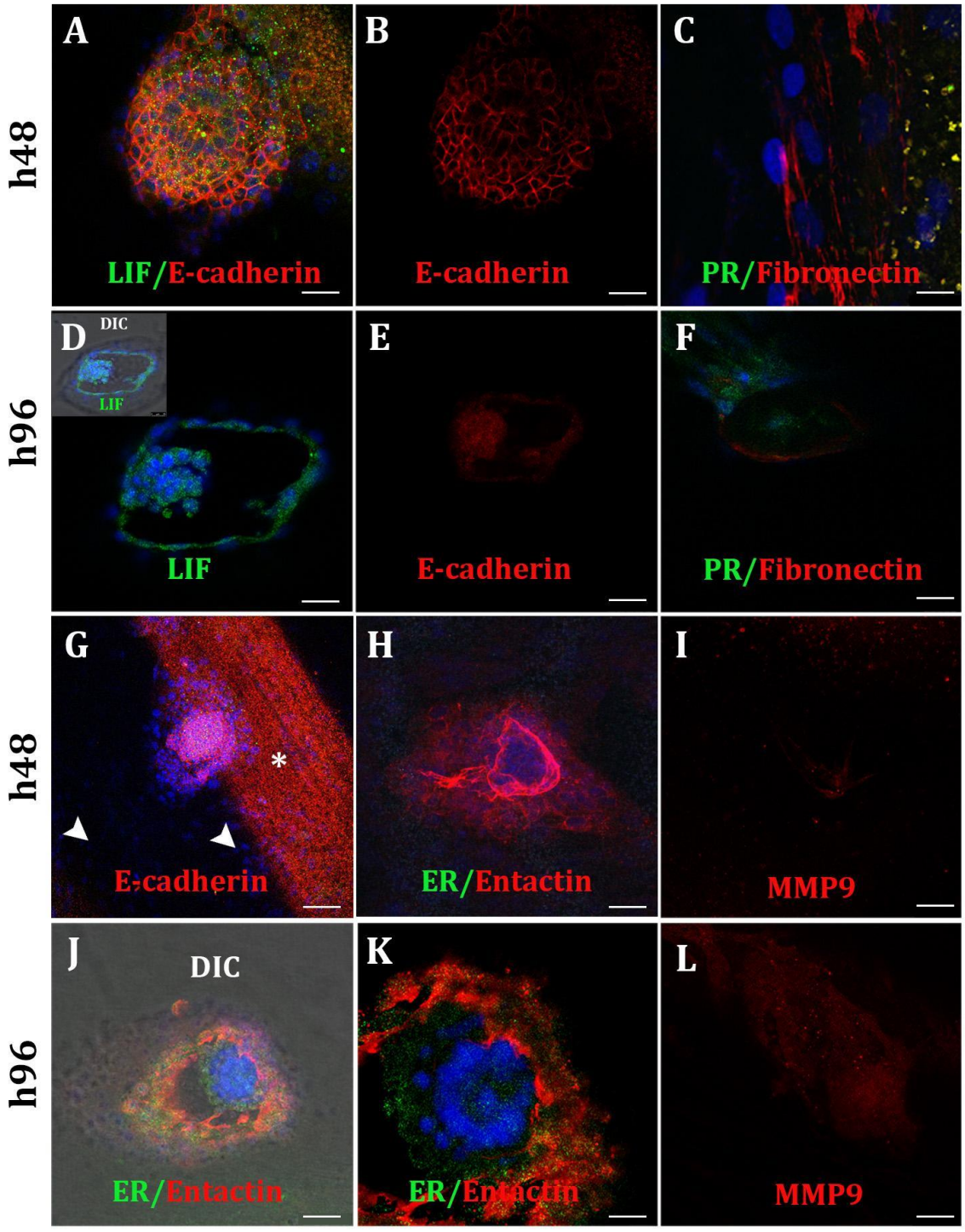


Figure 26. ***Immunofluorescent stainings in MSC group***. *E-cadherin (A, B, E) and fibronectin has decreased (C, F) during the culture period. The difference in MMP expression was not prominent. Entactin has decreased and ER has increased (H, J, K). PR has increased to some extent (C, F). The blastocysts were usually in in close contact with the underlying polymeric film (asterisk, G) and the MSC lying beneath (arrowheads, G). Blue (Hoechst): Nuclei. Bars= 25 µm (A-D, F), 40 µm (E, J, G, L), 10 µm (K), 100 µm (L).*

3.2 SEM Analysis

Scanning electron microscope (SEM) analysis of the experimental groups was performed to have information about the surface topography and composition of the film and cultured cells. The blastocyst on the routine IVF drop was also imaged to observe the surface structure of mouse blastocyst. The cell coated samples showed that the cells attached to the collagen-coated film fragment with the cellular extensions (Figure 27).

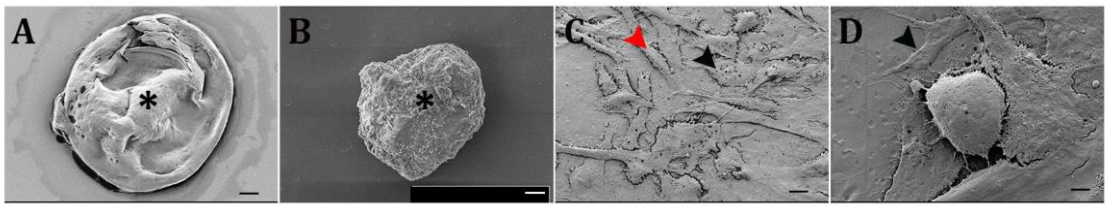


Figure 27. ***SEM analysis****. Routine IVF (A, asterisk) and blastocyst cultured on polymeric film (B, asterisk) were observed. Epithelial (C, black arrowhead) and stromal (C, red arrowhead) cells on the collagen-coated film can be observed. Cellular extensions of the MSCs on the collagen-coated film can be seen (D, arrowhead). Bars= 10 µm (A), 2 µm (B), 15 µm (C), 4 µm (D).*

3.3 Western Blot Analysis

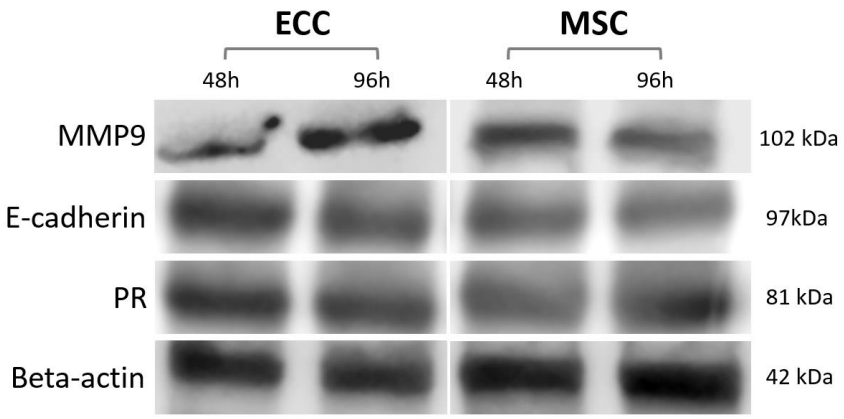


Figure 28. ***WB analysis****. The increase in MMP9 and the decrease in E-cadherin expression in ECC were prominent at the protein level. PR expression showed a slight decrease in expression in ECC. Increase in PR in the MSC group was evident.*

WB analysis was performed for the comparison of the protein expression of genes that have been found to have a significant change in gene expression (Chapter 4). There was a marked difference in MMP9 expression at the protein level between ECC and MSC group. E-cadherin decrease was more

prominent in the ECC group. On the other hand, an increase in expression of PR was more evident in the MSC group compared to ECC (Figure 28).

4. COMPARATIVE ANALYSIS OF EXPERIMENTAL GROUPS

The data obtained from each experimental group were compared based on their expression levels. The data of 6 experimental groups of routine IVF, film (F), ECC (E), MSC (M), film and ECC (FE), film and MSC (FM) were also represented with different colors. Biological replicates of the experimental groups were depicted as different geometrical shapes with 2 technical replicates and the corresponding color of the experimental groups. According to the results of heatmap expression of the data, box plot graphics were constructed for the interpretation of the groups established with and without the blastocyst.

The data on which cycle of gene expression was monitored during the repeats were run on the R Lab Statistics Platform. In the gene expressions, the increase and decrease in the 96th hour according to the 48th hour were determined. Since the aim was to observe the gene expression change of the selected markers, the heatmap was constituted according to the mean values of the -ΔΔCT values.

In order to evaluate the adhesion, invasion and functional status of the blastocyst, 23 different genes were examined for each group, 3 of which were biological and 2 technical replications of each biological repeat. LIF, ErbB1, ErbB2, HBEGF, ER, and PR were selected as functional; HOXA10, HOXA11, Wnt4, and IHH were selected as developmental; MMP9 and TIMP3 were selected as invasion; E-Cadherin, Fibronectin, L-Selectin, Laminin, Entactin, Integrin, Trophinin, Collagen (I, III, IV, V) were selected as adhesion genes. In addition to blastocyst cultured groups, the blastocyst free groups were also examined.

Blastocysts used in the experiments were from the mouse and the MSC and FM (Film+ MSC) cultures were constructed with human umbilical derived mesenchymal stem cells. The mouse and human primers designed for the 23 different genes and housekeeping gene of GAPDH were validated with the RT-PCR. According to the heatmap representation of the experimental data, the mouse and human primers designed for the corresponding markers gave almost the same expression pattern (Figure 29, 32). Based on this validation, the experimental setup was considered with the human primers for the groups with the MSCs and mouse primers for the other experimental groups.

Among 23 implantation markers, E-cadherin and fibronectin showed distinct expression pattern among all experimental repeats by decreasing in all experimental groups. L-selectin, LIF, Collagen V, III, HBEGF and ErbB1 also depicted similar expression as shown in dendrogram

representation of the markers. Trophinin, ER, integrin, PR and MMP9 expressions were upregulated mostly in ECC group. Collagen IV, I and Wnt4 showed almost no difference from 48 hours to 96 in the ECC group while they showed a decrease or stable expression in the film (F) and MSC (M) group. Hoxa11, IHH, Hoxa10, TIMP3, ErbB2, and entactin depicted an increase in expression distinctively in ECC group, on the other hand, their expressions were evaluated to be more stable in other experimental groups. The experimental groups of FM (film+ MSC) and FE (film+ ECC) showed almost similar expression pattern with a striking expression of MMP9 and laminin on FE group. The decrease in L-selectin, trophinin, Collagen III, V, and entactin was also evaluated in the FM group (Figure 29).

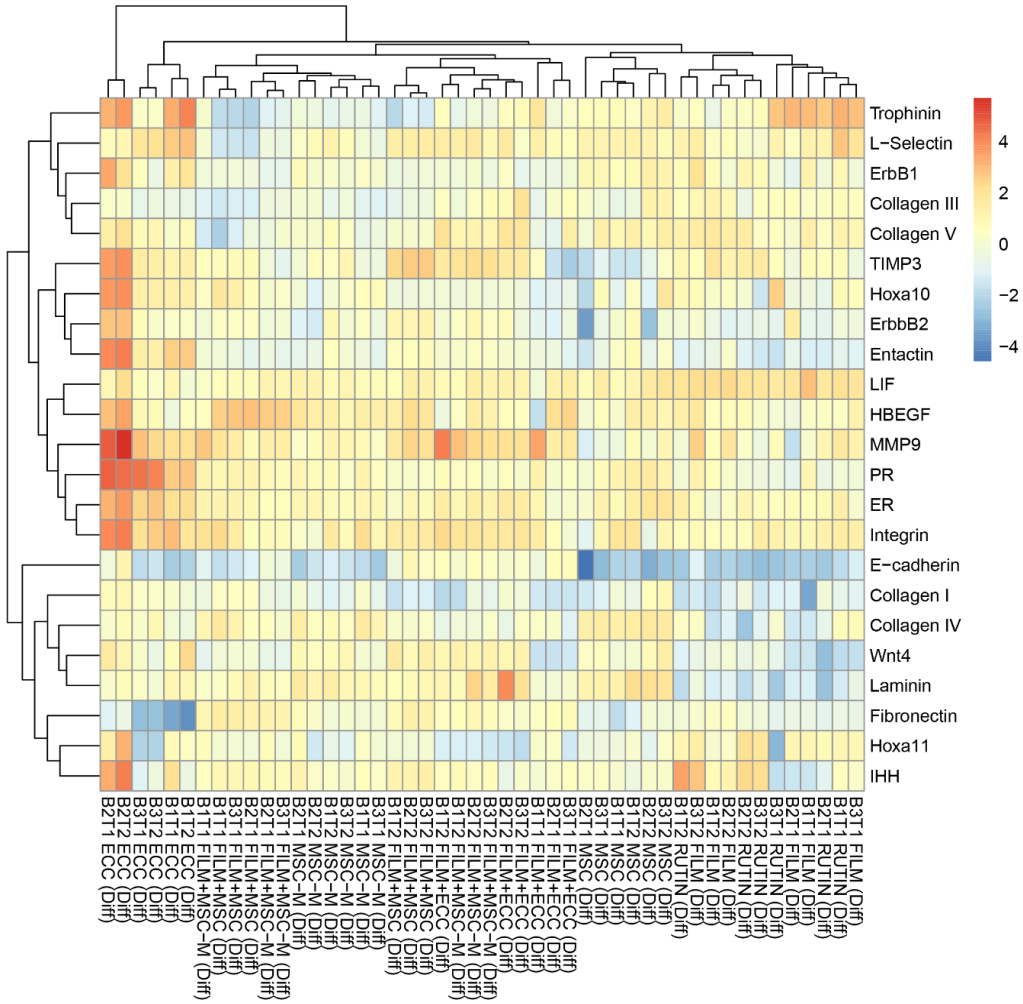


Figure 29. ***Heatmap representation of the differences between the 48th and 96th hours of expression of 23 genes in all groups with mouse and human primers.*** *Blue colour represents the down-regulation, red colour depicts the up-regulation of the gene from hour 48 to 96 for corresponding RT-PCR replicate. Dendrogram representation shows the hierarchical relationship between the experimental results. Scale bar (right-top) shows the mean of -ΔΔCT values unit representation of the colour codes depicted in the heatmap graph. RUTIN=Routine IVF, FILM=Film cultured blastocyst, MSC= Mesenchymal Stem Cell Culture of the blastocyst, ECC=Endometrial Co-Culture of the blastocyst, FM=Film cultured MSC, Film+ ECC=Film cultured ECC, Film+ MSC= Film cultured MSC, MSC-M= Mesenchymal Stem Cell Culture of blastocyst with mouse primers, FILM+MSC-M= Film cultured MSC with mouse primers.*

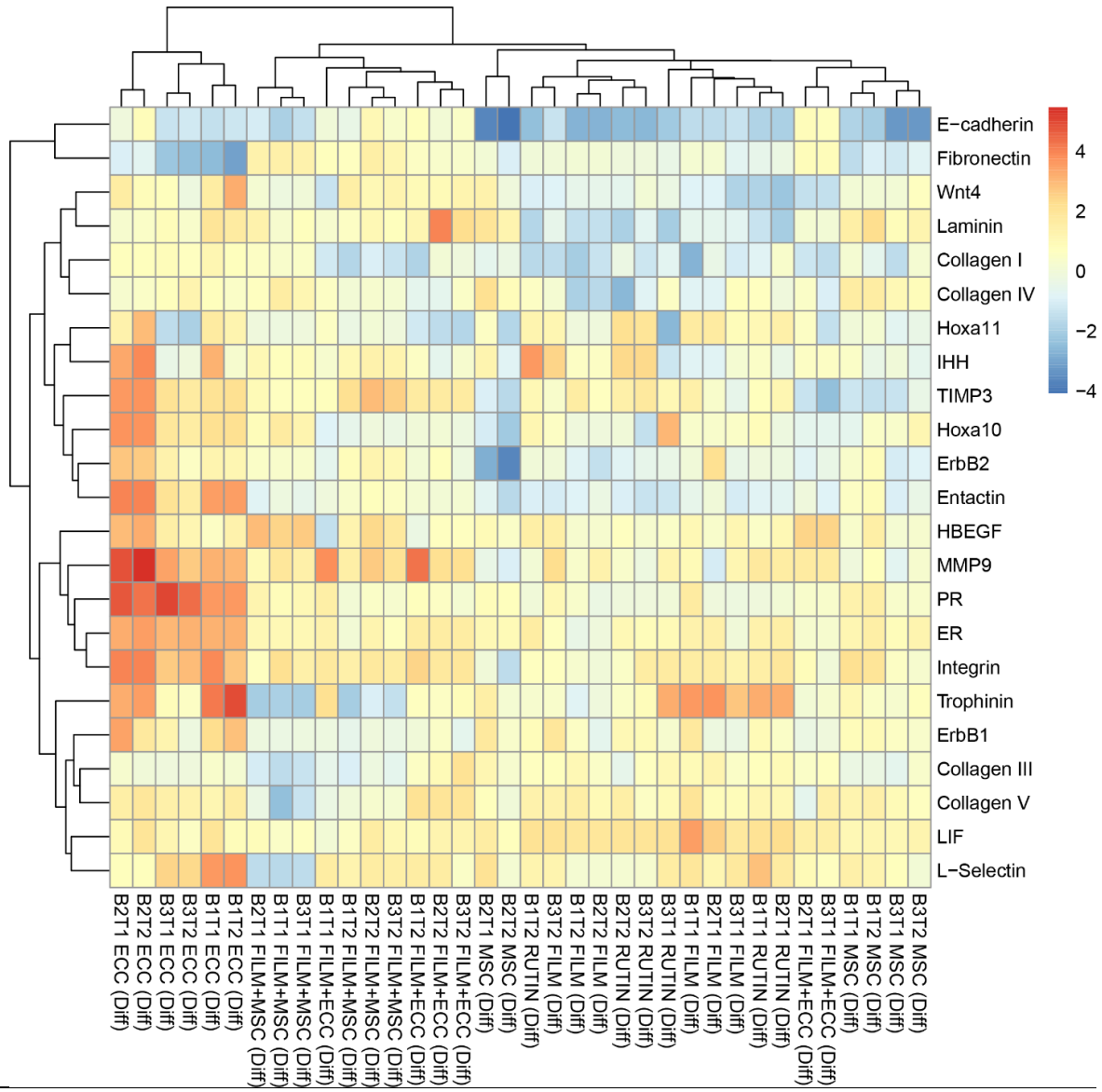


Figure 30. ***Heatmap representation of the differences between the 48th and 96th hours of expression of 23 genes in all groups.*** *Blue color represents the down-regulation, red color depicts the up-regulation of the gene from hour 48 to 96 for corresponding RT-PCR replicate. Dendrogram representation shows the hierarchical relationship between the experimental results. Scale bar (right-top) shows the mean of -ΔΔCT values unit representation of the color codes depicted in the heatmap graph. RUTIN=Routine IVF, FILM=Film cultured blastocyst, MSC= Mesenchymal Stem Cell Culture of the blastocyst, ECC=Endometrial Co-Culture of the blastocyst, FM=Film cultured MSC, Film+ ECC=Film cultured ECC, Film+ MSC= Film cultured MSC.*

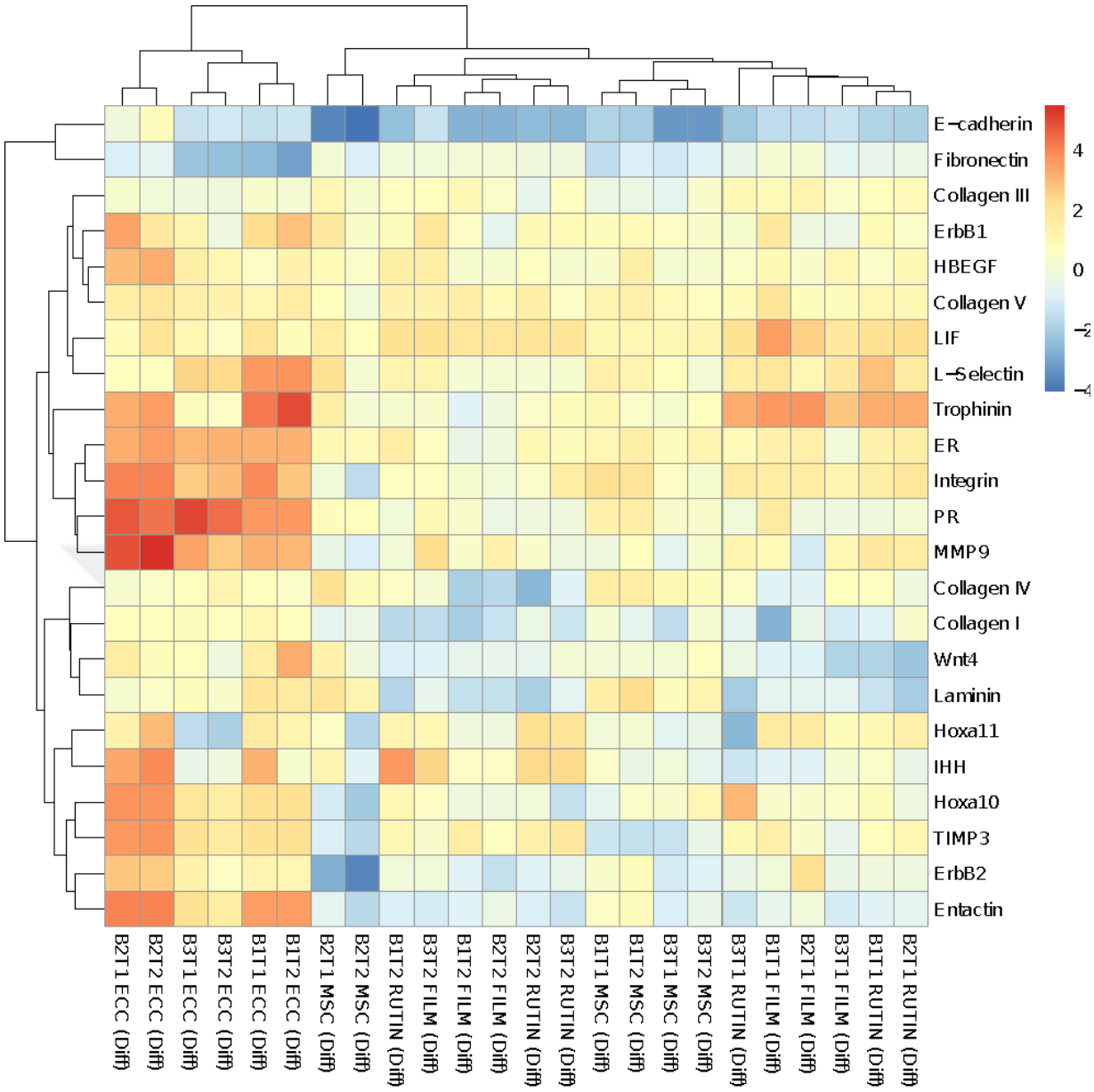


Figure 31. ***Heatmap representation of the differences between the 48th and 96th hours of expression of 23 genes in blastocyst cultured groups.*** *Blue color represents the down-regulation, red color depicts the up-regulation of the gene from hour 48 to 96 for corresponding RT-PCR replicate. Dendrogram representation shows the hierarchical relationship between the experimental results. Scale bar (right-top) shows the mean of -ΔΔCT values with the representation of the color codes depicted in the heatmap graph. RUTIN=Routine IVF, FILM=Film cultured blastocyst, MSC= Mesenchymal Stem Cell Culture of the blastocyst, ECC=Endometrial Co-Culture of the blastocyst.*

The expression pattern of the implantation related genes was also depicted with the four experimental groups which were constructed with the blastocyst. Based on the heatmap; trophinin, ER, integrin, PR, and MMP9 showed marked overexpression in comparison to 48h among other experimental groups. E-cadherin expression decrease was conspicuous in the MSC group. ErbB1, HBEGF, IHH, Hoxa10, TIMP3 and entactin gene expression was also evident in ECC group. Trophinin gene upregulation in routine and film cultured blastocyst was evident (Figure 31).

The experimental repeats were depicted in the boxplot representation of the data. Significant changes were depicted with green star compared to routine IVF group. A significant change in gene expression was also evaluated in comparison with the ECC group which was also depicted as a purple star (Figure 32,33,34).

Adhesion genes of fibronectin, integrin, laminin, trophinin, and entactin showed different levels of expression in experimental groups compared to routine IVF and ECC. When compared to routine IVF, the expression level of fibronectin at 96th hour decreased proportionally to the quarter of the 48th hour in ECC. On the other hand, an approximately two-fold increase in gene expression in FE (film +ECC) and FM (film+ MSC) group was evaluated which was significant to both routine IVF and ECC. In all groups except MSC, increase or stable expression of the fibronectin was significant according to ECC.

Integrin expression in ECC was the only group that shows a meaningful increase compared to routine IVF. The integrin showed approximately eight-fold increment in ECC group. In other experimental groups, integrin expression was also increased significantly according to the ECC group.

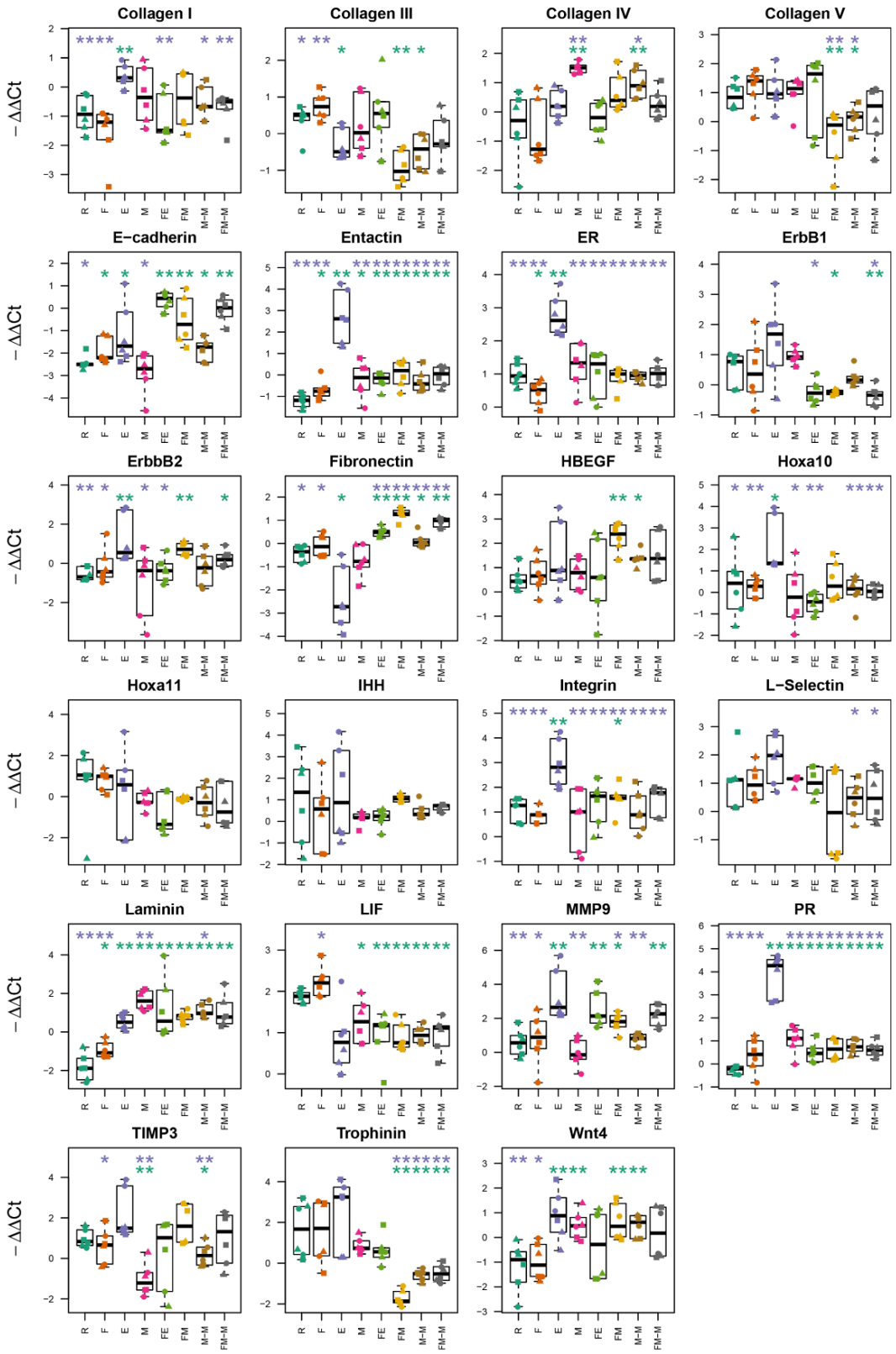


Figure 32. ***Boxplot representation based on the difference of Ct values of 48-96 (Ct_{48}-Ct_{96}) of all experimental groups with mouse and human primers.*** *The median levels of the -ΔΔCt values are also represented in each boxplot. Green stars are statistically significant compared to the R group and purple stars show statistical significance compared to group E. R=Routine IVF, F=Film cultured blastocyst, E=ECC, M=MSC, FE=Film cultured ECC, FM= Film cultured MSC, M-M=MSC with mouse primers, FM-M= Film cultured MSC with mouse primers. (**: p <0.01, *: p <0.05).*

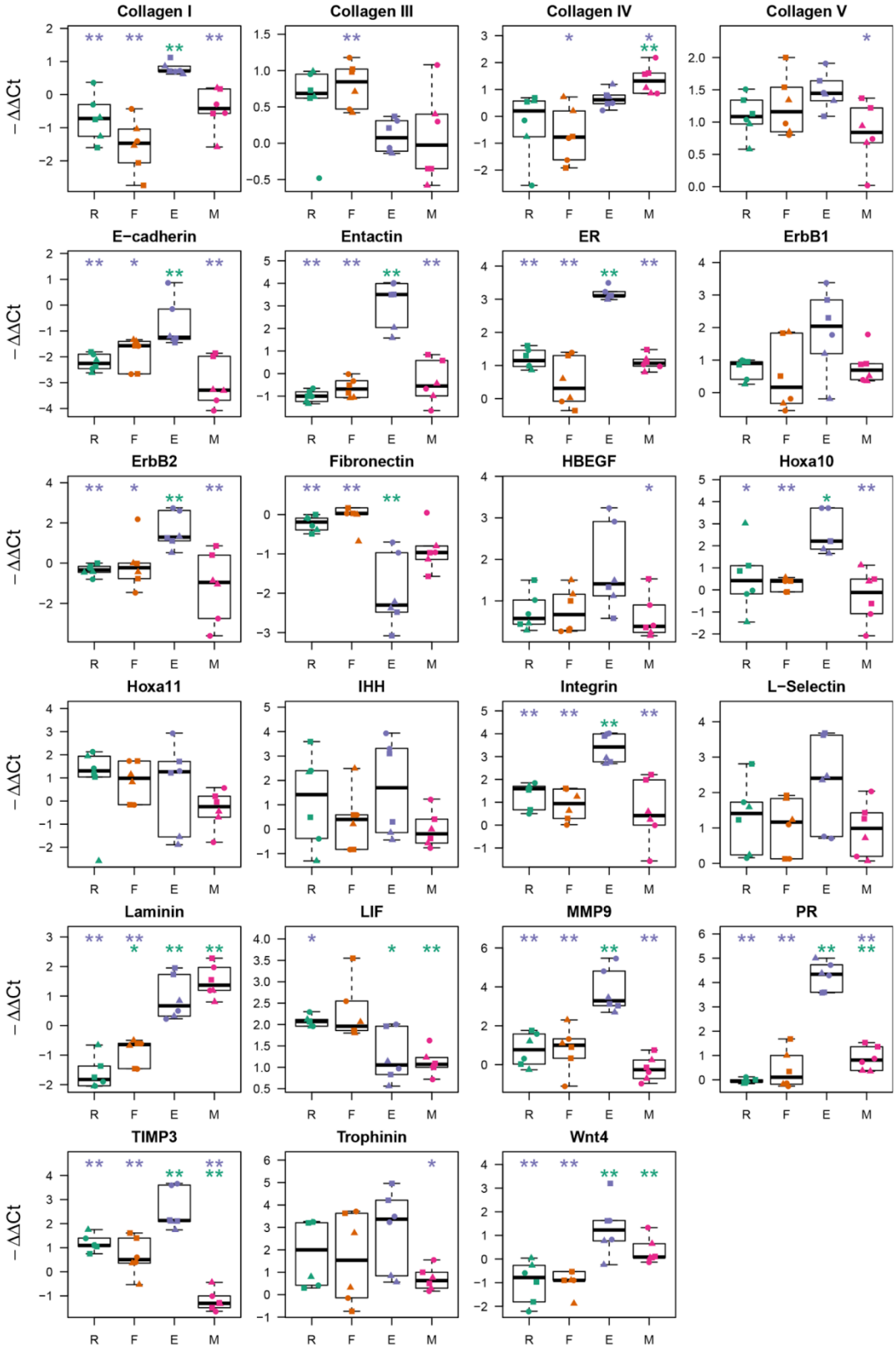


Figure 33. ***Boxplot representation based on the difference of Ct values of 48-96 (Ct_{48}-Ct_{96}) of 4 experimental groups.*** *The median levels of the -ΔΔCt values are also represented in each boxplot. Green stars are statistically significant compared to the R group and purple stars show statistical significance compared to group E. R=Routine IVF, F=Film cultured blastocyst, E=ECC, M=MSC, FE=Film cultured ECC, FM= Film cultured MSC, M-M=MSC with mouse primers, FM-M= Film cultured MSC with mouse primers. (**: p <0.01, *: p <0.05)*

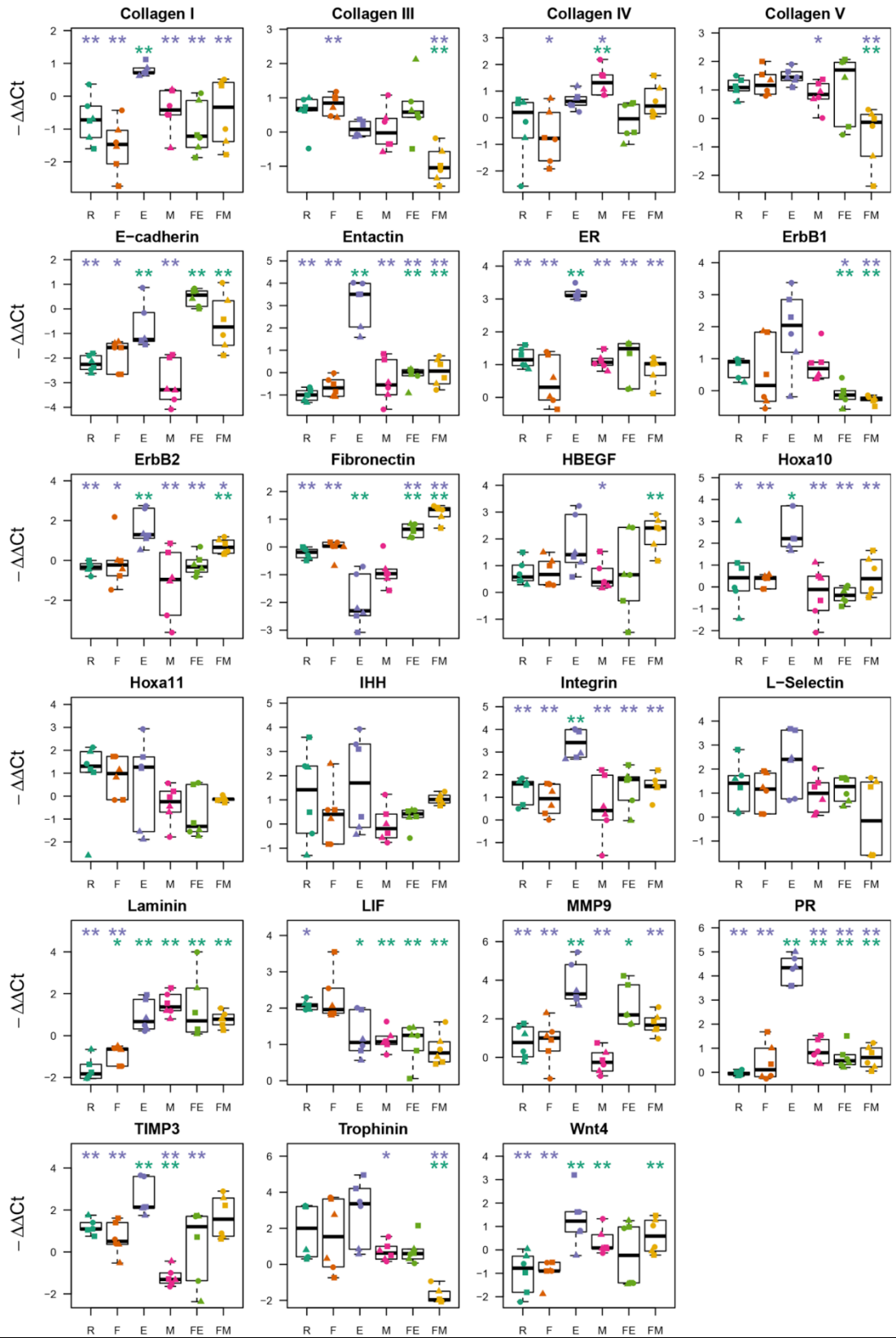


Figure 34. ***Boxplot representation based on the difference of Ct values of 48-96 (Ct_{48}-Ct_{96}) of 6 experimental groups.*** *Green stars are statistically significant compared to the R group and purple stars show statistical significance compared to group E. R=Routine IVF, F=Film cultured blastocyst, E=ECC, M=MSC, FE=Film cultured ECC, FM= Film cultured MSC, M-M=MSC with mouse primers, FM-M= Film cultured MSC with mouse primers. (**: p <0.01, *: p <0.05)*

Trophinin expression decreased four-fold in FM (film+ MSC) group and it was significant compared to both routine IVF and ECC. In the MSC group trophinin expression increase from 48th hour to 96th hour was lower than ECC however, it was significant compared to ECC. Other experimental groups also depicted an increase at 96th hour however, their expression changes were not significant in comparison to routine IVF and ECC.

Entactin expression showed different levels of significant changes in culture groups of FE and FM compared to both routine IVF and ECC. In the routine IVF group, two-fold decrease in entactin expression was statistically significant to ECC. In the ECC, entactin gene expression increase was significant compared to routine IVF group which was approximately twelve-fold of a 48th hour at the 96th hour.

Among collagens, Collagen type I and IV showed significant changes in ECC and MSC respectively compared to routine IVF. In the ECC, Collagen I expression showed a significant increase in expression compared to routine IVF. Except for ECC, Collagen I expression decreased significantly and when compared to ECC the expressions were significant.

Collagen III, IV, and V showed different gene expression profile with different significant levels compared to both routine IVF and ECC. Collagen III expression increased to two-fold in the film group (F) which was statistically significant compared to routine IVF. On the other hand, the two-fold decrease was also prominent in the FM group which was statistically significant in comparison to both ECC and routine IVF.

Collagen IV increase in expression was prominent with approximately two-fold in MSC (M) group which was statistically significant compared to ECC and more significant in comparison to routine IVF. The approximately two-fold decrease of collagen IV was also statistically significant in Film (F) group in comparison to ECC. Collagen IV was increased in all groups except film (F) group with a decrease to half at the 96th hour.

Collagen V expression showed an increase in expression in all groups except FM with a slight change in expression at the 96th hour. In the MSC group, Collagen V expression showed an increase in expression which as significant compared to ECC. The decrease in expression of Collagen V in the FM group was statistically significant to both routine IVF and ECC.

E-cadherin expression decreased in experimental groups of routine IVF, film culture, ECC, MSC, and film+ MSC (FM). It showed a significant decrease in gene expression from hour 48 to 96 in ECC and FM according to routine IVF. On the other hand, the increase of E-cadherin expression was also significant in the FE group compared to routine IVF. In the routine IVF group, E-cadherin

expression decreased four-fold which is statistically significant compared to ECC. Although film cultured blastocyst group (F) showed a significant decrease in expression compared to ECC, MSC and routine IVF culture group showed a more significant decrease when compared to ECC.

L-selectin expression showed no significant change in gene expression in any experimental group compared to both routine IVF and ECC. Only the FM group showed a slight decrease in expression. Although the expression increase of L-selectin was approximately six-fold, the increase in expression was not significant in ECC according to routine IVF.

HBEGF expression showed an increase in expression in all experimental groups with different level of significance. In the ECC group, HBEGF expression increased almost two-fold from 48^{th} to the 96^{th} hour. In the MSC group, HB-EGF expression was also increased meaningfully in comparison to ECC. HBEGF showed an approximately six-fold increase in expression in the FM group which was statistically significant compared to ECC.

HBEGF receptors of ErbB1showed an increase in expression in experimental groups of Routine IVF (R), film (F), ECC (E) and MSC(M). In the experimental groups of FE and FM, ErbB1 expression was almost stable which were significant according to routine IVF and ECC.

ErbB2 expression was increased approximately two-fold in the ECC (E) and FM group which was statistically significant compared to routine IVF. In the experimental groups of routine IVF (R), MSC (M) and film+ ECC (FE) the expression change was statistically significant compared to ECC. Also, the expression changes of the ErbB2 in the film (F) and film+ MSC (FM) groups were significant according to ECC.

ER expression showed the only significant expression in the ECC group compared to routine IVF with approximately eight-fold change. The increase in expression of ER was also significant in other experimental groups compared to ECC.

In comparison to routine IVF, all experimental groups except film group (F), showed increment in the expression of PR. In the ECC group, PR expression increase was statistically significant with an almost twenty-fold change in expression. In the groups of MSC, film+ ECC (FE) and film+ MSC (FM) increment of PR expression was significant according to routine IVF.

Among developmental genes of IHH, Wnt4, Hoxa10, and Hoxa11, only Hoxa10 and Wnt4 showed a significant change in gene expression according to routine IVF and ECC. IHH expression increased in all groups, however, the increase in expressions were not significant according to routine IVF and ECC.

Wnt4 expression was expressive in ECC (E), MSC (M), and film cultured MSC (FM) compared to routine IVF. In comparison to ECC, Wnt4 expression change was meaningful in routine IVF (R) and film culture (F) which showed a decrease in expression almost two-fold.

Hoxa10 expression was significantly increased in nearly four-fold in only ECC group compared to routine IVF. In the routine IVF, a slight increase of Hoxa10 was significant in comparison to ECC. Hoxa10 showed different gene expression profiles in the groups of the film (F), MSC (M), film+ ECC(FE) and film+ MSC (FM) which were significant in comparison to ECC.

Invasion related marker of MMP9 showed an increase in expression almost all groups. In the ECC group, MMP9 showed an almost eight-fold increase which was significant compared to routine IVF. The increase of MMP9 with an almost two-fold increase was also significant in the FE group compared to routine IVF. The expressions of MMP9 in the groups of routine IVF (R), film (F), MSC (M) and film+ MSC (FM) showed significant increment in comparison to ECC.

The increase in expression of TIMP3 was significant in the ECC group compared to routine IVF (R). On the other hand, TIMP3 expression decreased significantly in the MSC group compared to routine IVF and ECC. In the routine IVF, film (F) and film+ ECC (FE) group, TIMP3 expression increased from 48th hour to 96th hour which was expressive in comparison to ECC.

4.1 Cell Vitality

Since the biomaterial used in this study was novel, the cellular viability of blastocyst had been considered. For the viability analysis of cells in cultures at hour 48 and 96, staining was performed for each group using the live cell / dead cell kit. As a result of staining, the living cells absorbed green and the dead cells absorbed red fluorescence. The results were outlined in figure 35.

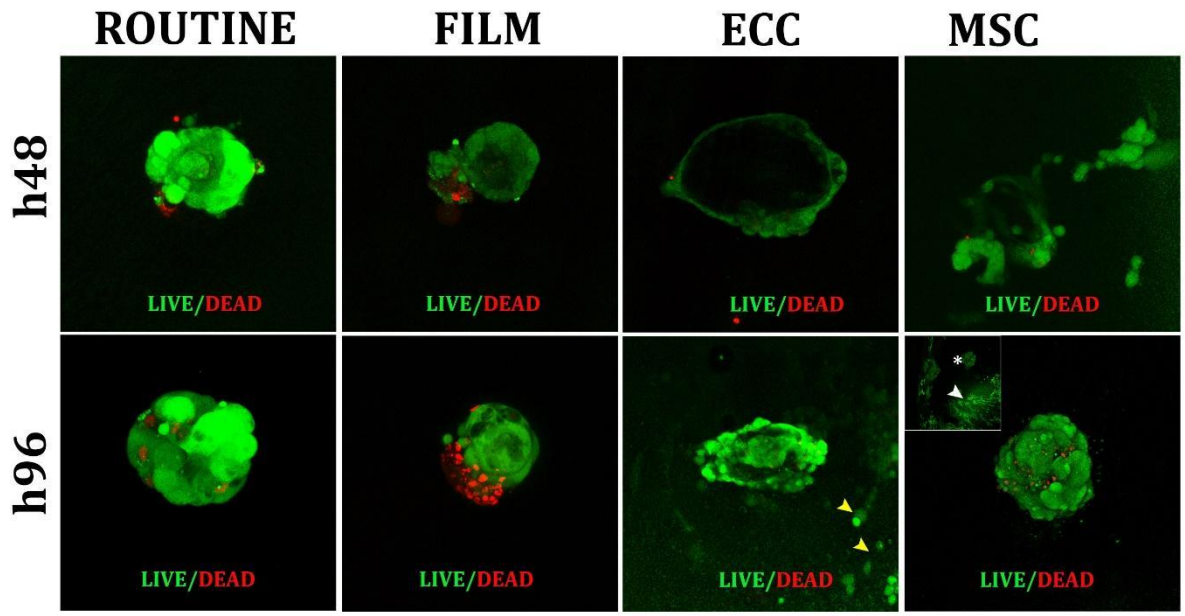


Figure 35. ***Evaluation of cell viability at hours 48 and 96 in experimental groups with the blastocyst***. *ECC and MSC groups may also show the viability of surrounding cells (green and white arrows, asterisk: blastocyst). Images are formed by the maximum projection of the optical sections taken on the z-axis in the confocal. Live: Green, Dead: Red.*

This experiment was carried out with three biological replicates and the results were plotted in figure 36. As can be seen from this graph, a small number of cells lost their viability in each group at 48 and 96 hours which was evident in the ECC group. ECC blastocyst group had more live cells in the blastocyst suggesting support of blastocyst viability when they are co-cultured.

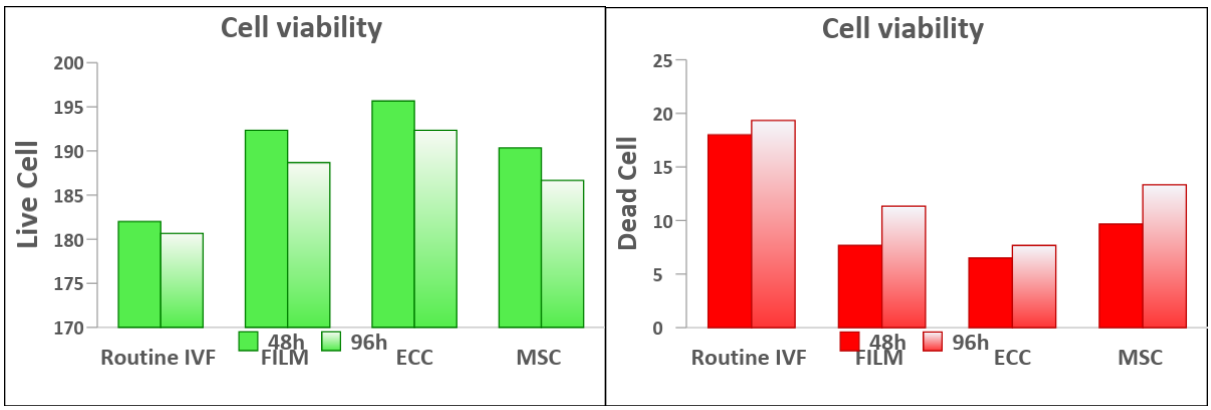


Figure 36. ***Evaluation of cell viability at hours 48 and 96 in experimental groups with the blastocyst.*** *For the viability comparison of the experimental groups, dead cells (stained red) were counted in each Z stack images. The viability assay was performed three times and average values of the dead and live cells were taken for the evaluation of the blastocyst within four different experimental groups.*

Chapter 4

DISCUSSION

Despite the developments in assisted reproductive technologies (ART) to increase the possibility of an embryo to implant, the implantation rate is almost 30%. Implantation failure of the embryo is the main reason and major restriction for ART. Since the implantation requires receptive endometrium and good quality blastocysts, complex molecular interactions among them are one of the reasons behind the unclarified comprehension of idiopathic infertility. Although well-qualified embryos are selected in clinical studies for the embryo transfer, the IVF success ratio is comparatively low due to the inadequate information of mammalian embryo development. Multiple embryos transferred to increase the chance of implantation, however, it may end with the complications of prematurity and low birth weight with multiple births.[109]

The literature knowledge includes several molecular techniques that have been applied to understand the certain aspects of the human implantation mechanism including reverse-transcription PCR (RT- PCR), multiplex assays and proteomics by mass spectrometry. *In vitro* culture models have been also constructed to mimic the in vivo environment of the implantation by converging more closely to the main stages of the implantation; apposition, attachment, and invasion. Ex vivo and *in vitro* models of human pre and post-implantation have been established within ethical boundaries and culture conditions. Improved implantation models might take place these restrictions and limitations to comprehend implantation machinery better.[109]

Co-culture systems were used in the creation of the *in vitro* models with the endometrial cells. In clinical studies, preimplantation embryos were taken from the patients that are diagnosed with the implantation failure.[110] The embryos were cocultured in the autologous monolayer of endometrial cells until the blastocyst stage (day 6) and transferred to observe the increase in implantation rate. 3D Matrigel culture system has been used to mimic 3D environment rather than a monolayer co-culture system on plastic or glass. Collagen has been used as an extracellular matrix protein in the multi-layered co-culture systems to observe the attachment of the trophoblast cells to the endometrial epithelial cells followed by the invasion through the endometrial stromal cells.[111]

We preferred to construct the experimental groups of ECC by considering showing cell-cell and cell-extracellular matrix interactions within 3D implantation models on the film structures. For imitation of the extracellular matrix scaffold, stromal cells of the endometrial cells are seeded within the Matrigel which an extracellular matrix preparation is rich in laminin, fibronectin, entactin. A layer of endometrial epithelial cells was grown on top of this stromal layer, resembling the uterine epithelium to mimic the endometrium environment. Since the optimum 3D culture has not been

revealed and MSCs have been demonstrated to have positive effects on the developing embryo, MSC culture was constructed on the film structures to examine the interaction of MSC with blastocysts and their possible effect on implantation.

In this study, it was concluded to use biomaterials that are used in many areas for construction of the 3D environment. Use of biomaterials for damaged tissues to increase functionality increases the hopeful results. Polymeric cell carriers were used among the biomaterials. Polylactic acid and polyglycolic acid mixture were used to form a three-dimensional film structure as a polymeric cell carrier. Polymeric carriers were used for the construction of implantation models for the first time. The purpose of this study was to construct different models to aid implantation of well-qualified embryos by use of polymeric carrier (film) and to find out the most effective model for adhesion and penetration of the blastocyst. We suppose that polymeric carrier of the film can be used as an implantation model when it is covered with the ECC or MSC by constructing a 3D environment. MSC was used to observe the efficacy of 3-dimensional tissue organization using immunosuppressive MSCs in polymer structures. We aimed to create 3-dimensional functional endometrium tissue culture using polymer structures. In the subsequent studies, these models can be used with easily degradable forms to assist in favor of ongoing pregnancy. We aimed to assess the capacity of the assembled polymer structures and the three-dimensional organization of cellular structures and blastocysts during implantation.

Routine IVF group was constructed to compare any difference between routine culture conditions and experimental groups in terms of possible expression differences and cell viability due to film. Film culture of blastocysts experimental group was established to observe attachment and viability of the blastocysts on the collagen-coated polymeric films. Blastocyst on ECC or MSC cultured film was set up to compare the possible effect of the underlying culture system for the healthy survival and development of the blastocysts. Without the blastocysts, ECC or MSC culture on film was also constructed to evaluate any changes that are under the control of the blastocysts.

The results obtained in this study were evaluated with different perspectives. For the evaluation of the experimental data, all markers were evaluated individually within the experimental groups and the expression changes were evaluated from 48h to 96h.

The embryonic development needs epithelial and mesenchymal interactions. These interactions are preliminarily controlled by the progesterone hormone that starts a complex molecular pathway including many developmental genes; IHH, Wnt4, Hoxa 10 and Hoxa 11.[112] Hoxa 10 expression increased four-fold in the ECC group compared to routine IVF ($p<0.01$, **). Since the ECC group had epithelial and stromal cells underlying the blastocyst, the Hoxa10 expression may come from co-

cultured cells to induce blastocyst development in a 3D environment. Almost stable Hoxa10 expression in the other experimental groups suggested that blastocyst was needed for the rise of the Hoxa10 expression level. However, although there was a slight increase in expression of Hoxa10, the significance of the expression changes in other groups compared to ECC may originate from lower expression at the 48thhour. According to the literature, Hoxa11 and IHH expression increase during the implantation process which in a way coherent with the experimental data. [39] However, since there was no significance in expression change of these genes, the developmental support the embryo may cease in some content due to vitro environment. Due to a decrease of Wnt4 expression in routine IVF and film culture, it can be suggested that cellular support was required for the expression of the Wnt4. A significant increment of Wnt4 in E, M, and FM group suggested that Wnt4 was expressed from endometrial cells during the implantation. As a progesterone receptor ligand, Wnt4 expression expected to be increased in the ECC to induce developmental support of the embryo. It was suspicious that the development markers were low despite the high expression of functional markers. The data clearly indicated that under the presence of the suitable environment *in vitro*, the blastocyst may be supported developmentally.

Hormone receptors of the ERα and PR showed an increase in expression in almost all experimental groups with the significant expression in ECC group in comparison to routine IVF. At the protein level, although MSC culture showed increase in PR expression better than ECC (Figure 28), according to the PCR results ECC depicted more PR expression than MSC group. PR expression showed an almost twenty-fold increase from 48th hour to 96th in the ECC indicated PR expression from the endometrial cells. ER expression was also showed an eight-fold increase from 48th hour to 96th in ECC which was also pointed ER expression from the endometrial cells. Although ER was positive in the nucleus and cytoplasm, the expression increase of ER in the ECC was validated with the IF results (Figure 25). In the qPCR analysis, the increase in these receptors may originate due to the presence of the estrogen and progesterone in the culturing medium. IF stainings of the ER and PR were not solely positive in the nuclear area, cytoplasmic portion of the cells was also positive. During the proliferative phase, ER and PR can also be positive in the cytoplasm of the cells of the human endometrium. The reason behind the cytoplasmic positivity may arouse from the progesterone and estrogen level of the culture medium since they relocate from cytoplasm to the nucleus for the expression of the receptors.[117] Increase in PR and ER significantly in the ECC group clearly indicated that the culture responded well enough to the hormone supplement. The increase of PR and ER from 48 to 96 hour suggested that the receptivity for the coming embryo was established in the ECC group. Both receptor expressions were necessary for the development of the embryo since they induce the downstream genes by regulating multiple signaling pathways including IHH, Wnt4, and HBEGF.

As a progesterone receptor ligand, HBEGF expression was also expected to be increased from hour 48 to 96. In the ECC group, the two-fold increase of HBEGF can be explained by the increase of PR. Although the increase of HBEGF in the ECC was two-fold and higher than MSC culture, the increment of HBEGF found to be significant in the MSC group compared to ECC. HB-EGF promotes blastocyst development, hatching and trophoblast outgrowth, therefore, it can be inferred that MSC culture showed a better response to the blastocyst growth in terms of HBEGF.

HBEGF receptors of ErbB1 and ErbB2 were also expected to be increased as an implantation marker. Expression change of ErbB1 found to be significant in FE and FM group when compared to routine IVF suggested endometrial cells or MSCs expressed ErbB1 even in the absence of the blastocysts. A significant increase of ErbB2 in the ECC indicated the requirement of the endometrial cell support for significant expression change of ErbB2. However, a significant increase of ErbB2 in the MSC culture compared to routine IVF conflicted the idea of endometrial cell requirement.

LIF is expressed in the preimplantation blastocyst and endometrium of glandular and luminal epithelium along with the stromal cells. Its expression is expected to be increased from 48 to 96h since it has a dual role in endometrial glands preparation for uterine receptivity and stromal expression during WOI to assist embryo attachment to the uterus. LIF production during pregnancy has an interdependent effect on hematopoiesis and immune modulation.[112] LIF expression change in ECC was significant compared to routine IVF indicated endometrial and stromal cell expression of LIF under the implantation area. Based on the experimental data it can be inferred that the blastocyst itself (routine IVF and film culture) produced LIF during the implantation and the coculture cells also supported LIF production significantly even in the absence of the blastocyst.

During the implantation period, E-cadherin shows distinct expression pattern for the proper embryo attachment by increasing during attachment and decreasing during the invasion. From 48h to 96h it was expected to decrease in E-cadherin expression for disassociation of the cells to allow blastocyst invasion under the co-cultured area. [50] Decrease in E-cadherin was validated with the IF results of the ECC and MSC culture of the blastocyst (Figure 25, 26). In the qPCR analysis, decrease in E-cadherin expression in all experimental groups was varying with the lower decreasing rate of ECC group which may be related to the expression of E-cadherin from epithelial cells of non-implantation areas. Although the E-cadherin expression decreased in MSC culture in qPCR analysis, WB results depicted slight change in expression at protein level (Figure 28). In the blastocyst cultures without the underlying cells, the decrease in E-cadherin expression may be related to preparation of losing the blastomeres for the possible implantation. Moreover, MMP9 increase in expression may be related to

the decrease in E-cadherin expression suggesting their mutual role in modulation of the endometrial cells. [51]

A fibrillar network of the endometrium changes to aid blastocyst implantation. Extracellular matrix proteins of the fibronectin showed a decrease in expression in ECC and MSC culture suggesting remodeling of the co-culture cells. Decrease in Fibronectin was validated with the IF results of the ECC and MSC culture of the blastocyst (Figure 25, 26). In comparison to the expression rate of ECC and MC, there was no significant difference between the decreasing rate of fibronectin expression. In comparison to routine IVF, the four-fold decrease in fibronectin expression was significant in ECC while the increasing rate was significant in FM and FE suggesting blastocyst requirement for fibronectin decrease in the culture.

Integrin binding to the extracellular matrix protein of fibronectin mediates the blastocyst attachment. [43] Almost eight-fold increase in integrin expression in the ECC group may be related with the attachment and development of the blastocyst to the cultured cells. Although the expression levels of the integrin were increased with the lower fold changes in the other experimental groups, the expression changes were significant compared to ECC. Since the small fold change of the gene expression was found to be significant, the expression of integrin in these groups may be lower at hour 48. However, a decrease of fibronectin and an increase in integrin expression was found to be skeptical that may arouse from the collagen-coated surface of the film structures.

L-Selectin (CD62L) initiates the interaction and facilitates the adhesion between the blastocyst and endometrial epithelium at the site of invasion.[46] The blastocyst cultured experimental groups demonstrated that L-selectin showed an increase in expression to promote attachment of the blastocyst. However, no significant change in expression between 48th and 96th was observed when the cultures are compared to both routine IVF and ECC.

Laminin expression expected to increase as the embryo develops to maintain the membrane integrity of the blastocyst. [55] The decrease in laminin from hour 48 to 96 in routine IVF and film culture was statistically significant in comparison to ECC. The decrease in laminin expression in these groups may be related with the loss of the membrane integrity of the blastomeres since the lack of the supporting cells underlying the blastocyst. Related with the same reason, the laminin expression in ECC or MSC groups increased suggesting support of the cells for the embryo implantation by maintaining the trophoblast and stromal cell differentiation and proliferation.[113] The expression difference of laminin in groups of FM and FE from the routine IVF can be explained as the formation of cellular adhesion structures of the 3D culture systems.

Trophinin has a crucial role during the attachment of the blastocyst to the epithelial cells. The trophinin expression expected to be increased to maintain the crosstalk between the embryo and endometrial cells. [44] In the FM group, the trophinin expression decreased significantly in comparison to both routine IVF and film culture since there was no blastocyst for the intercommunication. However, slight expression change of the FE group may be related with the epithelial cell expression of the trophinin. Although the expression of trophinin was increasing in the ECC group, the MSC group showed a significant change in expression of trophinin when compared to ECC which determines that MSC had lower expression of trophinin.

Entactin and fibronectin are found as an extracellular matrix protein in the Matrigel which is used for ECC construction as evaluated in the IF results. Since the graph shows the difference between 48 and 96 hours gene expression of the ECC, it is the gene expression result of the blastocyst and endometrial epithelial and stromal cells. Increase in laminin and certain collagen types may be related with the increase in entactin expression of the experimental groups which connects these proteins and from the basal membrane structure. Due to its role in trophoblast outgrowth during the implantation[56], its expression was expected to be increased in culture groups of the MSC and ECC rather than routine IVF (R) and film (F) groups. As the blastocyst lost its integrity during the culture with the decrease in entactin expression in the routine IVF and film groups, it was supported well in the ECC group. The decrease in entactin expression in the routine IVF was also showed in the IF results, especially on the blastocyst.

Fibrillar collagen types of collagen I, III and IV showed inconsistent expression when ECC experimental group was considered. Collagen type I expected to be widespread collagen under the inter-implantation site and since we can interpret the expression change from the data, it can be concluded that COL1A1 was overexpressed in ECC group proposing fibrillar formation under the implantation area. [63] The COL1A1 may be expressed from stromal cells of ECC and mesenchymal cells of the MSC culture for regulation of integrity of the culture. Due to the presence of extracellular matrix proteins within the Matrigel, the fibrillar collagen expression may be induced from the cells.

Collagen III expression was found to be consistent in ECC and MSC groups in both culture durations which was significantly decreased in the FM group. Since uterus has a major structural component as a collagen type III, ECC of mouse endometrial cells may induce expression of COL3A1 more than MSC culture.

Anchoring collagen type V was expected to be also expressed in collagen type I expressed area since it has a role in binding of collagen type I and V. Due to its critical role in stabilization, modulation, compartmentalization, and storage of the several growth factors, COL5A1 expression may be related

to these functions in the *in vitro* cultures. [58] COL5A1 expression increase was found to be increased in ECC and MSC culture compared to routine IVF suggesting its accumulation on decidual type MSCs.[61]

Implantation process requires proper endometrial remodeling for the invasive characteristics of the implanting blastocysts. MMPs and TIMPs are the major tissue remodeling enzymes for the regulation of the cyclic changes of the uterus and implantation of the embryo[114]. Although the MMP9 expression was increased at hour 96 in qPCR analysis, IF results of the MMP9 showed slight decrease in expression in the routine IVF group (Figure 23). In the ECC group, it was observed that MMP9 expression increase was significantly higher when compared with the routine IVF. The increase of MMP9 was also validated with the WB results (Figure 28). A significant change of MMP9 in ECC depicted MMP9 expression from the endometrial cells pointing their potential role in trophoblast invasion. Increase in MMP9 expression in routine IVF and film group may be related with the embryonic expression of the MMP9 demonstrating its invasive behaviour. [115] As a major MMP9 inhibitor, TIMP3 expression expected to be also increased for regulation of the MMP9 activity which was significantly expressed in ECC group in comparison to routine IVF. Although the rate of increase of TIMP3 expression was significant in the ECC group, the rate of decrease in MSC was also significant in comparison to routine IVF depicting conflicting result between the ECC and MSC group.[116]

CONCLUSION

In conclusion, it was found that three-dimensional co-culture systems on the polymeric cell carriers are more effective than two-dimensional cultures. Moreover, although MSC culture was found compatible with the blastocyst implantation, the ECC gave significant results in terms of functional, adhesion, invasion and developmental genes. The ECC culture was also found to be more effective for support of embryo viability. In the film culture experimental groups, the image acquisition was difficult due to positional problems and autofluorescence characteristics of the film. To sum up, the three-dimensional culture of ECC on the film was found to be more effective depicting implantation parameters and they can be used with biodegradable polymeric carriers to enhance implantation.

VITA

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BIBLIOGRAPHY

1. E T. The uterus and fertility. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/18155200. Published December 31, 2000. Accessed November 25, 2018.

2. Hawkins SM, Matzuk MM. The Menstrual Cycle. *Annals of the New York Academy of Sciences*. 2008;1135(1):10-18. doi:10.1196/annals.1429.018.

3. Zhang S, Lin H, Kong S, et al. Physiological and molecular determinants of embryo implantation. *Molecular Aspects of Medicine*. 2013;34(5):939-980. doi:10.1016/j.mam.2012.12.011.

4. Lee SK, Kim CJ, Kim D-J, Kang J. Immune Cells in the Female Reproductive Tract. *Immune Network*. 2015;15(1):16. doi:10.4110/in.2015.15.1.16.

5. King A. Uterine leukocytes and decidualization. *Human Reproduction Update*. 2000;6(1):28-36. doi:10.1093/humupd/6.1.28.

6. Helmestam M, Lindgren KE, Stavreus-Evers A, Olovsson M. Mifepristone-Exposured Human Endometrial Endothelial Cells In Vitro. *Reproductive Sciences*. 2013;21(3):408-414. doi:10.1177/1933719113497284.

7. Gil-Sanchis C, Cervelló I, Khurana S, Faus A, Verfaillie C, Simón C. Contribution of different bone marrow-derived cell types in endometrial regeneration using an irradiated murine model. *Fertility and Sterility*. 2015;103(6):1596-1605.e1. doi:10.1016/j.fertnstert.2015.02.030.

8. Challen GA, Little MH. A Side Order of Stem Cells: The SP Phenotype. *Stem Cells*. 2006;24(1):3-12. doi:10.1634/stemcells.2005-0116.

9. Molecular cues to implantation. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/15180948. Published December 31, 2000. Accessed November 25, 2018.

10. Gökçimen, A., & Temel, S. İmplantasyon ve moleküler etkileşimler. *SDÜ Tıp Fakültesi Dergisi*, *11*(4).

11. Wang. Roadmap to embryo implantation: clues from mouse models. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/16485018. Published December 31, 2000. Accessed November 25, 2018.

12. (PDF) Physiology of Embryo-Endometrial Cross Talk. ResearchGate. https://www.researchgate.net/publication/280068102_Physiology_of_Embryo-Endometrial_Cross_Talk. Published December 31, 2014. Accessed November 25, 2018.

13. Kim S-M, Kim J-S. A Review of Mechanisms of Implantation. *Development & Reproduction*. 2017;21(4):351-359. doi:10.12717/dr.2017.21.4.351.

14. JE L. Autophagy regulates embryonic survival during delayed implantation. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/21363932. Published December 31, 2000. Accessed November 25, 2018.

15. Zhang S, Lin H, Kong S, et al. Physiological and molecular determinants of embryo implantation. *Molecular Aspects of Medicine*. 2013;34(5):939-980. doi:10.1016/j.mam.2012.12.011.

16. Hamatani T, Daikoku T, Wang H, et al. Global gene expression analysis identifies molecular pathways distinguishing blastocyst dormancy and activation. *Proceedings of the National Academy of Sciences*. 2004;101(28):10326-10331. doi:10.1073/pnas.0402597101.

17. Zhang S, Lin H, Kong S, et al. Physiological and molecular determinants of embryo implantation. *Molecular Aspects of Medicine*. 2013;34(5):939-980. doi:10.1016/j.mam.2012.12.011.

18. Degradation of estrogen receptor α in activated blastocysts is associated with implantation in the delayed implantation mouse model. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/24442344. Published December 31, 2000. Accessed November 25, 2018.

19. Molecular complexity in establishing uterine receptivity and implantation. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/16143898. Published December 31, 2000. Accessed November 25, 2018.

20. L M. The control of implantation. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/4605229. Published December 31, 2000. Accessed November 25, 2018.

21. Zhang S, Lin H, Kong S, et al. Physiological and molecular determinants of embryo implantation. *Molecular Aspects of Medicine*. 2013;34(5):939-980. doi:10.1016/j.mam.2012.12.011.

22. Aplin. Embryo-epithelium interactions during implantation at a glance. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/28043966. Published December 31, 2000. Accessed November 25, 2018.

23. Activation of the epithelial Na+ channel triggers prostaglandin E_2 release and production required for embryo implantation. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/22729284?dopt=Abstract. Published December 31, 2000. Accessed November 25, 2018.

24. Deregulation of the serum- and glucocorticoid-inducible kinase SGK1 in the endometrium causes reproductive failure. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/22001908?dopt=Abstract. Published December 31, 2000. Accessed November 25, 2018.

25. Characterization of the uterine phenotype during the peri-implantation period for LIF-null, MF1 strain mice. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/15848385?dopt=Abstract. Published December 31, 2000. Accessed November 25, 2018.

26. Y L. Entosis allows timely elimination of the luminal epithelial barrier for embryo implantation. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/25865893?dopt=Abstract. Published December 31, 2000. Accessed November 25, 2018.

27. Olive DL. Book Review The Endometrium: Molecular, Cellular, and Clinical Perspectives (Reproductive Medicine & Assisted Reproductive Techniques.) Second edition. Edited by John D. Aplin, Asgerally T. Fazleabas, Stanley R. Glasser, and Linda C. Giudice. 879 pp., illustrated. London, Informa Healthcare, 2008. $559.95. 978-0-415-38583-1. *New England Journal of Medicine*. 2009;360(3):310-311. doi:10.1056/nejmbkrev0809333.

28. B. Cyclic decidualization of the human endometrium in reproductive health and failure. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/25141152?dopt=Abstract. Published December 31, 2000. Accessed November 25, 2018.

29. Mori M, Bogdan A, Balassa T, Csabai T, Szekeres-Bartho J. The decidua—the maternal bed embracing the embryo—maintains the pregnancy. *Seminars in Immunopathology*. 2016;38(6):635-649. doi:10.1007/s00281-016-0574-0.

30. Zonula occludens-1 and E-cadherin are coordinately expressed in the mouse uterus with the initiation of implantation and decidualization. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/10191061. Published December 31, 2000. Accessed November 25, 2018.

31. HAREM, İ. Ş. Desidual Hücreler. *Harran Üniversitesi Veteriner Fakültesi Dergisi*, *5*(2), 202-207.

32. Sharma S, Godbole G, Modi D. Decidual Control of Trophoblast Invasion. *American Journal of Reproductive Immunology*. 2016;75(3):341-350. doi:10.1111/aji.12466.

33. Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. *Nature Medicine*. 2012;18(12):1754-1767. doi:10.1038/nm.3012.

34. Fagerberg L, Hallström BM, Oksvold P, et al. Analysis of the Human Tissue-specific Expression by Genome-wide Integration of Transcriptomics and Antibody-based Proteomics. *Molecular & Cellular Proteomics*. 2013;13(2):397-406. doi:10.1074/mcp.m113.035600.

35. Salleh N, Giribabu N. Leukemia Inhibitory Factor: Roles in Embryo Implantation and in Nonhormonal Contraception. *The Scientific World Journal*. 2014;2014:1-10. doi:10.1155/2014/201514.

36. H S. Evidence for heterodimeric association of leukemia inhibitory factor (LIF) receptor and gp130 in the mouse uterus for LIF signaling during blastocy... - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/16452727. Published December 31, 2000. Accessed November 25, 2018.

37. He B, Ni Z, Kong S, Lu J, Wang H. Homeobox genes for embryo implantation: From mouse to human. *Animal Models and Experimental Medicine*. 2018;1(1):14-22. doi:10.1002/ame2.12002.

38. al. Msx homeobox genes critically regulate embryo implantation by controlling paracrine signaling between uterine stroma and epithelium. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/22383889. Published December 31, 2000. Accessed November 25, 2018.

39. K L. Indian hedgehog is a major mediator of progesterone signaling in the mouse uterus. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/16951680. Published December 31, 2000. Accessed November 25, 2018.

40. Wetendorf M, DeMayo FJ. The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signaling network. *Molecular and Cellular Endocrinology*. 2012;357(1-2):108-118. doi:10.1016/j.mce.2011.10.028.

41. Chen Q, Zhang Y, Lu J, et al. Embryo-uterine cross-talk during implantation: the role of Wnt signaling. *Molecular Human Reproduction*. 2009;15(4):215-221. doi:10.1093/molehr/gap009.

42. Zhang S, Lin H, Kong S, et al. Physiological and molecular determinants of embryo implantation. *Molecular Aspects of Medicine*. 2013;34(5):939-980. doi:10.1016/j.mam.2012.12.011.

43. Singh. Adhesion molecules in endometrial epithelium: tissue integrity and embryo implantation. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/19453302. Published December 31, 2000. Accessed November 25, 2018.

44. Sugihara K, Sugiyama D, Byrne J, et al. Trophoblast cell activation by trophinin ligation is implicated in human embryo implantation. *Proceedings of the National Academy of Sciences*. 2007;104(10):3799-3804. doi:10.1073/pnas.0611516104.

45. Feng Y, Ma X, Deng L, et al. Role of Selectins and their Ligands in Human Implantation Stage. *Glycobiology*. January 2017:cwx009. doi:10.1093/glycob/cwx009.

46. Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/12532021. Published December 31, 2000. Accessed November 25, 2018.

47. James JL, Carter AM, Chamley LW. Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation? *Placenta*. 2012;33(5):327-334. doi:10.1016/j.placenta.2012.01.020.

48. Tepass U, Truong K, Godt D, Ikura M, Peifer M. Cadherins in embryonic and neural morphogenesis. *Nature Reviews Molecular Cell Biology*. 2000;1(2):91-100. doi:10.1038/35040042.

49. Riethmacher D, Brinkmann V, Birchmeier C. A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *Proceedings of the National Academy of Sciences*. 1995;92(3):855-859. doi:10.1073/pnas.92.3.855.

50. Impaired down-regulation of E-cadherin and beta-catenin protein expression in endometrial epithelial cells in the mid-secretory endometrium of infe... - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/20410224. Published December 31, 2000. Accessed November 25, 2018.

51. Li Q, Wang J, Armant DR, Bagchi MK, Bagchi IC. Calcitonin Down-regulates E-cadherin Expression in Rodent Uterine Epithelium during Implantation. *Journal of Biological Chemistry*. 2002;277(48):46447-46455. doi:10.1074/jbc.m203555200.

52. Armant DR. Blastocysts don't go it alone. Extrinsic signals fine-tune the intrinsic developmental program of trophoblast cells. *Developmental Biology*. 2005;280(2):260-280. doi:10.1016/j.ydbio.2005.02.009.

53. Goossens K, Van Soom A, Van Poucke M, et al. Identification and expression analysis of genes associated with bovine blastocyst formation. *BMC Developmental Biology*. 2007;7(1):64. doi:10.1186/1471-213x-7-64.

54. Armant DR. Blastocysts don't go it alone. Extrinsic signals fine-tune the intrinsic developmental program of trophoblast cells. *Developmental Biology*. 2005;280(2):260-280. doi:10.1016/j.ydbio.2005.02.009.

55. Azimi M, Niimi T, Yoshida N, Kitagawa Y. *Cytotechnology*. 1999;31(1/2):185-193. doi:10.1023/a:1008084508489.

56. Yelian FD. Recombinant entactin promotes mouse primary trophoblast cell adhesion and migration through the Arg-Gly-Asp (RGD) recognition sequence [published erratum appears in J Cell Biol 1993 Jul;122(1):279]. *The Journal of Cell Biology*. 1993;121(4):923-929. doi:10.1083/jcb.121.4.923.

57. M. Expression of nidogen and laminin in basement membranes during mouse embryogenesis and in teratocarcinoma cells. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/2995165. Published December 31, 2000. Accessed November 25, 2018.

58. Iwahashi M, Muragaki Y. Increased type I and V collagen expression in uterine leiomyomas during the menstrual cycle. *Fertility and Sterility*. 2011;95(6):2137-2139. doi:10.1016/j.fertnstert.2010.12.028.

59. Temporal changes to uterine collagen types I, III and V in relation to early pregnancy in the rat. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/7624506. Published December 31, 2000. Accessed November 25, 2018.

60. Blankenship. Loss of laminin and type IV collagen in uterine luminal epithelial basement membranes during blastocyst implantation in the mouse. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/8540630. Published December 31, 2000. Accessed November 25, 2018.

61. DD C. Differential temporal and spatial expression of mRNA encoding extracellular matrix components in decidua during the peri-implantation period. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/1391307. Published December 31, 2000. Accessed November 25, 2018.

62. Oefner CM, Sharkey A, Gardner L, Critchley H, Oyen M, Moffett A. Collagen type IV at the fetal–maternal interface. *Placenta*. 2015;36(1):59-68. doi:10.1016/j.placenta.2014.10.012.

63. Spiess K, Teodoro WR, Zorn TMT. Distribution of Collagen Types I, III, and V in Pregnant Mouse Endometrium. *Connective Tissue Research*. 2007;48(2):99-108. doi:10.1080/03008200601166194.

64. Matrix metalloproteinases: a review. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/8435466. Published December 31, 2000. Accessed November 25, 2018.

65. Yoshii N, Hamatani T, Inagaki N, et al. Successful implantation after reducing matrix metalloproteinase activity in the uterine cavity. *Reproductive Biology and Endocrinology*. 2013;11(1):37. doi:10.1186/1477-7827-11-37.

66. Mikołajczyk M, Skrzypczak J, Wirstlein P. No correlation between pinopode formation and LIF and MMP2 expression in endometrium during implantation window. *Folia Histochemica et Cytobiologica*. 2012;49(4):615-621. doi:10.5603/fhc.2011.0084.

67. Gurung S, Werkmeister JA, Gargett CE. Inhibition of Transforming Growth Factor-β Receptor signaling promotes culture expansion of undifferentiated human Endometrial Mesenchymal Stem/stromal Cells. *Scientific Reports*. 2015;5(1). doi:10.1038/srep15042.

68. Torry DS, Leavenworth J, Chang M, et al. Angiogenesis in implantation. *Journal of Assisted Reproduction and Genetics*. 2007;24(7):303-315. doi:10.1007/s10815-007-9152-7.

69. Cohen PE, Zhu L, Pollard JW. Absence of Colony Stimulating Factor-1 in Osteopetrotic (csfmop/csfmop) Mice Disrupts Estrous Cycles and Ovulation1. *Biology of Reproduction*. 1997;56(1):110-118. doi:10.1095/biolreprod56.1.110.

70. Slayden OD. Effects of Keratinocyte Growth Factor in the Endometrium of Rhesus Macaques during the Luteal-Follicular Transition. *Journal of Clinical Endocrinology & Metabolism*. 2000;85(1):275-285. doi:10.1210/jc.85.1.275.

71. Global gene expression analysis identifies molecular pathways distinguishing blastocyst dormancy and activation. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/15232000. Published December 31, 2000. Accessed November 25, 2018.

72. Krege JH, Hodgin JB, Couse JF, et al. Generation and reproductive phenotypes of mice lacking estrogen receptor. *Proceedings of the National Academy of Sciences*. 1998;95(26):15677-15682. doi:10.1073/pnas.95.26.15677.

73. Hou, Q. Estrogen receptor and progesterone receptor genes are expressed differentially in mouse embryos during preimplantation development. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;90(20):9460. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC47588/. Accessed November 25, 2018.

74. Degradation of estrogen receptor α in activated blastocysts is associated with implantation in the delayed implantation mouse model. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/24442344. Published December 31, 2000. Accessed November 25, 2018.

75. Jacobsen BM, Horwitz KB. Progesterone receptors, their isoforms and progesterone regulated transcription. *Molecular and Cellular Endocrinology*. 2012;357(1-2):18-29. doi:10.1016/j.mce.2011.09.016.

76. Large MJ, DeMayo FJ. The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling. *Molecular and Cellular Endocrinology*. 2012;358(2):155-165. doi:10.1016/j.mce.2011.07.027.

77. Acceleration of trophoblast differentiation by heparin-binding EGF-like growth factor is dependent on the stage-specific activation of calcium infl... - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/10654598. Published December 31, 2000. Accessed November 25, 2018.

78. Co-existence of heparin-binding epidermal growth factor-like growth factor and pinopodes in human endometrium at the time of implantation. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/12149409. Published December 31, 2000. Accessed November 25, 2018.

79. Ullrich. Signal transduction by receptors with tyrosine kinase activity. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/2158859. Published December 31, 2000. Accessed November 25, 2018.

80. Miller, M. M., McMullen, P. D., Andersen, M. E., & Clewell, R. A. (2017). Multiple receptors shape the estrogen response pathway and are critical considerations for the future of in vitro-based risk assessment efforts. *Critical reviews in toxicology*, *47*(7), 570-586.

81. Nagamura-Inoue T. Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility. *World Journal of Stem Cells*. 2014;6(2):195. doi:10.4252/wjsc.v6.i2.195.

82. Umbilical Cord as Prospective Source for Mesenchymal Stem Cell-Based Therapy. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/27651799. Published December 31, 2000. Accessed November 25, 2018.

83. AR W. Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/21960725. Published December 31, 2000. Accessed November 25, 2018.

84. Nagamura-Inoue T. Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility. *World Journal of Stem Cells*. 2014;6(2):195. doi:10.4252/wjsc.v6.i2.195.

85. Talwadekar MD, Kale VP, Limaye LS. Placenta-derived mesenchymal stem cells possess better immunoregulatory properties compared to their cord-derived counterparts–a paired sample study. *Scientific Reports*. 2015;5(1). doi:10.1038/srep15784.

86. Umbilical Cord as Prospective Source for Mesenchymal Stem Cell-Based Therapy. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/27651799. Published December 31, 2000. Accessed November 25, 2018.

87. Umbilical Cord as Prospective Source for Mesenchymal Stem Cell-Based Therapy. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/27651799. Published December 31, 2000. Accessed November 25, 2018.

88. Umbilical cord mesenchymal stromal cell transplantations: A systemic analysis of clinical trials. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/28964742. Published December 31, 2000. Accessed November 25, 2018.

89. Le Bouteiller P, Bensussan A. Up-and-down immunity of pregnancy in humans. *F1000Research*. 2017;6:1216. doi:10.12688/f1000research.11690.1.

90. Dekel N, Gnainsky Y, Granot I, Mor G. REVIEW ARTICLE: Inflammation and Implantation. *American Journal of Reproductive Immunology*. 2009;63(1):17-21. doi:10.1111/j.1600-0897.2009.00792.x.

91. AR W. Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/21960725. Published December 31, 2000. Accessed November 25, 2018.

92. Umbilical Cord as Prospective Source for Mesenchymal Stem Cell-Based Therapy. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/27651799. Published December 31, 2000. Accessed November 25, 2018.

93. Mesenchymal Stem Cells (MSCs) Therapy for Recovery of Fertility: a Systematic Review. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/28884412. Published December 31, 2000. Accessed November 25, 2018.

94. Mesenchymal Stem Cells (MSCs) Therapy for Recovery of Fertility: a Systematic Review. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/28884412. Published December 31, 2000. Accessed November 25, 2018.

95. HS T. Molecular markers of implantation: clinical implications. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/11396649. Published December 31, 2000. Accessed November 25, 2018.

96. Weimar CHE, Post Uiterweer ED, Teklenburg G, Heijnen CJ, Macklon NS. Reprint of: In-vitro model systems for the study of human embryo–endometrium interactions. *Reproductive BioMedicine Online*. 2013;27(6):673-688. doi:10.1016/j.rbmo.2013.10.004.

97. Weimar CHE, Post Uiterweer ED, Teklenburg G, Heijnen CJ, Macklon NS. In-vitro model systems for the study of human embryo–endometrium interactions. *Reproductive BioMedicine Online*. 2013;27(5):461-476. doi:10.1016/j.rbmo.2013.08.002.

98. Wang. Roadmap to embryo implantation: clues from mouse models. - PubMed - NCBI. Nih.gov. https://www.ncbi.nlm.nih.gov/pubmed/16485018. Published December 31, 2000. Accessed November 25, 2018.

99. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. *Nature Reviews Genetics*. 2006;7(3):185-199. doi:10.1038/nrg1808.

100. Tagami T, Ozeki T. Recent Trends in Clinical Trials Related to Carrier-Based Drugs. *Journal of Pharmaceutical Sciences*. 2017;106(9):2219-2226. doi:10.1016/j.xphs.2017.02.026.

101. Villanova JCO, Oréfice RL, Cunha AS. Aplicações farmacêuticas de polímeros. *Polímeros*. 2010;20(1):51-64. doi:10.1590/s0104-14282010005000009.

102. Athanasiou KA, Mauli Agrawal C. Orthopedic polymeric biomaterials: applications of biodegradables: Biomaterials. *Biomaterials*. 2000;21(24):2493. doi:10.1016/s0142-9612(00)00198-8.

103. Carletti E, Motta A, Migliaresi C. Scaffolds for Tissue Engineering and 3D Cell Culture. *Methods in Molecular Biology*. October 2010:17-39. doi:10.1007/978-1-60761-984-0_2.

104. Evans ND, Gentleman E, Polak JM. Scaffolds for stem cells. *Materials Today*. 2006;9(12):26-33. doi:10.1016/s1369-7021(06)71740-0.

105. Bao Ha TL, Minh T, Nguyen D, Minh D. Naturally Derived Biomaterials: Preparation and Application. *Regenerative Medicine and Tissue Engineering*. May 2013. doi:10.5772/55668.

106. Mondrinos MJ, Koutzaki S, Jiwanmall E, et al. Engineering Three-Dimensional Pulmonary Tissue Constructs. *Tissue Engineering*. 2006;0(0):060116055635001. doi:10.1089/ten.2005.12.ft-1.

107. Lin AS., Barrows TH, Cartmell SH, Guldberg RE. Microarchitectural and mechanical characterization of oriented porous polymer scaffolds. *Biomaterials*. 2003;24(3):481-489. doi:10.1016/s0142-9612(02)00361-7.

108. To P. blastocyst – PCOS To Mommy. Wordpress.com. https://pcostomommy.wordpress.com/tag/blastocyst/. Published January 18, 2015. Accessed November 25, 2018.

109. Niakan, K. K., Han, J., Pedersen, R. A., Simon, C., & Pera, R. A. R. (2012). Human pre-implantation embryo development. Development, 139(5), 829-841.

110. Lindenberg, S., Nielsen, M.H., Lenz, S., 1985. *In vitro* studies of human blastocyst implantation. Annals of the New York Academy of Sciences 442, 368–374.

111. Simon, C., Mercader, A., Garcia-Velasco, J., Nikas, G., Moreno, C., Remohi, J., Pellicer, A., 1999. Coculture of human embryos with autologous human endometrial epithelial cells in patients with implantation failure. The Journal of Clinical Endocrinology and Metabolism 84, 2638–2646

112. Nasef, A., Mazurier, C., Bouchet, S., François, S., Chapel, A., Thierry, D., ... & Fouillard, L. (2008). Leukemia inhibitory factor: Role in human mesenchymal stem cells mediated immunosuppression. Cellular immunology, 253(1-2), 16-22.

113. Autio-Harmainen H, Sandberg M, Pihlajaniemi T and Vuorio E: Synthesis of laminin and type IV collagen by trophoblastic cells and fibroblastic stromal cells in the early human placenta. Lab Invest 64: 483-491, 1991.

114. K., Saikat, et al. "Alteration in Endometrial Remodeling: A Cause for Implantation Failure in Endometriosis?" *Endometriosis - Basic Concepts and Current Research Trends*, 2012.

115. Plaks, V., Rinkenberger, J., Dai, J., Flannery, M., Sund, M., Kanasaki, K., ... & Werb, Z. (2013). Matrix metalloproteinase-9 deficiency phenocopies features of preeclampsia and intrauterine growth restriction. *Proceedings of the National Academy of Sciences*, *110*(27), 11109-11114.

116. Arpino, V., Brock, M., & Gill, S. E. (2015). The role of TIMPs in regulation of extracellular matrix proteolysis. *Matrix Biology*, *44*, 247-254.

117. BAYARD, FRANCIS, et al. "Cytoplasmic and nuclear estradiol and progesterone receptors in human endometrium." *The Journal of Clinical Endocrinology & Metabolism* 46.4 (1978): 635-648.

118. Karahuseyinoglu, S., Cinar, O., Kilic, E., Kara, F., Akay, G. G., Demiralp, D. Ö., ... & Can, A. (2007). Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. *Stem cells*, *25*(2), 319-331.