Enhancement of Implantation Potential of Mouse Blastocysts Via Use of Three-Dimensional Endometrial Co-Cultures on Natural and Artificial Materials

by

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ABSTRACT

Implantation of an embryo is a fundamental and important process of reproduction in humans. The implantation process is composed of various complex molecular and cellular steps. Embryo implantation occurs in a very short period which is called “window of implantation”. Parameters of the successful implantation are related to the blastocyst itself and the receptivity of the uterus. During the window of implantation, highly complicated biological events take place, and despite the developments in assisted reproduction recurrent implantation failures and spontaneous abortions are still inevitable, which in turn on implies that, it is still necessary to bring new approaches to assisted reproduction applications to provide an increase of success in ART.

The first aim of this widescale project is to generate a new attitude to enhance the implantation potential of mouse blastocysts by creating applicable three-dimensional (3D) embryo culture models. In this thesis, it is aimed to construct a 3D environment to promote the implantation of a blastocyst by providing an imitated natural profile of the endometrium *in vitro*. In relation with this objective, natural collagen sponges and artificial polymeric sponges were used as constructions covered with epithelial and stromal cells to mimic the endometrium itself. When the blastocysts were cultured with this newly created model, the expression of the implantation related genes and the morphological changes of the blastocyst were examined and compared. The experimental groups were designed as follows; routine IVF group which refers to the culture of embryo itself in a culture medium, blastocyst on collagen sponge coated with endometrium derived epithelial and stromal cells, blastocyst on artificial sponge covered with collagen under the endometrium derived epithelial and stromal cells and the sponge groups with cells but without blastocyst as control groups for 48 hours and 96 hours as incubation periods.

As a final approach, it was expected to be a new impact to understand the complicated process of the embryo implantation and compose a 3D culture model that can be applicable for embryo transfers in ART. By this purpose, it is shown that usage of sponges with endometrial co – culture cells as 3D models is effective result to support implantation of an embryo more successfully.

Keywords: Embryo, blastocyst, endometrium, implantation, artificial sponges, natural sponges, epithelial cells, stromal cells, 3 – D models

ÖZET

Embriyo implantasyonu, insanlarda üreme sisteminin önemli ve başlıca süreçlerinden biridir. İmplantasyon süreci, oldukça karmaşık moleküler ve hücresel aşamalardan oluşur. Embriyo implantasyonu, çok kısa bir sürede gerçekleşen ve "İmplantasyon Penceresi" adı verilen bir zaman diliminde gerçekleşir. Başarılı bir implantasyonun en önemli iki parametresi arasında blastosistin implantasyona uygunluğu ve uterusun alma yeteneği sıralanabilir. Üremeye yardımcı tekniklerdeki (ÜYTE) gelişmelere rağmen, tekrarlayan implantasyon başarısızlıkları ve spontan düşükler hala kaçınılmazdır. Bu da ÜYTE'deki başarı artışını sağlamak ve yeni yaklaşımlar getirmenin hala gerekli olduğu anlamına gelmektedir.

Bu geniş ölçekli projenin ilk amacı, uygulanabilir üç boyutlu (3D) embriyo kültür modelleri oluşturarak fare blastosistlerinin implantasyon potansiyelini artırmak için yeni bir tutum oluşturmaktır. Bu tezde, endometriyumun yapısının *in vitro* olarak oluşturularak blastosistin implantasyon potansiyelinin arttırılması amaçlanmıştır. Bu amaç doğrultusunda, doğal kolajen ve yapay polimerik süngerler endometriyal epitel ve stroma hücrelerinin hücre dışı matriks elemanlarıyla kaplanmasına bağlı olarak endometriyumu taklit edecek yapılar haline getirilmiştir. Blastosistlerin oluşturulan bu modellerle kültürlenmesi sonrası, implantasyonla ilgili genlerin değişimi ve blastosistin morfolojik değişiklikleri incelenip karşılaştırılmıştır.

Deney grupları, 48. ve 96. saatler olmak üzere, boş kültür ortamında embriyonun kendisinin kültür edildiği rutin IVF grubu, endometriyum türevi epitel ve stroma hücreleriyle kaplı kolajen sünger üzerinde blastosist, endometriyal epitel ve stroma hücreleri altında kolajen ile kaplı yapay sünger üzerinde blastosist ve kontrol olarak sadece hücreli süngerler şeklinde oluşturulmuştur.

Bu modellemenin, embriyo implantasyonunun karmaşık sürecini anlamak ve ÜYTE'de embriyo transferleri için uygulanabilecek bir üç boyutlu kültür modeli oluşturmak için yeni bir etki olması hedeflenmiştir. Bu amaçla yapay polimerik ve doğal kolajen köpüklerin endometriyal hücreler ile kaplanması sonucu oluşturulan üç boyutlu modellemelerin, bir embriyonun implantasyonunu daha başarılı bir şekilde desteklemek için etkili sonuç verdiği gösterilmiştir.

Anahtar Kelimeler: Embriyo, blastosist, endometriyum, implantasyon, yapay köpük, doğal köpük, epitel hücreler, stroma hücreleri, üç boyutlu modeller.

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NOMENCLATURE

ART	Assisted Reproductive Technology
BSA	Bovine Serum Albumin
CAMs	Cell Adhesion Molecules
DMEM	Dulbecco's Modified Eagle's Medium
DPBST	Dulbecco's Phosphate Buffered Saline
ECC	Endometrial Co-Culture
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ET	Embryo Transfer
FBS	Fetal Bovine Serum
FCS	Fetal Charcoal Stripped Serum
FET	Frozen Embryo Transfer
FSH	Follicular Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
HBEGF	Heparin Binding Epidermal Growth Factor
HBSS	Hanks Balanced Salt Solution
hCG	Human Chorionic Gonadotropin
HOX	Homeobox Genes
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
IGF	Insulin Like Growth Factor
IF	Immunofluorescent Staining
IL	Interleukin
IVM	*in vitro* Maturation
IVF	*in vitro* Fertilization
LH	Luteinizing Hormone
LIF	Leukemia Inhibitory Factor

MMP	Matrix Metalloproteinase
MUC	Mucin
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PGA	Polyglycolic Acid
PGD	Preimplantation Genetic Diagnosis
P (L – D, L) LA	Poly (lactic – co – glycolic acid)
PMSG	Pregnant Mare Serum Gonadotropin
PN	Pronucleus
PR	Progesterone Receptor
RIF	Recurrent Implantation Failure
RT – PCR	Reverse Transcriptase Polymerase Chain Reaction
SEM	Scanning Electron Microscope
STED	Stimulated Emission Depletion Microscopy
TGF - β	Transforming Growth Factor β
UV	Ultraviolet
WOI	Window of Implantation
2D	Two Dimensional
3D	Three Dimensional
i.u.	International Unit
i.p.	Intraperitoneal Injection

To my sturdy mother,

For teaching me to stand on my own feet first.

CHAPTER 1

Review of Literature

1. UTERUS

Paramesonephric ducts (Müllerian Ducts) are the primordial structures of the female reproductive tracts. Female reproductive system in humans is consisted of a pair of ovaries, uterine tubes, vagina, and a uterus. When the uterus of humans is pear – shaped, the mouse uterus is consisting of two uterine horn forming a "V" shape. Uterus has 3 main parts: cervix, fundus, and body. The layers of a uterus can be listed as: endometrium, myometrium, and perimetrium from inside to outside respectively.

The endometrium (uterine mucous membrane) is the layer which is involved in the implantation process. The myometrium is a layer consisted of bundles of smooth muscle layers, and is the thickest part of the uterus.[1] Perimetrium, or serosa layer, functions to cover the uterus as the outer layer.

1.1. Endometrium

The inner most layer of the uterus, the endometrium is the area where the implantation occurs. The endometrial structure is composed of functional and basal layers. Functional layer is thick and superficial, whereas the basal layer is deep and narrow. The changes in the blood levels of progesterone and oestrogen affect the functional layer and this layer is lost partially or totally in every menstrual cycle. The functional layer regenerates itself after menstruation. Basal layer is not affected by hormonal changes in the blood.[2]

The endometrium is lined by simple columnar epithelium beneath which lamina propria is found. Lamina propria holds the glandular portion that is mainly composed of simple branched tubular glands.

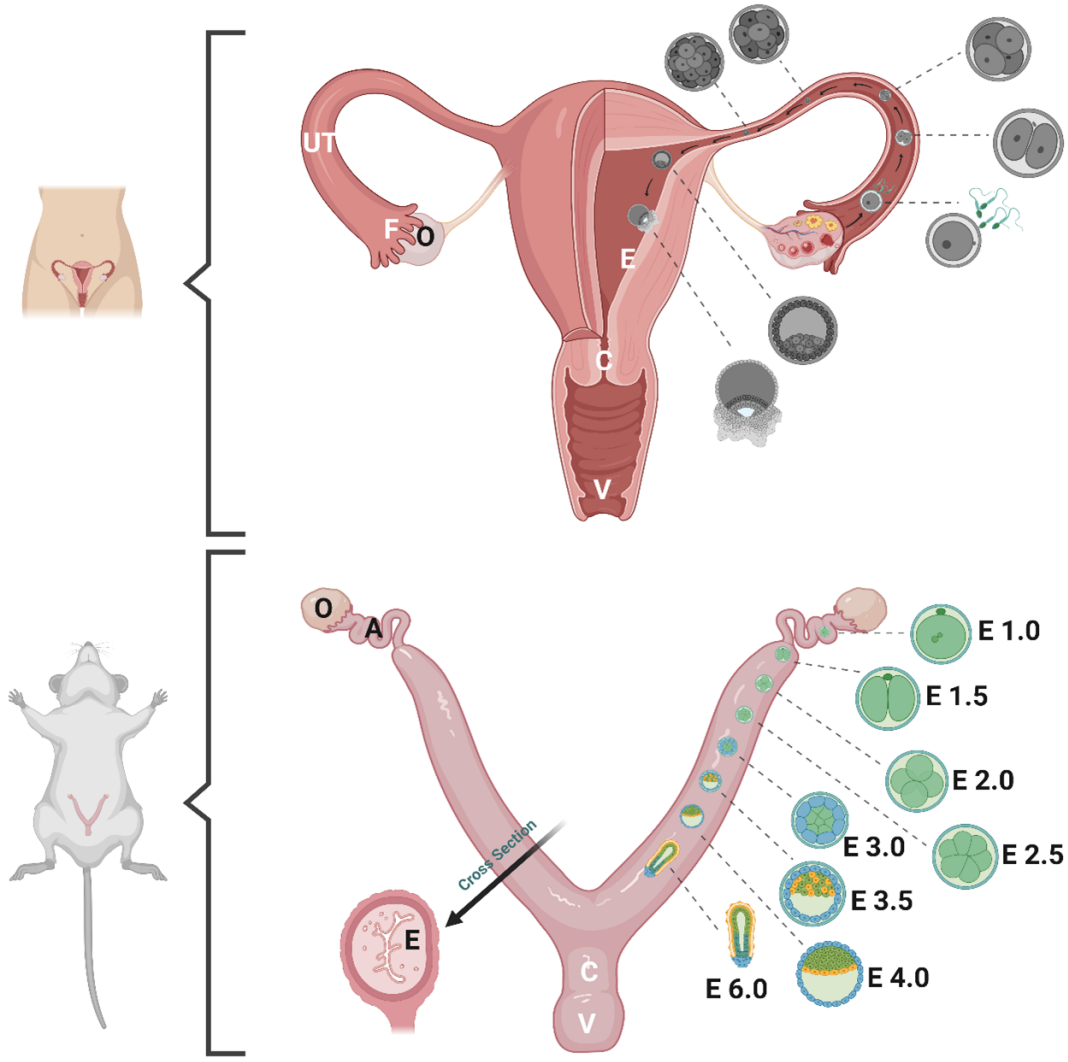

Figure 1. ***Comparison of Reproductive System in Humans and Mice*** The uterus of humans are pear – shaped whereas the mice uterus is consisted of uterine horns. Human reproductive system consists of ovaries (O), fimbriae (F), uterine tubes (UT), vagina (V), cervix (C), and the lining endometrium (E) of the uterus. The development of an implanting embryo from an ovulated oocyte is also shown. The oocyte meets with the sperm to form the zygote. The zygote develops into a 2- cell stage, 4 - cell stage, 8 - cell stage, a morula, and finally an implanting blastocyst, respectively. The reproductive system of a mouse consists of vagina (V), cervix (C), a pair of ovaries (O), the endometrium layer of uterus (E), and the ampulla region (A). As in humans, the development process of an embryo is shown in the figure. The timeline of a mouse embryo is indicated as E1.0, E1.5, E2.0, E2.5, E3.0, E3.5, E4.0, E6.0 corresponds to the timeline of the humans until day 6. *This figure is created via Biorender.com.*

1.1.1. Epithelial Cells

Steroid hormone regulation of the implantation related events in mice is well defined. In the beginning of the pregnancy, luminal and glandular epithelium is proliferated under the effect of the oestrogen hormone. By the 4th day of pregnancy, the proliferated epithelium differentiates by the effect of progesterone which is secreted by corpus luteum.[3]

1.1.2. Stromal Cells

Stromal cells are the important compartments of decidualization process during implantation. Decidualized stromal cells regulate the immunological activity and vascularization of the uterus upon implantation. Endometrial stromal cells show tighter cytoskeleton and have less metabolic activity when compared to decidualized stromal cells.[4] During implantation, the endometrial stromal cells surround the invading blastocyst and constitute an interaction with the embryo. Endometrial stromal cells have numerous functions, for example recognizing the embryo, regulating the immune cells, and the activity of vascular endothelial cells.

2. EMBRYO IMPLANTATION

Implantation of an embryo is a process consisting of different complex steps and molecular pathways. Embryo implantation occurs in three main steps: apposition, adhesion, and invasion. During these steps, the embryo must be competent, and the uterus needs to be in a receptive stage. The receptivity of the uterus occurs during a limited time interval which is called as the “window of implantation” (WOI). The uterus undergoes some morphological, genetic, and biological changes to become receptive for the implanting embryo. When the embryo itself becomes competent, prepared, and positioned for implantation; the trophoblast cells interact with the luminal epithelium of the endometrium. The first contact between the blastocyst and the endometrium is low and is called as the apposition.

Pinopodes are the structures only formed during the WOI, as the uterus is highly receptive to the blastocyst and ready for implantation. Pinopodes appear like projections on the surface of luminal epithelial cells and help the blastocyst to adhere to the surface epithelium of the endometrium. As the uterine receptivity is highly dependent on progesterone and oestrogen levels, the developmental changes regarding pinopodes are also sensitive to hormonal changes. When the uterus begins to enter

the receptive phase, the number of pinopodes increases and decreases as the uterus passes out the receptive phase.

As the blastocyst hatches from its zona pellucida, the luminal epithelium homes the blastocyst within a nidus, and the blastocyst invade from the luminal epithelial cells through the underlying stromal cells. During attachment and invasion; activation, and inhibition of several molecules such as cytokines and enzymes play critical roles for implantation.[5]

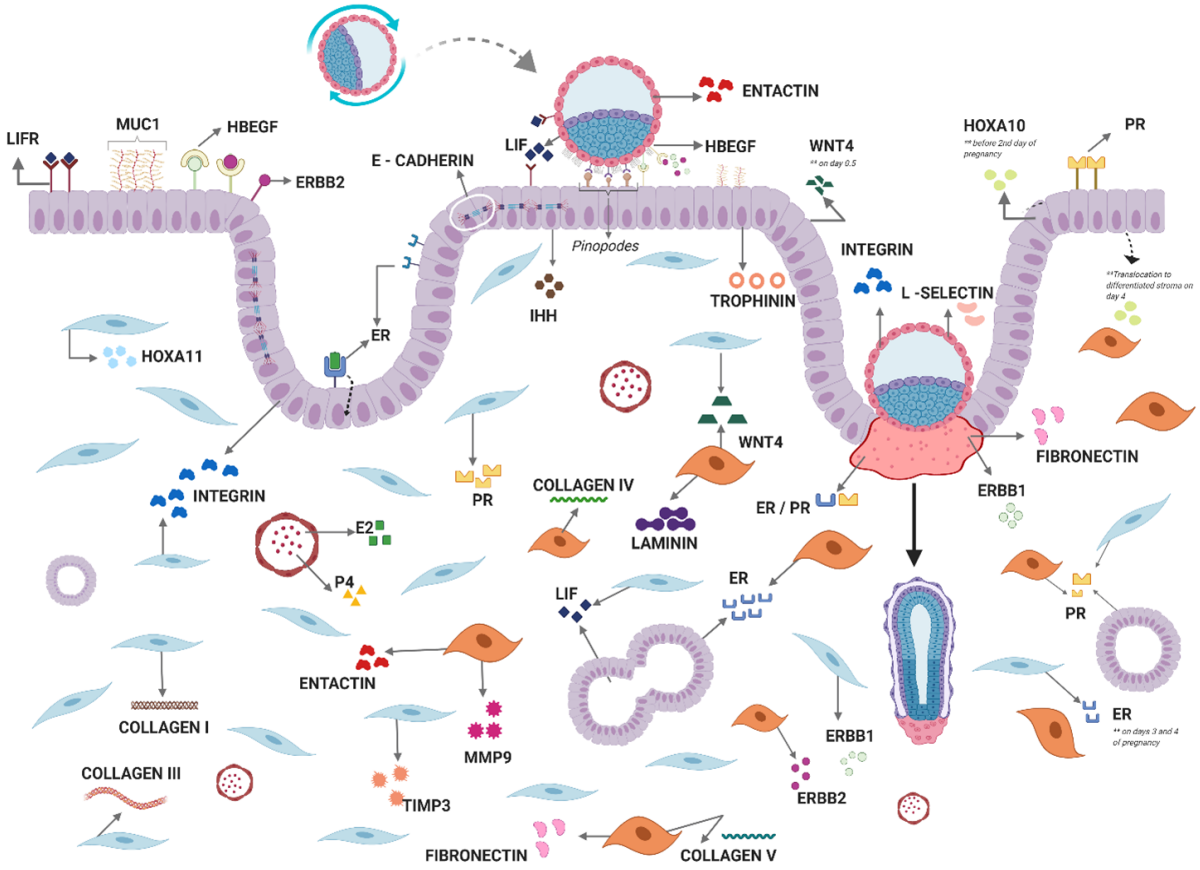

Figure 2. ***Molecular mechanisms and expression of molecules during implantation.*** The figure shows the expression pathways of implantation related genes and mechanisms. An embryo takes its position, attaches to the endometrium surface and implants through inside. Many molecules contribute to these processes. The surface epithelium (lining purple cells) secretes leukemia – inhibitory factor receptor, oestrogen receptors, mucin 1, heparin – binding EGF like growth factors, ERBB2, ERBB1 WNT4 (on day 0.5 of the pregnancy), homeobox A10 (before 2nd day of pregnancy), progesterone receptors, and trophinin. The oestrogen receptor is translocated to nucleus from cell surface in the presence of oestrogen. Glandular epithelium (gland shaped purple cells) expresses leukemia – inhibitory factor in addition of oestrogen and progesterone receptors. Underlying stromal cells (blue cells) express laminins, WNT4 molecules, progesterone, and oestrogen receptors (on days 3 and 4 of pregnancy), ERBB1, leukemia – inhibitory factor, integrin, homeobox A11, TIMP metallopeptidase inhibitor 3, and collagens I & III. The decidualized stromal cells are figured in orange. These decidua cells secrete laminin, WNT4, entactin, matrix metallopeptidase 9 (MMP9), fibronectin, ERBB2, progesterone and oestrogen receptors, collagens IV & V. Besides, leukemia – inhibitory factor receptors, entactin, HBEGF, and ERBB1 are expressed from an attaching embryo. The syncytiotrophoblast cells of an implanting embryo can express the oestrogen and progesterone receptors in addition to fibronectin and ERBB1. L – selectin and integrin are also expressed from the trophoblastic cells of an implanting embryo. E- Cadherin is a junction molecule which is responsible for cell-to-cell attachment. Oestrogen and progesterone reach to the implantation area and its environment via the blood stream. *This figure is created via Biorender.com.*

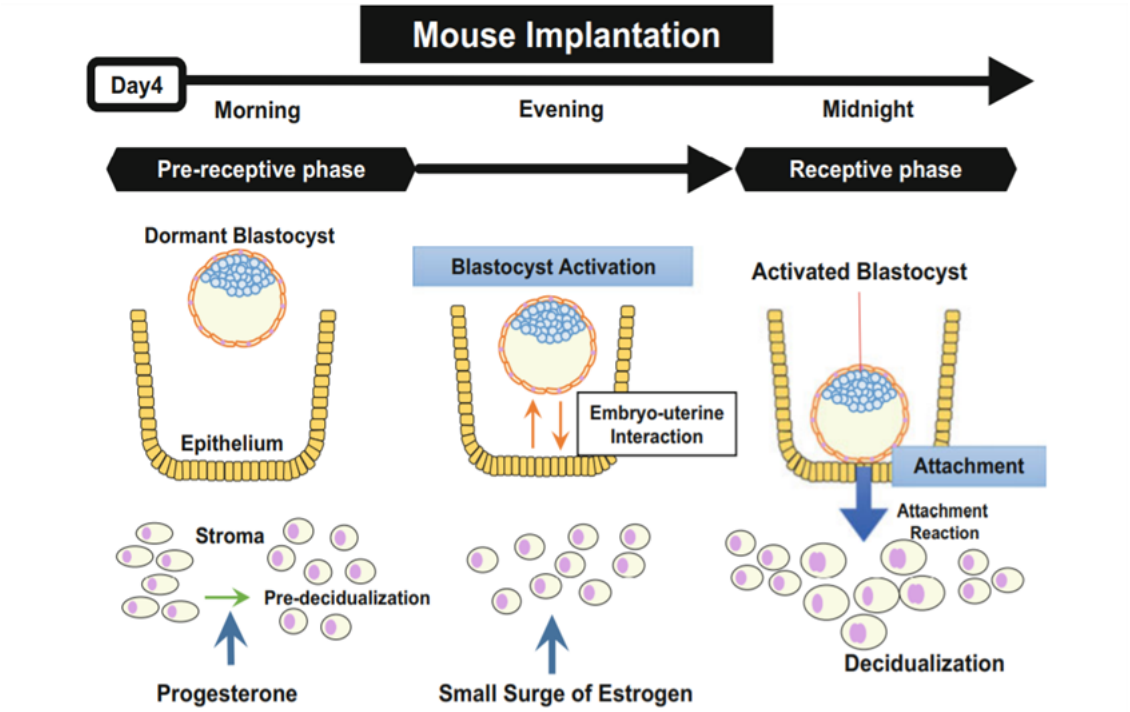

Figure 3. ***Schematic Presentation of Mouse Embryo Implantation*** [9] On day 4 of the oestrous cycle of mice, blastocyst activation starts to occur, and the embryo interacts with the uterus itself under the effect of a small surge of oestrogen hormone. By the end of day 4, attachment reaction consists, and this leads to decidualization.

2.1. Blastocyst Competency and Activation

A blastocyst needs to be competent to and active to successfully implant. Blastocyst activation can be defined as "programming of blastocyst to an implantation – competent state."[6] Golgi and endoplasmic reticulum functions are more apparent in an activated blastocyst when compared to dormant ones. [7] Dormant blastocysts are considered as non – active and less mitogenic. Oestrogen induces a series of complicated events related to transcriptional and translational pathways, during which an activated blastocyst possesses a full potential of the energy. [8]

2.2. Uterus Receptivity and Window of Implantation

When implantation initiates in mammals with placenta, the endometrium alters its state and begins to differentiate. The receptivity of the uterus can be defined as the accommodation capacity for competent blastocyst. [10, 11] The state of the uterine sensitivity in humans can be divided into three

main stages: pre-receptive, receptive, and nonreceptive. However, in mice, the oestrous cycle is irregular and can be categorized into 4 subgroups: proestrus, oestrous, metestrus, and dioestrus. In mouse, the oestrus cycle is not stable like humans and lasts for approximately 4 days. Day 4 is accepted as the receptive phase of the endometrium in mice. In humans, the menstrual cycle lasts from 28 to 30 days. Between days 20 to 24, the uterus is in secretory phase and the endometrium is receptive under the effect of progesterone and oestrogen.[12]

There are several molecular and cellular pathways which have roles during this short period known as the window of implantation. Progesterone and oestrogen are the major hormones that regulate the receptivity and sensitivity of the uterus for implanting blastocysts.

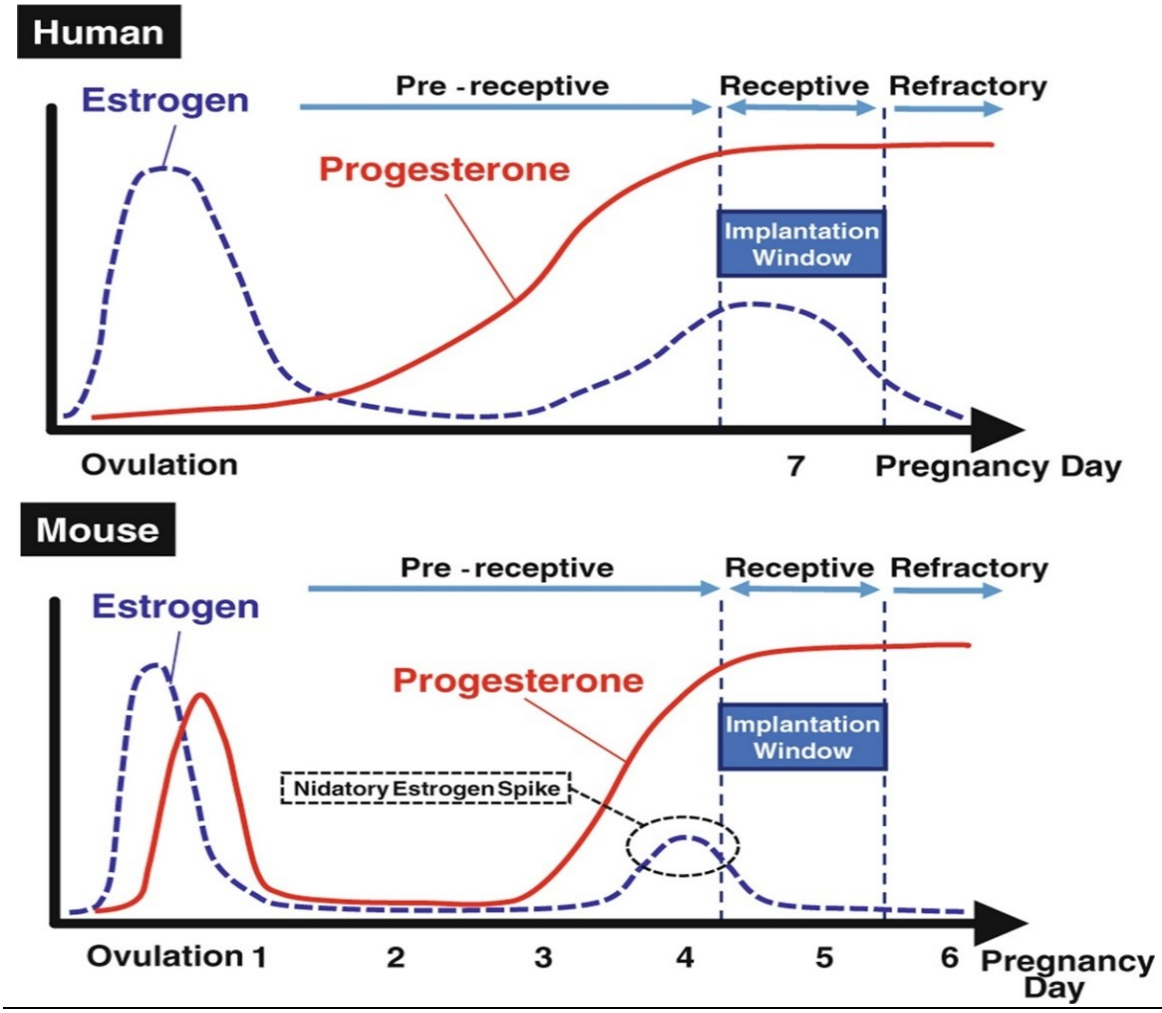

Figure 4. ***Hormone Effects During Phases of the Uterus for Human and Mice.***[13] Both for humans and mice, oestrogen and progesterone are the main functional hormones. In humans, progesterone is starting to be in the area from pre- receptive phase, whereas, in mouse it is also used during ovulation. In mouse, just before the implantation window, oestrogen spikes a small peak. Both for mouse and human, oestrogen and progesterone are functional during window of implantation.

2.3. Decidualization

Decidualization is a process when the endometrial stromal cells undergo morphological and functional changes dramatically to form decidual cells. Morphogens, cytokines, transcriptional factors, biochemicals are responsible from this complex procedure.[14] In the afternoon on day 5 of the mouse pregnancy, stromal cells differentiate and form primary decidual zone. By the days 6 and 7, decidual cells proliferate and fully form the decidual zone. [15] After the 8th day, secondary decidual zone is replaced by placental and embryonic development. [16] Besides its roles as supporting the

embryo development or regulating the trophoblast invasion, the decidua is also responsible for angiogenesis of maternal blood vessels that feed the developing embryo. [17]

3. SIGNALING MOLECULES AND PATHWAYS DURING IMPLANTATION

Implantation is a process consisting of serial complex molecular and cellular pathways and mechanisms. There are several genes or cellular molecules which have functions during this process. These molecules can be categorized as having functional or developmental roles and/or being important in adhesion and invasion steps, as described in *Table 1*.

FUNCTIONAL	DEVELOPMENTAL	ADHESION	INVASION
• LIF • ErbB1 • ErbB2 • ER • PR	• HOXA 10 • HOXA 11 • WNT 4 • IHH	• E-Cadherin • Laminin • Fibronectin • Entactin • Integrin • L-Selectin • Trophinin • Collagen I • Collagen III • Collagen IV • Collagen V	• MMP9 • TIMP3

Table 1. ***Categorization of the Implantation Related Genes***

3.1. Cytokines

Cytokines are the multifunctional peptides that help immune system as chemical messengers and immunomodulating agents by being involved in autocrine and paracrine systems. [18] It has been studied that cytokines have some important roles for a successful implantation. [19] Previous studies showed that after IVF treatment, the abnormal cytokine expression from embryos and endometrium itself may cause implantation failure. [20]

The cytokine activities can be categorized into two groups: pro - inflammatory and anti – inflammatory, due to their stimulation and reduction effects on inflammation respectively. [21] Invasion of a blastocyst and angiogenesis may be positively affected by a transient exposure to pro – inflammatory effect of a cytokine, whereas a long exposure time may cause some pregnancy defects.

3.1.1. LIF

Leukaemia Inhibitory Factors are the members of interleukin – 6 (IL – 6) family as cytokines. LIF binds to its receptor β (LIFR - β) which surrounds the membrane and glycoprotein 130 (gp130) to regulate several different cellular functions. LIF has multiple roles during implantation: formation of receptivity of the uterus, development of blastocyst, interactions between uterus and embryo, invasion of trophoblast. [22]

On day 4 of the pregnancy of a mice, LIF is found in the uterine lumen and epithelial cells.[23] When the expression of LIF is suppressed in mice, there is a development to blastocyst stage from a zygote, but no implantation occurs. [24, 25] Besides, according to further pathways of LIF signalling, gp130 knockout mice embryo cannot complete the second gestation period while LIFR–β may have successful implantation but can face with abnormalities about placenta or neuronal defects. [26]

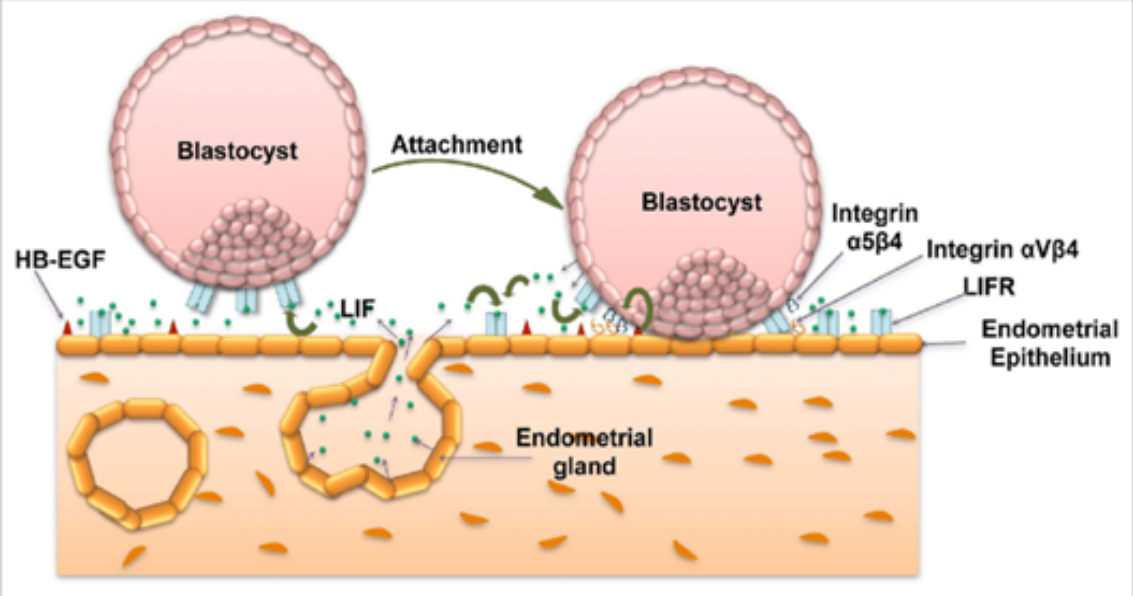

Figure 5. ***LIF mediated signaling pathways in the attachment of a blastocyst.***[27] Endometrial luminal and glandular epithelium, and blastocyst express LIF. Pinopodes are also formed during the time of implantation. HBEGF signaling on epithelial cells leads to the expression of integrins by trophoblast cells. All these events orchestrate the initiation of the blastocysts attachment which is also correlated with LIF expression by itself to have a role in autocrine and paracrine pathways.

3.2. Developmental Genes

Development of a good quality embryo is a process which is started primarily by the fertilization a qualified an oocyte with a high-quality sperm. Along with the quality of the oocyte having a major role for development of a good blastocyst, the period after fertilization is also important for determination of a proper embryo.[28]

Transcription factors and their related signaling pathways are crucial for embryonic development. Homeobox transcription factors and the hedgehog family of morphogens are the ones which attracted a prevalent attention for the studies because of their widespread effects on the developmental and the pathophysiological effects during embryogenesis. [29-31]

3.2.1. Homeobox Transcription Factors

Homeobox genes are known as genes that regulate specific nuclear proteins which act as transcription factors. In mammals, HOX, MSX, EMX, and HMX are determined as subgroups of homeobox genes which were initially identified in Drosophila. [32] Homeobox genes are regulated directly or indirectly by ovarian oestrogen (E2) and progesterone (P4) that are secreted periodically during reproductive cycles.

The largest family of homeobox genes is Hox genes family which represents the embryonic development in mice. [33] According to their chromosomal rearrangements and genomic loci, Hox genes are clustered into 4 groups. The expression patterns of Hox gene clusters are commonly overlapping in mice and human. [34]

Hoxa10 and Hoxa11 are the genes that have similar expression patterns which also supports that they have similar roles during the process of implantation. Before day 1.5 of mouse blastocyst implantation, Hoxa10 is expressed in luminal and glandular epithelium, then its expression location is translocated to the underlying stroma on day 4. [35] Similarly, uterine stromal cells express Hoxa11 which may be responsible for female infertility in the absence of this gene. [36] Both Hoxa10 and Hoxa11 play crucial and similar roles in decidualization process and the development of female reproductive tract in mice. [37, 38]

During peri-implantation process, the expression levels of cell adhesion molecules such as β3 integrin is upregulated in endometrial epithelial cells by Hoxa10. Additionally, Hoxa9, HOXD10, and

HOXB6 are other highly expressed genes involved in the functions of mid-secretory phase of human endometrium during menstrual cycle. [39]

3.2.2. WNT

In mammals, cell – cell interactions during embryogenesis and development are regulated by Wnt proteins which constitute a large cysteine – rich family. [40] Up to today, in mouse and other vertebrates, almost nineteen different Wnt genes were identified.

Through the preimplantation embryo development in mouse, many Wnt ligands, receptors, and regulators are highly expressed. [41-43] MAPK and Ca^{++} pathways, which are known to be crucial for unproblematic and normal preimplantation embryo development, are also controlled by Wnt proteins. [44, 45]

Evidence by several expression studies have proved that Wnt ligands may function in blastocyst competency, blastocyst activation, and morula to blastocyst transition.[46] Besides having roles during implantation process in rodents, Wnt signaling pathways also have roles during embryo – uterine interactions during early stages of pregnancy in sheep.[47]

3.2.3. IHH

Hedgehog signaling is important for the development of several different tissues including gonads. [48, 49] Indian Hedgehog, encoded by IHH, is a morphogen which regulates the cellular communication between endometrial epithelium and stromal cells during embryo implantation under the control of progesterone (P4). [50, 51] Although the IHH expression is increased during the early pregnancy and proestrus in adult rats, the oestrogen exposure downregulates the IHH expression in immature ones. [52, 53] P4 regulated gene, IHH, has its highest expression level on day 2.5 during preimplantation period and is expressed in the mouse uterine luminal epithelium.[54]

3.3. Cell Adhesion Molecules

After a blastocyst positions itself to the endometrium, it adheres itself onto the lining epithelium by the help of adhesion molecules. Integrins, selectins, trophinins and cadherins are the adhesion molecules.

3.3.1. Integrins

Integrins are the members of multi adhesive glycoproteins that are considered to be functional in uterine receptivity. They are formed by the bonds between the subunits, α and β, and found in plasma membrane. During early proliferative phase of the endometrium, especially β1 integrin is expressed by the glandular epithelium, while stromal cells also secrete integrin in the mid - secretory phase.

Due to the adhesive function of integrin, the lack of β1 subunit of integrin may cause implantation failure and deficiency of adhesion and invasion of a mouse blastocyst. [55]

3.3.2. Selectins

Selectins are calcium – dependent glycoproteins located on plasma membranes. There are several types of selectins such as L – Selectin, P – Selectin, and E – Selectin. P and E – Selectins have roles in immune recognition and trophoblast invasion, whereas L – Selectins are responsible for the adhesion of the embryo to the uterus during the interface period. L – Selectin is mostly expressed on circulating white blood cells. In humans, L – Selectin ligands are highly upregulated by uterine endometrial epithelial cells during the time of window of receptivity on the maternal side, likewise, trophoblasts of a blastocyst express L – Selectin at the fetal side of implantation.[56]

3.3.3. Trophinins

Trophinin is a membrane protein which mediates the homophilic binding. They are especially expressed in human luminal epithelium between days 16 and 17 of the early secretory phase. [57] During implantation, bystin, another cytoplasmic protein, directly binds to trophinin. Despite the trophinin expression is specific to mammals, bystin is also expressed in eukaryotes. [58] Ovarian hormones are the mediators of trophinin expression, independent of the existence or the absence of an implanting blastocyst in mouse.

3.3.4. Cadherins

E – Cadherin is a calcium – dependent transmembrane glycoprotein which is responsible for adhesion of the epithelial cells. Out of 3 types of cadherin, such as, N – Cadherin, P – Cadherin, and E – Cadherin; E – Cadherin has roles for regulating the attachment of an implanting embryo to the endometrial epithelium in mice.

E – Cadherin is expressed in the uterine epithelial cells and the embryo at the peri – implantation state. At morula stage, E – Cadherin has a crucial role for formation of blastocyst as a result of compaction of the morula.[59]

3.4. Extracellular Molecules

Extracellular Matrix (ECM) is a 3D environment composed of molecules such as enzymes, proteoglycans, collagen, and fibrous proteins. This network provides structural and biochemical support and helps cells for migration, proliferation, differentiation, and adhesion.

3.4.1. Fibronectins

Being member of the extracellular matrix proteins, fibronectin functions in adhesiveness and binds to integrins which are membrane receptor proteins. Facilitation of the implantation may be caused by one of the functions of fibronectins, as adhesiveness. It has been shown that when fibronectin and laminin are coated individually on tissue culture plates, mouse blastocyst attachment is improved *in vitro.*[60]

3.4.2. Laminins

Laminins are glycoproteins which function as building blocks of the connection between the intracellular and extracellular compartments. [61] Laminin is composed of three different domains named as α, β, and γ chains. The implantation related role of the laminin is to provide the integrity of the basal membrane. During menstrual cycle and early period of the pregnancy, laminin has been determined dynamically in the endometrium.[62]

3.4.3. Entactin

Entactin is a glycoprotein component of basement membrane. The role of entactin is to act in connection of the networks formed by laminins and collagens. On day 4 of the pregnancy, entactin is expressed in the decidualized stromal cells. It has been found not only on the stromal cells, but also on the trophoblastic cells of an attaching embryo. Besides, entactin is detectable on compacted morulae (8 to 16 cell stage) firstly.[63]

3.4.4. Collagens

Collagens are the major fibers of the extracellular environment in a tissue. During decidualization of the underlying stroma, implantation, and pregnancy, collagen fibers play crucial roles. According to

studies on rats, smooth muscle cells in myometrium layers secrete collagen under the effect of oestrogen hormone in females with normal oestrous cycle. [64] In mice, collagen types I, III, and V are detected in the endometrial stroma during days 5 to 8 of pregnancy.[65] Secretion of growth factors, proliferation and migration of cells can be considered as the major roles of collagen IV in tissues. Collagen IV is found in the decidualized zone in a receptive uterus. [66]

3.5. Enzymes

During implantation, especially the invasion process is highly influenced by two major enzyme families: matrix metalloproteinases and tissue inhibitors of metalloproteinases. Enzymes are secreted for digesting the extracellular matrix environment to ease the invasion of the adherent embryo.

3.5.1. MMPs

Matrix metalloproteinases are the members of the proteolytic enzymes which are zinc – dependent. The degradation of the extracellular matrix can be carried out by MMPs. There are more than 20 subtypes of MMPs, but MMP2 and MMP9 are the types of matrix metalloproteinases commonly found in human endometrium. Especially MMP2 and MMP9 degrade collagen I, IV, V, laminin, and fibronectin, all of which are expressed in the implantation area. The hyper activeness of MMPs may be the cause of impairing effect on endometrium, such as inflammation. [67] Therefore, there must be a balance between the activation and inhibition levels of MMPs.

3.5.2. TIMPs

Tissue inhibitors of metalloproteinases (TIMPs) are the inhibitors of MMPs. Generally, it is accepted that all TIMPs have capability to inhibit all types of MMPs, but however, MMP9 activities are especially inhibited by TIMP1 and TIMP3.[68] TIMP3 regulates the invasion process of cytotrophoblasts. As mentioned above, TIMPs are necessary to balance the MMP activity to prevent unfavorable effects on endometrium.

3.6. Growth Factors

Signaling proteins which can induce proliferation and differentiation in cells are leading to growth factors. Several species such as rodents, cow or sheep express genes for fibroblast growth factors, epidermal growth factors, insulin receptors, and insulin – like growth factors by their early-stage embryos. The action of epidermal growth factors (EGF) and leukemia inhibitory factor (LIF) are responsible for development of an embryo. [69]

Trophoblast cells express heparin – binding epidermal growth factor (HBEGF) – like growth factor which is responsible from synchronization of the signals between the endometrium and the trophoblastic cells of an implanting blastocyst.[70]

3.7. Hormone Receptors

Oestrogen and Progesterone are the hormones that are produced from the cholesterol in the ovary and are highly functional during the reproduction processes. Absence of both progesterone and oestrogen causes some abnormalities related ovulation and fertility. Implantation and development of an embryo may be adversely affected by the elimination of progesterone and oestrogen hormones from pregnant animals. It is also found that the expression levels of ER and PR genes are different in a preimplantation embryo. During preimplantation stage, PR gene is not expressed until blastocyst stage, whereas ER can be first detectable in fertilized eggs.[71] When these hormones bind to related receptors, they reveal their function. As hormones bind to their receptors, the receptors get activated as transcription factors to express their relevant target genes.[72]

3.7.1. ER

Oestrogen is a steroid hormone that functions in establishment of female reproduction system, growth of ovarian follicles, inhibition Follicle Stimulating Hormone (FSH) to avoid development of multiple mature follicles, stimulation of pituitary gland to release the Luteinizing Hormone (LH).

There are two different isoforms of oestrogen receptors (ER) which are ERα and ERβ. Previous studies about these receptors have shown that ERβ has high level distribution especially in ovary, epididymis, and prostate, whereas ERα has a wider expression area.

In the proliferative phase of the uterus, increment of oestrogen receptor alpha (ERα) occurs in response to increasing oestrogen levels, while during WOI, there is a decrement of this expression.[73] ERα is expressed in the luminal epithelium on the first day of pregnancy in mouse.[74] During menstrual cycle and pregnancy, ERβ is a highly upregulated receptor in endometrial epithelial cells. [75]

3.7.2 PR

During early stages of pregnancy, progesterone has crucial roles in the receptivity of the uterus for blastocyst attachment, embryo – uterine interactions, and decidualization.[76] Congruently to ER, Progesterone Receptor (PR) has two isoforms as PRα and PRβ. They are expressed by the same responsible gene; however, they have different start codons. [77, 78]

PR signaling pathways and mechanisms provide knowledge about the regulation of uterine function by paracrine signaling pathways.[79] According to previous studies, which were carried out with "progesterone receptor knockout mouse models", the mice became infertile related to various defects in ovary, uterus, embryo implantation, and decidualization. [80] For proper embryo implantation and descendent decidualization of stromal cells, PR has crucial anti-inflammatory and anti-proliferative roles in uterine epithelium. MUC1, an epithelial cell-surface glycoprotein, is inhibited significantly under the effect of PRα in relation to attachment and implantation of the embryo. [81, 82].

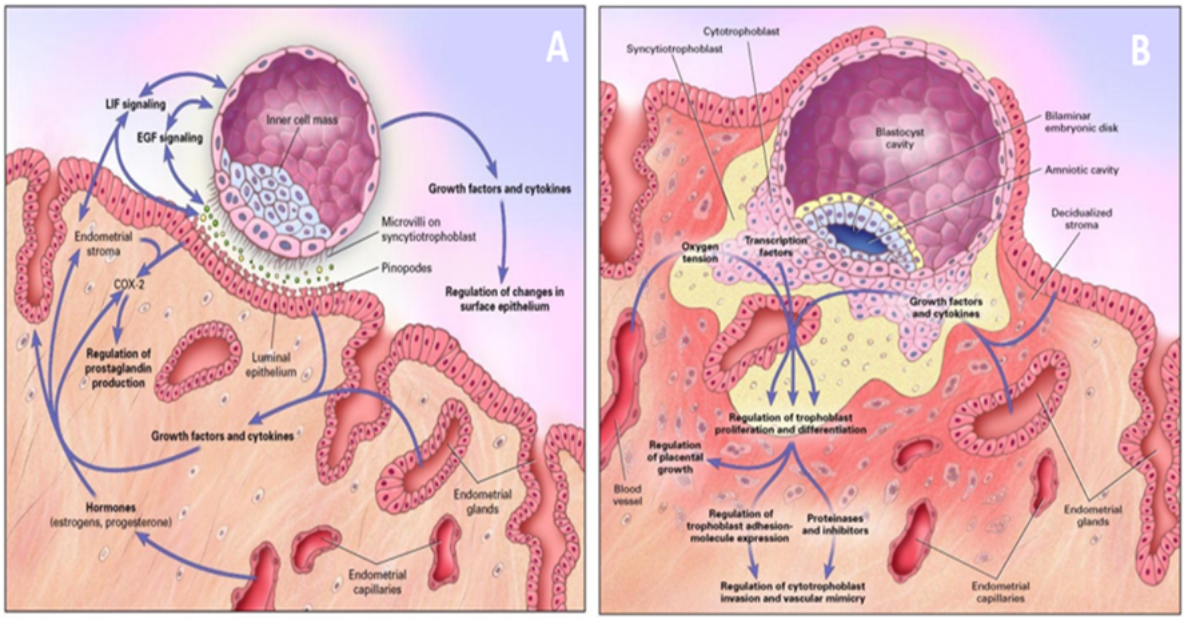

Figure 6. ***Blastocyst Apposition, Adhesion (A), and Implantation (B)*** *[83]* The signaling pathways during blastocyst apposition, adhesion, and implantation steps.

4. IMPLANTATION FAILURE AND ASSISTED REPRODUCTIVE TECHNOLOGY

Successful clinical pregnancy is composed of serial and synchronized events as implantation, decidualization, placentation, ongoing pregnancy, and finally live birth. In humans, conception occurs naturally only 30% of the time, even when the couple is young per cycle. The successful conception ratio per cycle and clinically acceptable pregnancy rates depend on multiple factors including maternal age, the quality and quantity of sperm of male, uterine pathology, and health of the uterus. Failed pregnancies which occur 75% of the time, are typically due to the implantation failure.[84]

Implantation beyond WOI may result in spontaneous miscarriage. Implantation failure linked miscarriages can also be related to embryo quality, uterine abnormalities, maternal or paternal genetic abnormalities or these miscarriages may be due to the problems related to hormonal, metabolic and immunological responses, or the embryo itself, as well.

Clinically infertility is defined as the absence of clinically defined pregnancy after 1 year of innate unprotected sexual intercourse. [85] One out of seven couples around the world consults doctors because of infertility issues. Currently, the use of Assisted Reproductive Technologies (ART) is increasing because infertility rates getting higher worldwide.

4.1. Recurrent Implantation Failure and Miscarriage

Even though Recurrent Implantation Failure (RIF) does not have a precise definition, it can be referred to as a situation in which the implantation of an embryo repeatedly failed to reach a stage which is recognizable via pelvic ultrasonography by a gynecologist. Recurrent implantation failure involves the reasons about the embryo, the uterus, and the environment.

Pregnancy loss before week 20 to 23 is termed as a “miscarriage.” The miscarriage can be either the loss of the embryo or the death of the fetus. Common reasons for miscarriage include infections, maternal medical conditions such as diabetes or thyroid disease, immune system protection responses, maternal age, cervical inefficiency, endometriosis, and misplaced embryo (ectopic pregnancy).

4.2. Assisted Reproductive Technologies (ART)

Assisted Reproductive Technologies are the combination of several different techniques which are used to provide solutions for infertility. These techniques can be listed as in vitro fertilization (IVF), in vitro maturation (IVM), artificial insemination, preimplantation genetic screening (PGS), preimplantation genetic diagnosis (PGD), cryopreservation, embryo transfer (ET), frozen embryo transfer (FET), intracytoplasmic sperm injection (ICSI) and intracytoplasmic morphologically selected sperm (IMSI).

One of the most used techniques is IVF which can be defined as controlling the menstrual cycle by suppressing the natural hormonal loop and supplementing the women by several functional hormones such as gonadotropins for superovulation. The oocytes and sperms are collected from the

couple and manipulated by ICSI or conventional IVF outside the body to let the sperm fertilize the egg for formation of the blastocyst.

5. IN VITRO CULTURE MODELS FOR IMPLANTATION

As the implantation process is highly complex, different culturing models are used to mimic the three-dimensional environment of the endometrium. Two – dimensional and three- dimensional systems are mostly used ones especially combined with several different techniques such as gel-based co-culture systems. As a human embryo cannot be used for further investigations due to the ethical concerns, animal models are the primary surrogates. Mouse embryos, trophoblastic spheroids from cell lines, primary trophoblasts, or first trimester placental explants are widely used to imitate the human embryo characteristics during adhesion and invasion. In addition, to construct the in vitro remodeling of the endometrium, epithelial cell lines or primary cultures and primary endometrial stromal cells or cell lines can be used as endometrium surrogates. [86]

5.1. Challenges About Culture Models

Studying implantation of an embryo with in vitro culture models is challenging since using human embryo maybe unethical depending on the regulation of the countries and animal models may not correspond outstanding to human processes. The major aim on studying in vitro models of implantation is to mimic the implantation environment outside the organism to enable understanding the implantation steps in detail.

6. POLYMERIC CELL CARRIERS & BIOMATERIALS

Polymeric carriers are the biomaterials widely used in pharmaceutical and clinical industries due to their biocompatibility, biosafety and non – toxicity. Biomaterials are commonly used for tissue formation studies as well as drug delivery experiments, regenerative medicine, or clinical applications. Depending on their crosslink formation capability as junction type networks, hydrogels are the commonly used materials as polymeric carriers. [87] Biodegradable materials categorized into two classes: natural and synthetic, due to their formations and sources. While metals, nonbiodegradable or biodegradable polymers or ceramics can be listed under the title of synthetic biomaterials; silk, collagen, cellulose, chitin or decellularized tissue - derived biomaterials are among as the natural ones. The preference to use either polymeric or natural materials for construction of scaffolds must be based on the required cellular attachment and proliferation capacity according to the experiment or

application. The biochemical properties of used biomaterials can be adjusted according to the interested research area in terms of the effect on cell survival, migration, and differentiation potential. [88]

6.1. Natural Materials

Generally, collagens, fibrinogens, silk are the commonly used proteins for construction of natural biomaterials in addition to polysaccharides such as starch and hyaluronic acid derivatives. [89] As the tissues have the extracellular matrix proteins and components, the naturally derived biomaterials are applicable for tissue regeneration, organoid formation, and replacement. As other synthetic and artificial biomaterials, natural ones are also used to mimic the natural Extracellular Matrix (ECM). When compared to synthetic and polymeric materials, natural materials have some specific advantageous points such as shortness of degradation period. Besides, purification and the control of quality are the main points which can be considered as difficulties and disadvantages.

Collagen is one of the main components of ECM and is found in almost all types of connective tissue. It is also usually preferred to be used as a natural biomaterial due to its biocompatibility and degradation potential. According to previous research, due to their acellular ECM structure and biocompatibility collagen based – biomaterials are not expected to cause immune response effect. [90] Collagen derived biomaterials are used for regeneration of cartilage and treating for cornea defects.

Matrigel is also used to mimic the 3D microenvironment. It is composed of MMPs, growth factors to support cell proliferation, Type IV Collagen, laminin, and heparin sulphate glycoprotein. Matrigel is a basement membrane which is soluble and extracted from Engelbrecht – Holm – Swarm (EHS). [91]

6.1.1. Synthetic Materials

Polyglycolic acid (PGA), polylactic acid (PLA) and the copolymer polylactide-co-glycoside (PLGA), poly (vinyl alcohol) and poly (ethylene glycol), (PEG)-polyester copolymers are the chemicals that are generally used for synthetic biomaterial construction. [92] Polymer – based biomaterials must be adjusted appropriately in terms of their temperature and pH sensitivity for providing the highest level of biosafety and to minimize the toxicity effects.

For 2D culturing models, artificial environments play roles as bio – adhesive surface, and polymeric biomaterials such as hydrogel microarrays function in the attachment and proliferation of stem cells. [93] Due to their superiority, which are biocompatibility, biodegradability, minimal risk of

immunogenicity and toxicity, polylactic acid (PLA) and poly (lactic-co-glycolic acid) (PLGA) are preferable as biomaterials to be used in the studies about regeneration, remodeling, and tissue – engineering applications. Also, they are preferred to be used as polymeric materials among researchers because their physical and mechanical properties are adjustable easily according to protocol needs by arranging the molecular weight, structure, and compositional properties.

Besides the usage of polymeric materials as scaffolds in cell culture methods, they are also used for the applications like drug delivery nanoparticles in defiance of their potential behaviors in systems of the organism. PLGA is an approved biocompatible polyester to be used in drug delivery systems for cancer treatments and are also a better choice due to their tumors and DNA targeting agents.

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 foetal bovine serum (12676029), foetal bovine serum (FBS) (Biochrom AG, Berlin) Penicillin-Streptomycin-Amphotericin B Solution, L-Glutamine, RIPA Lysis and Extraction Buffer were obtained 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 purchased from Sigma (St. Louis, MA, USA). Life Global sequential embryo culture medium was from Cooper's Surgical (Denmark). Corning Matrigel Basement Membrane Matrix (354234) was provided from Corning (NY 14831, USA). Quick-RNA Kit (Macharey-Nagel) was from Zymo Research (USA). Anti-tubulin (T9026) was purchased from Sigma, USA. Anti-E Cadherin (ab76055), Anti-LIF (ab113262), Anti-MMP9 (ab38898), Anti-pan cytokeratin (ab6401), Anti-Vimentin (ab92547), Anti-Fibronectin (ab2413), Anti-Integrin alpha 5 (ab150361), Anti-Entactin (ab14511), Anti-Collagen I (ab34710), Anti-Collagen IV (ab6586), Anti-Trophinin (ab78117), Anti-EGFR (ab2430) antibodies were purchased from Abcam (UK). Anti-ERα (ab32063) anti-PR antibodies (ab2765) and Anti- HBEGF (sc28908) were purchased from Abcam and Santa Cruz Biotechnology (USA), respectively. Oregon 488, Alexa Fluor 488, 568, 594 dyes, Hoechst 33342, and Live/Dead Viability/Cytotoxicity kit (L3224) were obtained from Life Technologies (Thermo Fisher Scientific Inc., MA, 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-cm^2 and 10 cm diameter tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) were used in cell culture experiments. Haematoxylin (105174), Eosin (109844), Entellan (107961) and PAS staining kit (101646) were purchased from Merck, Germany. Genipin was obtained from (G4796, Sigma, St. Louis, MA, USA) Sigma.

2. BLASTOCYST PREPARATION

2.1. Preparation of Animals

The animals used in the experiments were obtained from Koç University Research Center for Translational Medicine, Animal Research Facility with the Ethical Committee approval number 2015/15. The animals were fed ad-libitum and hold in light and dark 12 hours both.

2.2. Superovulation of Female Mice

CB6F1 (C57BL/6 X BALB/C) female 6-8 weeks mice were injected with 10 IU of hCG hormone after 48 hours following the 10 IU of PMSG injection to stimulate the superovulation. After injection of hCG, the female mice were mated with CB6F1 (C57BL/6 X BALB/C) male mice in 1:1 ratio per cage. The night after, the females with vaginal plug formation were detected and sacrificed with cervical dislocation method (Fig. 7).

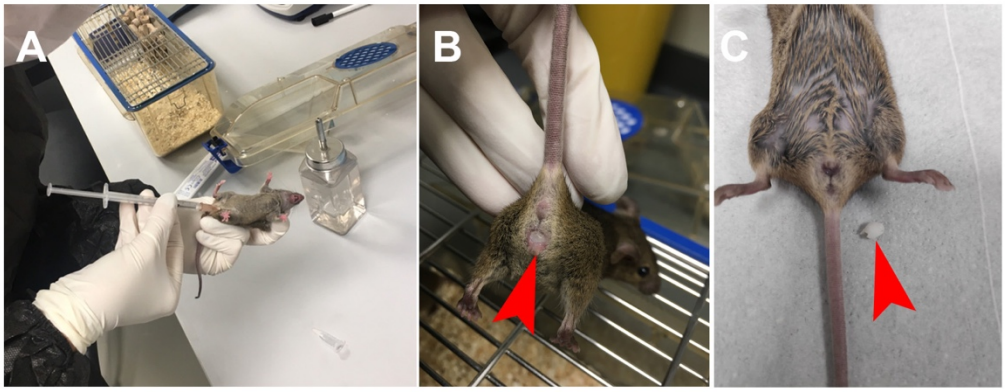

Figure 7. ***Superovulation of Female Mice.*** Female mice were injected with hCG following the PMSG hormone by i.p. injection method (A). Detection of vaginal plug of pregnant mice (B, arrowhead). Observation of vaginal plug outside after scarification of mice (C, arrowhead).

2.3. Isolation and Culture of Blastocyst

The ampulla regions of the uterus were taken to medium supplemented with HSA to manipulate the embryos in ambient atmosphere. The pronuclear (PN) zygotes were isolated from the ampulla region under stereo microscope. The zygotes were kept in hyaluronidase which is used for removal of cumulus cells and is a salt buffer solution containing human serum albumin and hyaluronidase enzyme. Then, they were rinsed in medium supplemented with HSA to remove the hyaluronidase and transferred to time lapse and single step embryo culture medium in 10 µl culture drops under oil in a 37 ° C incubator supplemented with 6 % CO_2. All the mediums except medium supplemented with HSA were pre-equilibrated at 37°C and 6 % CO_2 before usage (Fig.8).

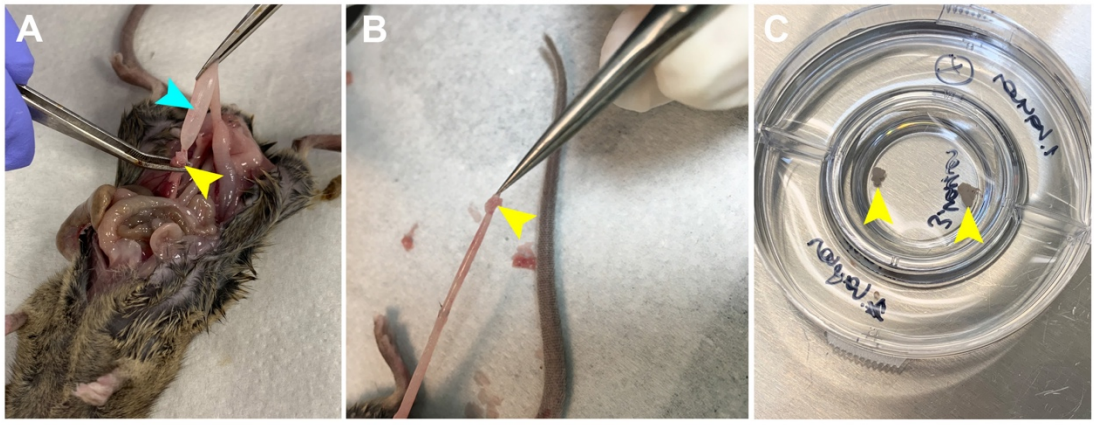

Figure 8. ***Isolation of the Blastocyst*****.** The V – shaped uterine horns can be seen in A (blue arrowhead). The ampulla region (the region between uterus and oviduct) is detected (A, yellow arrowhead) and separated from the uterus (B, yellow arrowhead). The ampullas were washed in buffered medium on a heated plate (C, yellow arrowheads).

2.4. Selection of the Blastocyst

The embryos which reached to day 4 and 5 were evaluated according to their qualities regarding Gardner's blastocyst scoring criteria. The blastocysts with many trophectoderm cells and good inner cell mass were selected for further culturing experiments (Fig. 9).

	Gardner's Criteria	Istanbul Consensus
Stage of Development	1 (Cavity < Half Volume)	1 (Early)
	2 (Cavity > Half Volume)	
	3 (Cavity = Volume)	2 (Blastocyst)
	4 (Expanded)	**3 (Expanded)**
	5 (Hatching)	**4 (Hatched / Hatching)**
	6 (Hatched)	
ICM Quality	**A (Many Tight Cells)**	**1 (Good)**
	B (Several Loose Cells)	2 (Fair)
	C (Few Cells)	
Trophoectoderm Quality	**A (Many Cohesive Cells)**	**1 (Cohesive)**
	B (Few Loose Cells)	2 (Loose)
	C (Few Large Cells)	3 (Very Few)

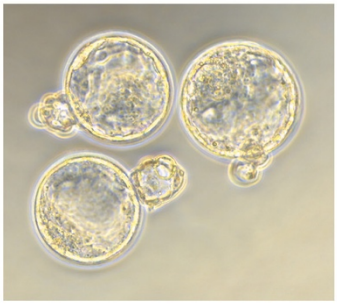

Figure 9. ***Selection Criteria for Blastocysts.*** In stages of development section, 4 and 5 in Gardner's criteria corresponds to 3 and 5 in Istanbul Consensus. For ICM Quality, A corresponds to 1 and for trophectoderm quality, A corresponds 1. Some of the selected hatching blastocysts can be seen on the right image.

3. NATURAL AND ARTIFICIAL SPONGE PREPARATION

3.1. Natural Collagen Sponge Preparation

Collagen solution of 1% w/v was prepared in 0.05 M acetic acid (30 mg collagen in 3 ml 0.05 M acetic acid), stirred for 2 minutes, and incubated for 48 hours at room temperature. 750 µl of polymer solution poured into 24 well plate. The samples were frozen at -20°C overnight. Then collagen was lyophilized (0.140 mbar or 133 x 10^{-6} bar, -80°C) for 8 hours.

For sterilization process, alcohol sterilization was performed but after evaporation of the alcohol, the sponges lost their shape and formation. Several protocols have been tried to obtain the best condition. Firstly, consecutively alcohol at +4°C, evaporation, and UV sterilization was applied, then alcohol at +4°C, rinsing with DPBS, evaporation, and UV sterilization methods were tried, respectively. But for both protocols, sponges got thinner like paper. So that, only UV sterilization was found the optimum sterilization. Also, the sterilized sponge pieces were directly stained with Hoechst staining to observe if there is any staining for any type of contamination.

To create the crosslinking, Genipin was used in concentrations as 0.3% and 0.1% to obtain the best formation. But for both concentrations, Genipin gave its blue color into the culturing medium after 24 hours. Also, it has been found toxic to the cells as cells did not attach to the surface and RNA concentration was found very low in optimization experiments. So that, both sterilization and crosslinking were done by UV methods and sponges were poured without genipin (Figure 10).

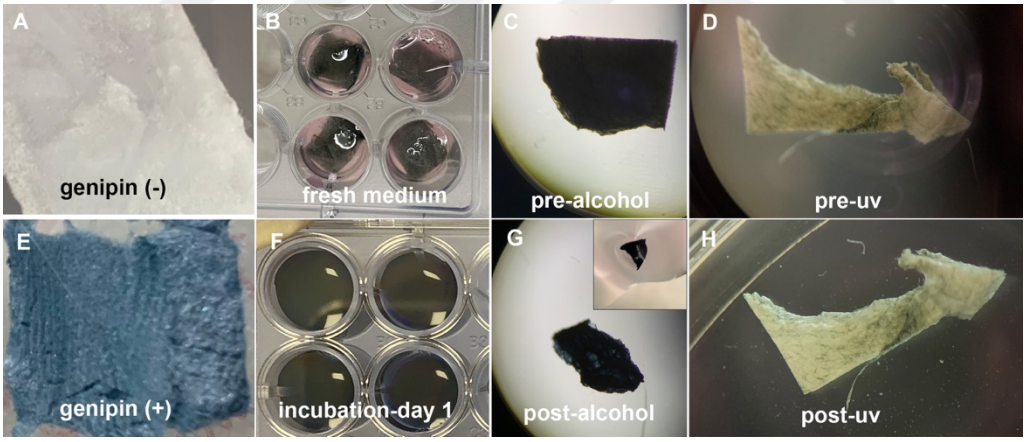

Figure 10. ***Crosslinking experiments for natural sponge.*** Genipin which is a cross linker caused blue staining of the medium (A, B, E, F). Alcohol used for sterilization caused a significant decrease in the volume of the sponge (C, G). Only uv light was used for both cross linking and sterilization (D, H).

3.2. Artificial Polymeric Sponge Preparation

Poly (L – Lactic acid – co – D, L – Lactic Acid) (PLDLLA) and Poly (D, L – Lactic acid – co – Glycolic Acid) (PLGA) polymers mixed in 1:1 ratio and 4-1% solution was prepared in dioxane. To find the best pore size, 4%, 3.5%, 3%, 2.5%, 2%, 1.5%, 1% concentrations were prepared, poured into petri dishes and was frozen at -80 and -20 °C**.** Then the materials were evaluated under scanning electron

microscope. The pores on the air facing side of the samples and the pores on the glass bottom side of the samples gave similar and trustable results. Depending on this, -20 °C and 2% concentration were selected, and optimization has been done according to the parameters of glass bottom side surface of the samples (Figure 10-12, Tables 2, 3). To obtain the equal porousness, ready - to – use sculptures were used for pouring process.

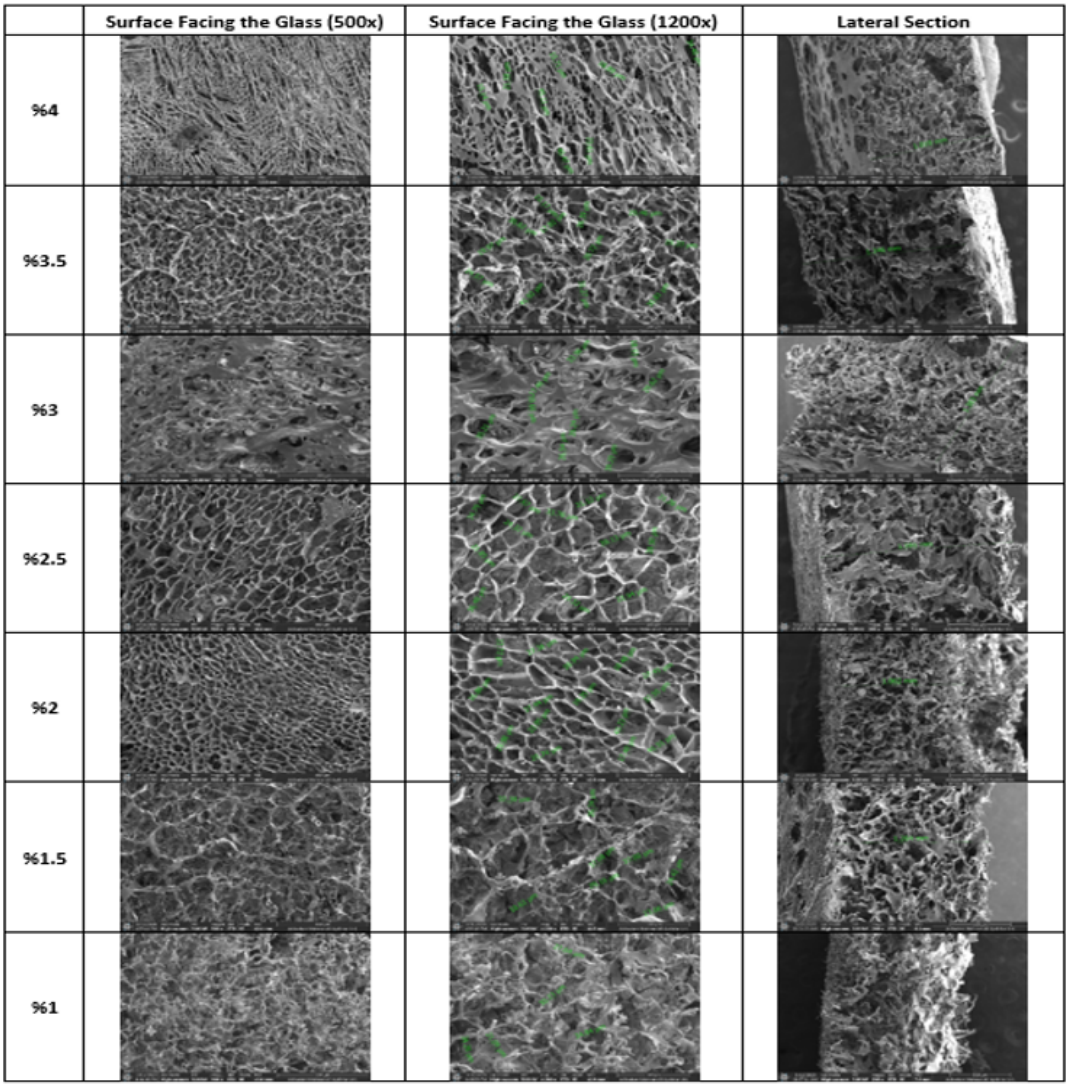

Figure 11. ***The glass facing surface images of polymeric sponges by freezing at -80 °C and with different concentrations.***

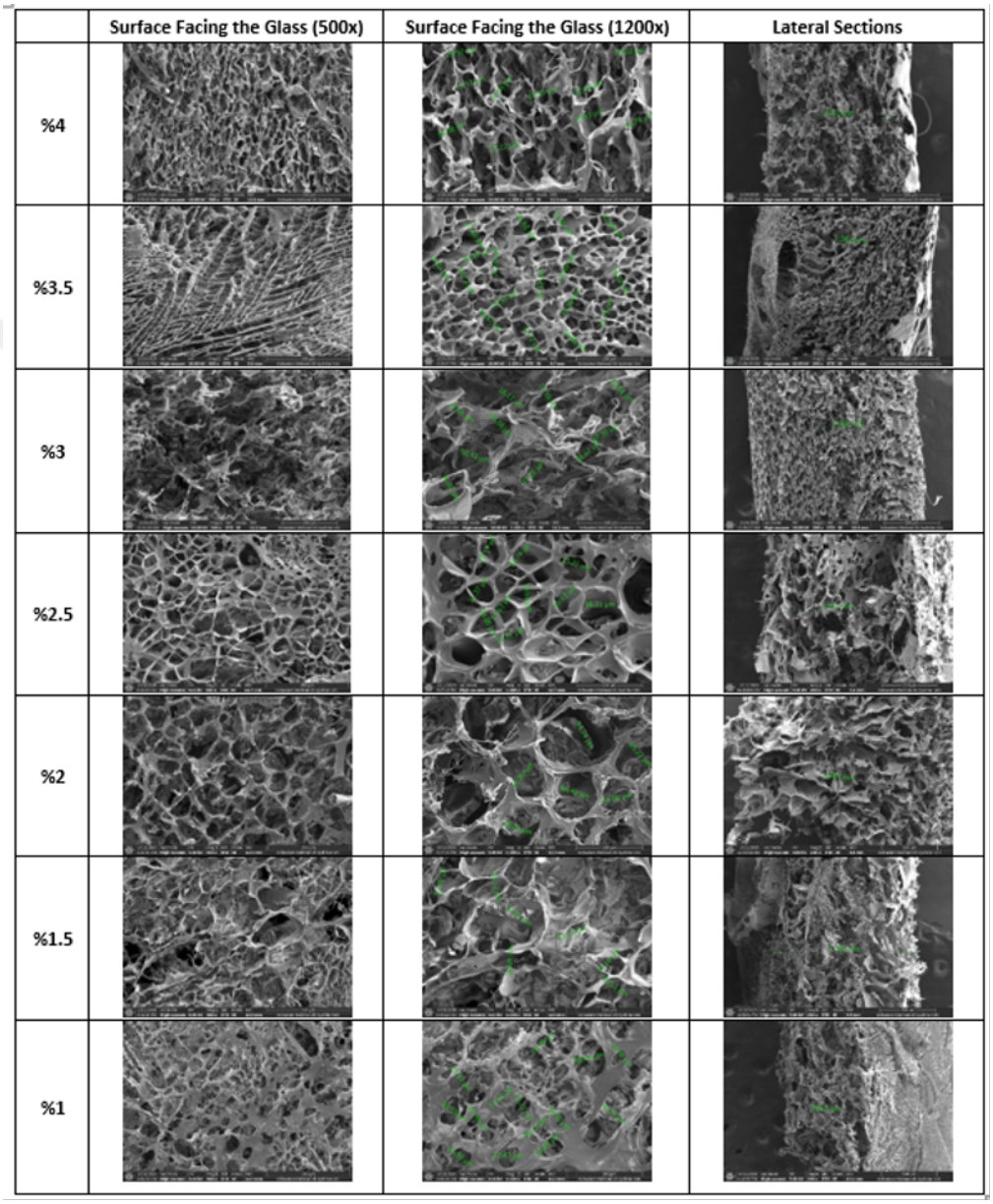

Figure 12. ***The glass facing surface images of polymeric sponges by freezing at -20 °C and with different concentrations.***

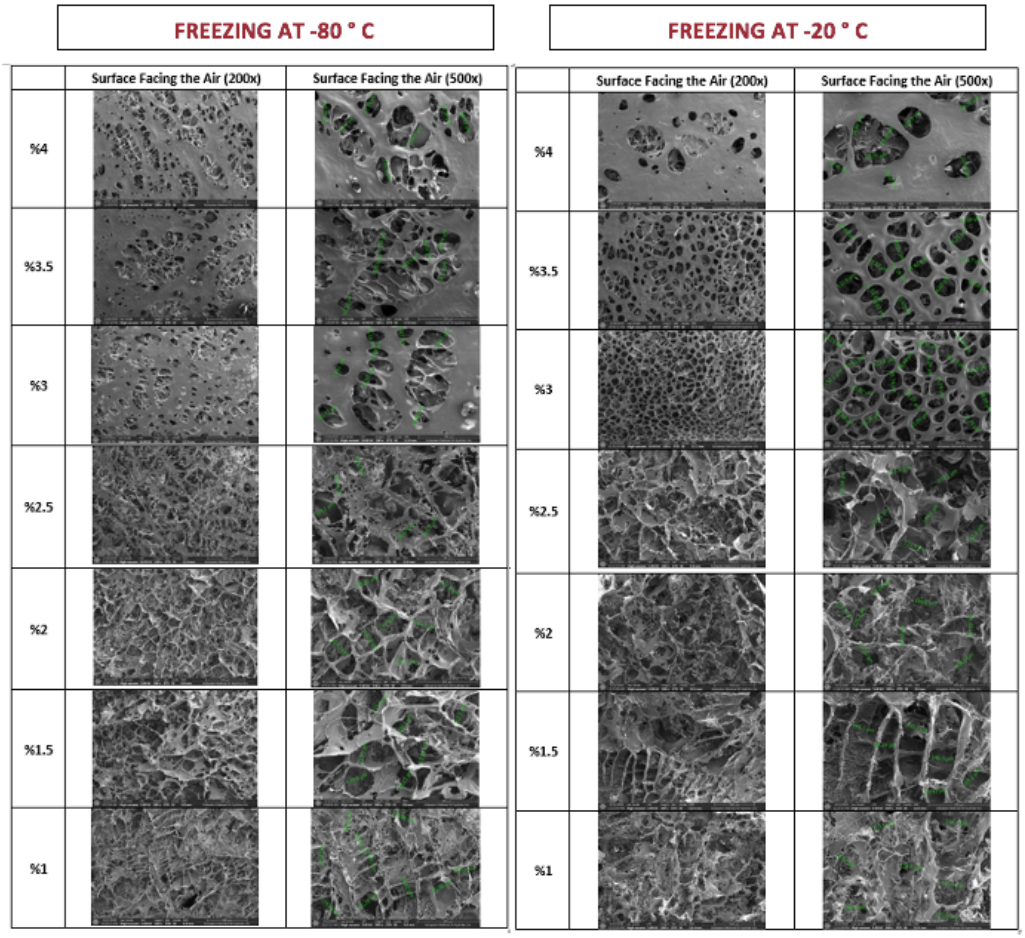

Figure 13. ***The air facing surface images of polymeric sponges by freezing at -80 °C and -20 °C with different concentrations.***

	Surface Facing the Glass Average Pore Diameter (µm)	Surface Facing the Air Average Pore Diameter (µm)	Lateral Section Average Pore Diameter (µm)
%4	**20.9** ±10.7	**76.91** ±15.8	**46.78** ±10.22
%3.5	**27.04** ±9	**48.56** ±13.82	**50.35**±12.11
%3	**24.2** ±7.80	**50.29** ±10.64	**42.19** ±15.81
%2.5	**38.45** ±10.64	**46.74** ±9.2	**39.8** ±14.6
%2.5	**30.536** ±7.72	**110.99** ±10.88	**69.04** ±14.28
%2	**29.81** ±7.34	**113.85** ±10.52	**89.31** ±11.51
%1.5	**27.34** ±5.15	**121.46** ±16.51	**90.38** ±15.15
%1	**31.63** ±7.16	**113.19** ±15.94	**87.94** ±9.66

Table 2. ***The pore sizes of the samples prepared in -80 °C with different concentrations.***

	Surface Facing the Glass Average Pore Diameter (μm)	Surface Facing the Air Average Pore Diameter (μm)	Lateral Section Average Pore Diameter (μm)
%4	**22.24** ±6.19	**38.39** ±7.61	**58.42** ±13.49
%3.5	**20.33** ±4.27	**75.34** ±11.22	**45.29** ±11.98
%3	**30.68** ±11.22	**83.07** ± 9.95	**43.53** ±10.77
%2.5	**36.67** ±10.10	**58.53** ±8.99	**80.4** ±14.44
%2.5	**33.85** ±10.57	**104.49** ±14.47	**90.53** ±9.01
%2	**47.39** ±10.85	**130.9** ±7.69	**121.28** ±12.86
%1.5	**54.01** ±15.3	**125.92** ±16.9	**141.21** ±11.75
%1	**30.7** ±7.12	**164.48** ±15.02	**92.45** ±10.68

Table 3. ***The pore sizes of the samples prepared in -20 °C with different concentrations.***

4. PREPARATION OF CULTURE SYSTEMS

4.1. Isolation and Culture of Epithelial and Stromal Cells from Endometrium of Mouse Uterus

To obtain epithelial and stromal cells from uterus, the horn shaped uterus was opened longitudinally, and the epithelial sheet of the endometrium was wriggled. The whole tissue was chopped into small pieces and incubated in an enzymatic solution consisting of 0.25% trypsin and 10 mg/mL collagenase/dispase at 4 °C for 2 hours and at room temperature for 1 hour. After the incubation period, the uterine tissues were centrifuged for 3 minutes at 300 x g. The supernatant was kept in another falcon tube and the remaining tissues were vortexed with ice cold HBSS until the epithelial sheets were released and observed. The epithelial sheet was aspirated and centrifuged for 3 minutes at 600 x g. The remaining pellet was added into the stored supernatant and incubated at 37°C for 30 minutes on a shaker. The centrifuged epithelial sheet pellets were resuspended with fresh DMEM F12: Ham's medium supplemented with 10 % FCS, 1% PS, 2 mM L-glutamine, 20 ng/ml EGF, 7.14 nmol/L β-Oestradiol, 63.5 nmol/L Progesterone. After resuspension, the resuspended tissues were filtered through a 100 μm cell strainer and the passed resuspension was again filtered by a 40 μm pore sized nylon mesh. The epithelial cells remained on the surface of the 40 μm mesh were

backwashed with a medium and seeded onto a 35mm culture dish. The filtered cells were also seeded. The incubated tissues were also centrifuged and filtered with the use of the same method to get the epithelial cells from the upper surface of the mesh and stromal cells from the filtered part and both parts were seeded in a 35 mm culture dish (Figure 13).

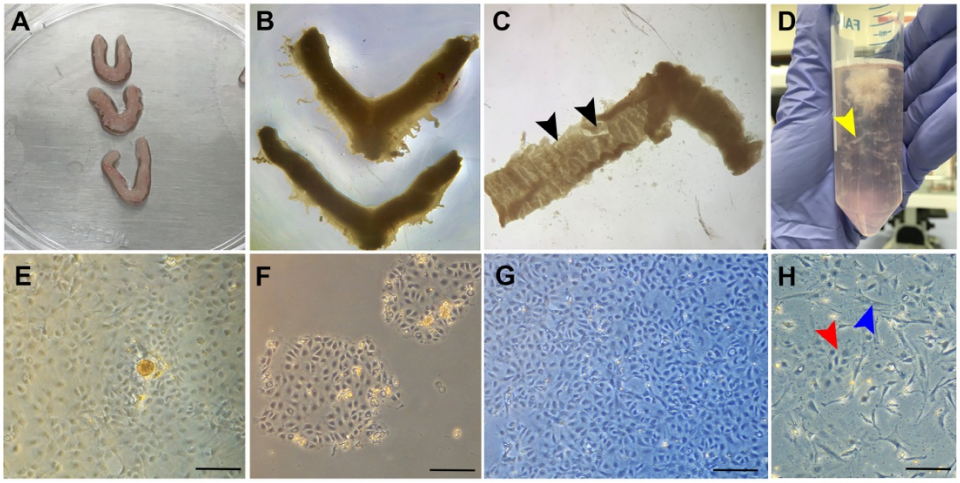

Figure 14. ***Isolation and Culture of Endometrial Cells*****.** Uteri were obtained from female mice (A, B). Under stereomicroscope, uterus horns were opened longitudinally, and epithelial sheet was wriggled (C, black arrowheads). After incubation, the chopped pieces were vortexed, and the epithelial sheet was released (D, yellow arrowhead). The seeded tissue pieces of epithelial area were left for explant tissue culture (E). The epithelial cells were isolated (F, G). In the co-culture area epithelial cells (H, red arrowhead) and stromal cells (H, blue arrowhead) can be identified. Scale bars: E-G=200 µm, H: 100 µm.

5. ESTABLISHMENT OF EXPERIMENTAL GROUPS

5.1. Routine IVF Group

In this group of the experiments, the blastocysts were cultured in a Life Global Culturing Medium drop for 48 hours and 96 hours. The drop was covered with light paraffin oil to avoid evaporation (Figure 15). The incubator was 37°C and was adjusted to 6% carbon dioxide.

5.2. THREE-DIMENSIONAL (3D) CULTURE MODELS

5.2.1. Construction of ECC Coated Artificial Polymeric Sponge

The artificial polymeric sponge pieces were sterilized with 70% ethanol and the alcohol was evaporated overnight under the hood and exposed to UV light for 2 hours. Lyophilized collagen was dissolved in 2% acetic acid and final concentration was 2 mg/ml. After sterilization, sponge pieces were covered with collagen (2.5 µl/cm^2) and dried in the incubator for 1 hour following the drying procedure by evaporation under the hood for 1 hour. Freshly isolated primary or early passage (1-4

passages) endometrial epithelial and stromal cells were thawed. Matrigel was diluted in 1:4 ratio with a serum free media. The thawed endometrial stromal cells (10^5 cells/per 10 µl) were mixed with Matrigel dilution and overlaid on sponge pieces (10 µl/piece) and left in a 37 °C incubator for 30 minutes for gelation process. Endometrial epithelial cells were seeded on the top of the Matrigel to mimic the endometrium organization. The Matrigel coated sponges were incubated in DMEM F12: Ham's medium supplemented with 10 % FCS, 1% PS, 2 mM L-glutamine, 20 ng/ml EGF, 7.14 nmol/L β-Oestradiol, 63.5 nmol/L Progesterone for 48 hours and 96 hours.

5.2.2. Construction of ECC Coated Natural Collagen Sponge

The natural collagen sponge pieces were sterilized with UV light for 2 hours. After sterilization, sponge fragments were wetted with medium. Freshly isolated primary or early passage (1-4 passages) endometrial epithelial and stromal cells were thawed. Matrigel were diluted in 1:4 ratio with a serum free media on ice. The thawed endometrial cells (10^5 cells/per 10 µl) were mixed with Matrigel dilution and overlaid on sponge pieces (10 µl/piece) and left in a 37 °C incubator for 30 minutes for gelation process. Endometrial epithelial cells were seeded on the top of the Matrigel to mimic the endometrium organization. The Matrigel coated sponges were incubated in DMEM F12: Ham's medium supplemented with 10 % FCS, 1% PS, 2 mM L-glutamine, 20 ng/ml EGF, 7.14 nmol/L β-Oestradiol, 63.5 nmol/L Progesterone for 48 hours and 96 hours.

5.2.3. Construction of ECC Coated Artificial Polymeric Sponge with Blastocyst

The artificial polymeric sponge pieces were sterilized with 70% ethanol and the alcohol was evaporated under the hood overnight and exposed to UV light for 2 hours. Lyophilized collagen was dissolved in 2% acetic acid and final concentration was 2 mg/ml. After sterilization, sponge pieces were covered with collagen (2.5 µl/cm^2) and dried in the incubator for 1 hour following the drying procedure by evaporation under the hood for 1 hour. The sponge fragments were wetted with medium. Freshly isolated primary or early passage (1-4 passages) endometrial epithelium and stromal cells were thawed. Matrigel were diluted in 1:4 ratio with a serum free media on ice. The thawed endometrial cells (10^5 cells/per 10 µl) were mixed with Matrigel dilution and overlaid on sponge pieces (10 µl/piece) and left in a 37 °C incubator for 30 minutes for gelation process. Endometrial epithelial cells were seeded on the top of the Matrigel to mimic the endometrium organization. The blastocysts were placed onto the Matrigel coated sponges by mouth pipetting under stereomicroscope. The sponge pieces were incubated in DMEM F12: Ham's medium

supplemented with 10 % FCS, 1% PS, 2 mM L-glutamine, 20 ng/ml EGF, 7.14 nmol/L β-Oestradiol, 63.5 nmol/L Progesterone for 48 hours and 96 hours.

5.2.4. Construction of ECC Coated Natural Collagen Sponge with Blastocyst

The natural collagen sponge pieces were sterilized with 70% ethanol and the alcohol was evaporated overnight under the hood and exposed to UV light for 2 hours. After sterilization, sponge fragments were wetted with medium. Freshly isolated primary or early passage (1-4 passages) endometrial epithelium and stromal cells were thawed. Matrigel were diluted in 1:4 ratio with a serum free media on ice. The thawed endometrial cells (10^5 cells/per 10 µl) were mixed with Matrigel dilution and overlaid on sponge pieces (10 µl/piece) and left in a 37 °C incubator for 30 minutes for gelation process. Endometrial epithelial cells were seeded on the top of the Matrigel to mimic the endometrium organization. The blastocysts were placed onto the Matrigel coated sponges by mouth pipetting under stereomicroscope. The sponge pieces were incubated in DMEM F12: Ham's medium supplemented with 10 % FCS, 1% PS, 2 mM L-glutamine, 20 ng/ml EGF, 7.14 nmol/L β-Oestradiol, 63.5 nmol/L Progesterone for 48 hours and 96 hours.

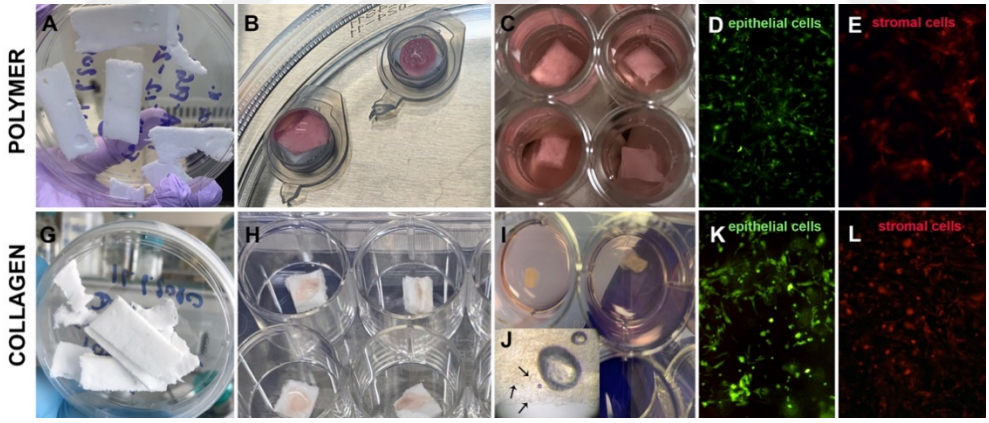

Figure 15. ***Construction of the Experimental Groups.*** Polymeric sponge pieces (A-E) were coated with Collagen Type 1, and cells were suspended in Matrigel and seeded as drops on the top of the sponges (B). The cell - covered sponge pieces sunk into the medium (C). Epithelial cells with GFP and stromal cells with FMC covered the surface of the sponges (D, E). Matrigel was put as drop on the top of the natural collagen sponges (G-L). Three blastocysts can be seen in J (arrows). Epithelial cells with GFP and stromal cells with FMC covered the surface of the sponges (K, L). The reason for use of GFP and FMC is only to show the presence of the cells.

6. EXPERIMENTAL GROUPS

6.1. Routine IVF Group

The blastocysts were cultured in Life Global Culturing Medium drop for 48 hours and 96 hours. The drops were covered with light paraffin oil to prevent evaporation. After incubation periods, 10

µl pre – warmed (37°C) PBS droplets were prepared in 35 mm culture dishes to remove the paraffin oil from blastocysts. Approximately 2 blastocysts were used for each interested marker. For RT – PCR experiments, the blastocysts were harvested with RNA lysis buffer for RNA isolation according to manufacturer's protocols and kept in -80 °C freezer. For each marker, one blastocyst is fixed with 4% PFA and then stored at +4 °C.

6.2. 3D Endometrial Co – Culture on Artificial Polymeric Sponge

3D environment with co – culture of endometrial epithelial and stromal cells has been constructed by using Matrigel. 3D constructions with endometrial cells were created on the top of the collagen coated artificial polymeric sponges and cultured for 48 and 96 hours. After incubation periods, endometrial cells covered with sponge pieces were trypsinized to detach cells and harvested with RNA lysis buffer for RNA isolation according to manufacturer's protocols and kept in -80 °C freezer. The samples of collagen coated artificial polymeric sponges were fixed with 4% PFA and stored at +4 °C for further immunofluorescence staining.

6.3. 3D Endometrial Co – Culture on Natural Collagen Sponge

3D environment with co – culture of endometrial epithelial and stromal cells has been constructed by using Matrigel. 3D constructions with endometrial cells were created on the top of the natural collagen sponges and cultured for 48 and 96 hours. After incubation periods, endometrial cells covered with sponge pieces were trypsinized to detach cells and harvested with RNA lysis buffer for RNA isolation according to manufacturer's protocols and kept in -80 °C freezer. The samples of natural collagen sponges were fixed with 4%PFA and stored at +4 °C for further immunofluorescence staining.

6.4. 3D Endometrial Co – Culture on Artificial Polymeric Sponge with Blastocyst

3D environment with co – culture of endometrial epithelial and stromal cells has been constructed by using Matrigel. 3D constructions with endometrial cells were created on the top of the collagen coated artificial polymeric sponges and cultured for 48 and 96 hours. The blastocysts were placed on collagen and cell coated artificial polymeric sponge by using a mouth pipette and cultured for 48 and 96 hours. After incubation periods, endometrial cells covered with sponge pieces that hold blastocyst were trypsinized to detach cells and harvested with RNA lysis buffer for RNA isolation according to manufacturer's protocols and kept in -80 °C freezer. The samples of collagen coated artificial

polymeric sponges were fixed with 4%PFA and stored at +4 °C for further immunofluorescence staining.

6.5. 3D Endometrial Co – Culture on Natural Collagen Sponge with Blastocyst

3D environment with co – culture of endometrial epithelial and stromal cells has been constructed by using Matrigel. 3D constructions with endometrial cells were created on the top of the natural collagen sponges and cultured for 48 and 96 hours. The blastocysts were placed on cell coated natural collagen sponges by using a mouth pipette and cultured for 48 and 96 hours. After incubation periods, endometrial cells covered with sponge pieces that hold blastocyst were trypsinized to detach cells and harvested with RNA lysis buffer for RNA isolation according to manufacturer's protocols and kept in -80 °C freezer. The samples of natural collagen sponges were fixed with 4%PFA and stored at +4 °C for further immunofluorescence staining.

7. EVALUATION OF IMPLANTATION POTENTIAL

All interested markers were evaluated according to the methods below. SEM, Immunofluorescence Staining, qPCR methods were used for observation and evaluation of the related genes. The methodology of the techniques is outlined in Figure 16.

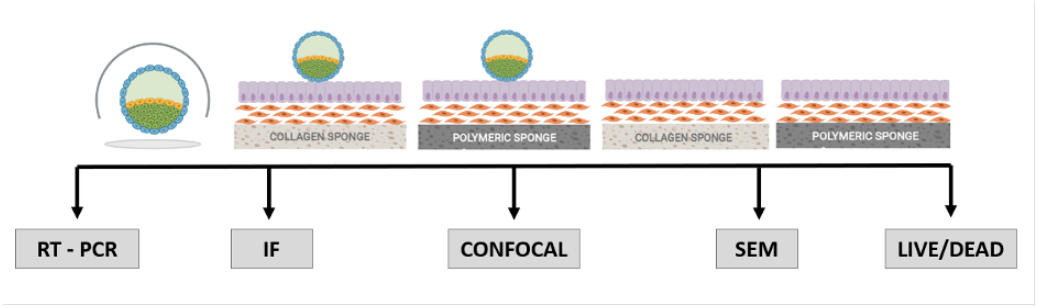

Figure 16. ***The outline of methodology.*** Three experimental groups with blastocyst along with two groups without blastocyst were evaluated for 48 and 96 hours by RT – PCR, Western Blotting, SEM, Immunofluorescence Staining, Confocal Imaging and Live/Dead Cell Assays. *(This figure is created via biorender.com)*

7.1. Immunofluorescence Staining

For routine IVF group, the blastocysts were fixed with 4% paraformaldehyde in room temperature for 20 minutes. They were washed with DPBS after fixation to rinse the paraformaldehyde. Blastocysts were incubated with 0.1% Triton-X for permeabilization at room temperature for 10 minutes. They were washed with DPBST (w/Tween-20). The blastocysts were kept in blocking

solution (Superblock) for blocking at room temperature for 20 minutes. Then, they were taken into the 1° Antibody droplets which were concentrated in 1:100 ratio. The blastocysts were kept in +4°C, overnight. After washing with DPBS, blastocysts were incubated in fluorochrome-conjugated 2° antibody (Alexa 488/Alexa 594/Dylight 549) droplets for 90 minutes in 37°C incubator. The blastocysts were washed with DPBS and placed in a 10 µl drop of 1:2000 diluted Hoechst in PBS: Glycerol (1:1) on a slide. The high mounting method was used to eliminate the compression of the blastocysts.

For groups with sponges, the scaffolds with blastocysts were fixed with 4% paraformaldehyde in room temperature for 20 minutes. They were washed with DPBS after fixation to rinse the paraformaldehyde. Scaffolds were incubated with 0.1% Triton-X for permeabilization at room temperature for 15 minutes. They were washed with DPBST (w/Tween-20). The scaffolds were kept in blocking solution (Superblock) for blocking at room temperature for 20 minutes. Then, they were taken into the 1° Antibody which were concentrated in 1:100 ratio. The scaffolds were kept in +4°C, overnight. After washing with DPBS, the scaffolds were incubated in fluorochrome-conjugated 2° antibody (Alexa 488, Alexa 594, Dylight 549) droplets for 90 minutes in 37°C incubator. The scaffolds were washed with DPBS and stained with 1:2000 diluted Hoechst in PBS: Glycerol (1:1). The scaffolds were turned upside down to face the glass bottom petri dishes for imaging (Figure 17). For different purposes, cryosections were also obtained from natural and artificial sponges and routine HE and PAS stainings were performed. Images are obtained by a Leica dmi8/SP8 (Leica, Germany) confocal laser scanning microscope.

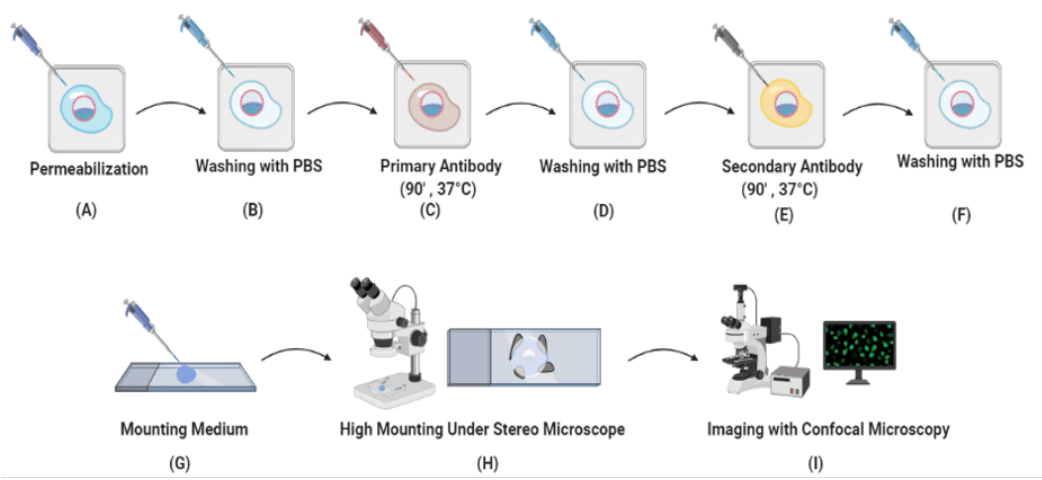

Figure 17. ***The Methodology for Immunofluorescence Staining.*** *(This figure is created via biorender.com)*

7.2. SEM Analysis

After appropriate incubation periods, the samples were washed in 0.2M Sorensen's Buffer. 2.5% of glutaraldehyde prepared in 0.1M Sorenson's Buffer was used to fix the cells at room temperature for 2 hours. Cells were again washed with Sorenson's Buffer for 3 times and dried at RT overnight (airdry). The samples were vacuumed and covered with 5 nm Au. The SEM images were captured by ZEISS EVO LS 15 with electron detector at KUYTAM, Koç University. The electrons were accelerated with 4keV and the working distance was about approximately 8 to 12 mm.

7.3. Live/Dead Cell Analysis

Live/Dead Cell Analysis was used to measure viability situation of the cells after 48 and 96 hours of incubation. LIVE/DEAD™ Viability/Cytotoxicity Kit was (Thermo Fisher Scientific, USA) used to perform the experiments and the manufacturers protocols were followed. 20 µl of 2mM EthD-1 were mixed 10 ml of DPBS and homogenised by pipetting. Then, 5 µl of 4mM calcein-AM was added and vortexed briefly. The cells and sponge fragments were incubated with the prepared solution for 30 to 45 minutes in the dark. After the incubation period, 10 µl of fresh LIVE/DEAD® cell reagent solution was added to the glass bottom petri dishes. Blastocysts were placed into the drops by mouth pipetting and the sponge pieces were faced carefully with the drop by using fine forceps. Leica DMi8/ SP8 TCS-STED microscope was used for imaging. Red cells correspond to dead ones whereas the live cells appear green.

7.4. qRT-PCR

The blastocysts were incubated for 48 and 96 hours in a culture dish in routine IVF group. Then, they were transferred to RNA Lysis Buffer. For sponge scaffolds, the cells were detached with trypsin and the collected pellet was lysed with RNA Lysis Buffer. RNA was isolated with Quick-RNA® Kit (Macharey-Nagel) according to manufacturer's instructions. The RNA amount was quantified with spectrophotometric read at 260 nm by Nanodrop (Thermo Scientific). M-MLV Reverse Transcriptase enzyme was used to convert RNA into cDNA. mRNA expression levels of interested genes were detected by using Light Cycler® 480 SYBR Green I Master. List of primers can be found in Table 4.

GENES	FORWARD PRIMERS (5' – 3')	REVERSE PRIMERS (3' – 5')
CDH1	ATCCTCGCCCTGCTGATT	ACCACCGTTCTCCTCCGTA
MMP9	TCCCCAAAGACCTGAAAACC	CTGCTTCTCTCCCATCATCTG
LIF	AAACGGCCTGCATCTAAGG	AGCAGCAGTAAGGGCACAAT
ESR1	TCCAGCAGTAACGAGAAAGGA	AGCCAGAGGCATAGTCATTGC
ITGAV	AGCCAGAGGCATAGTCATTGC	CATCTCCATTGGTATCAGTGGC
HBEGF	CTTAGTGGAACCTCGCTGTC	AGAAAGAGCTTCAGCATCACC
ERBB1	ATGAAAACACCTATGCCTTAGCC	TAAGTTCCGCATGGGCAGTTC
ERBB2	ACCGACATGAAGTTGCGACTC	AGGTAAGCTCCAAATTGCCCT
WNT4	AGACGTGCGAGAAACTCAAAG	GGAACTGGTATTGGCACTCCT
HOXA10	GGCAGTTCCAAAGGCGAAAAT	GTCTGGTGCTTCGTGTAAGGG
HOXA11	ATTGAGCCCGCCACTAAATGG	TCGAAAAACTGGTCGAAAGCC
IHH	CTCTTGCCTACAAGCAGTTCA	CCGTGTTCTCCTCGTCCTT
FN1	GATGTCCGAACAGCTATTTACCA	CCTTGCGACTTCAGCCACT
LAMA	ACTATGCCGTCAGCGATACAG	GGCACCAGCTTTGAATAATACGA
NID1	CCCAGCTTCGGCTCAGTAG	AACGGGGATAAGTCTTCTCGAT
PGR	GCCAGCCAGAGCCCACAATA	TGTGCTGCCCTTCCATTGCC
SELL	TGCAGAGAGACCCAGCAAG	CAGACCCACAGCTTCAGGAT
TROP	AACTGCCTAACAAGGGAAGAGT	CCACCAAAGCTAATACCAGCA
COL I	GCTCCTCTTAGGGGCCAC	ATTGGGGACCCTAGGCCAT
COL IV	CTGGCACAAAAGGGACGAG	ACGTGGCCGAGAATTTCACC
COL III	ACGTAGATGAATTGGGATGCAG	GGGTTGGGGCAGTCTAGTG
COL V	TTGGAAACCTTCTCCATGTCAGA	TCCCCAGTGGGTGTTATAGGA
TIMP3	ACACGGAAGCCTCTGAAAGTC	ACTTTGTGGAGAGGTGGGAC
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Table 4. ***List of primers and their sequences used in qPCR.***

7.5. Statistical Analysis

The expression levels of the interested genes were compared for 48 and 96 hours of incubation. Experiments were repeated three times independently of each other. To measure the statistical significance levels of gene expressions in terms of time dependency and differences between groups, Wilcoxon's Signed Rank Test was performed. To normalize the gene expression levels, GAPDH was selected as housekeeping gene. Subtracting Ct value of the housekeeping gene from the target gene [Ct (a target gene) − Ct (GAPDH)] was done for normalization. The formula [Ct48(target gene) −Ct48(GAPDH)]- [Ct96(target gene) −Ct96(GAPDH)], which is ΔΔCT, was the used to detect the expression difference from 48th hour to 96th hour. The results were presented as -ΔΔCt to observe the gene expression change relative to the 48th-hour expression, normalized to GAPDH. Significance level was set at 5% ($p < 0.05$).

CHAPTER 3

Results

1. CHARACTERIZATION OF ENDOMETRIAL CELLS

Isolated endometrial epithelial and stromal cells were characterized according to their specificity as positivity for cytokeratin 18 (CK18) and vimentin. Cytokeratin 18 is a cytoskeletal intermediate filament expressed almost in all tissues including endometrium. Vimentin is regarded as a mesenchymal marker. Validation of their expressions were evaluated by RT – PCR analysis. As expected, isolated epithelial cells gave positive result for CK18 whereas they were negative for vimentin. The other way around, stromal cells were positive for vimentin and negative for CK18. Besides the PCR results, IF staining was also performed to visualize the island formation of epithelial cells and vimentin positivity of stromal cells (Figure 18).

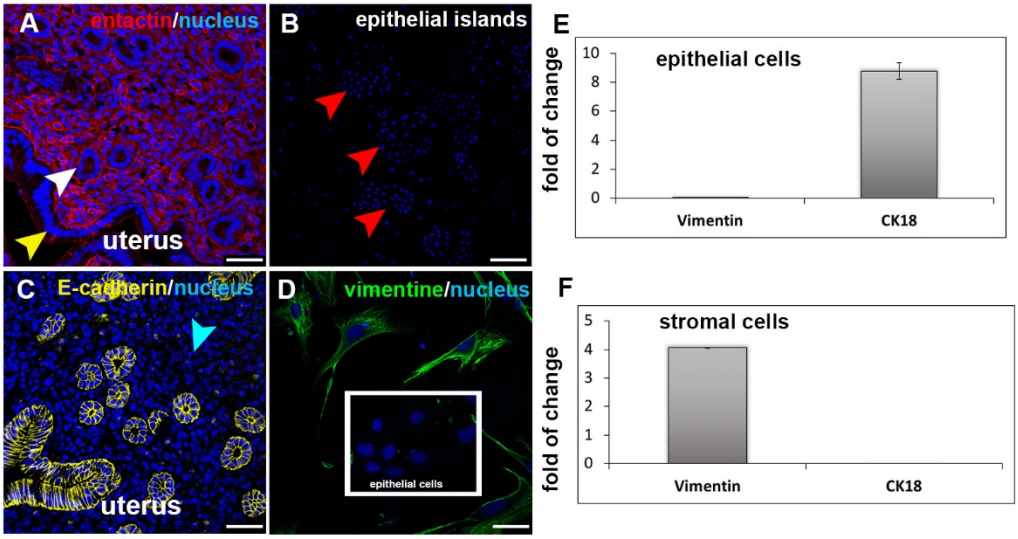

Figure 18. ***Characterization of the isolated cells.*** The luminal (A, yellow arrowhead) and the glandular (A, white arrowhead) cells and the stromal cells (C, blue arrowhead) were isolated and seeded separately (B). During the isolation epithelial cells made up of islands surrounded by vimentin positive stromal cells (D). qPCR results also show this deep discrimination (E, F). The uterus was stained by entactin and E-cadherin to show the histological structure of the tissue.

2. EVALUATION OF EXPERIMENTAL GROUPS

Experimental groups were constructed as follows: Routine IVF (R), blastocyst cultured on 3D endometrial cells coated polymeric sponge (P), blastocyst cultured on 3D endometrial cells coated collagen sponge (C), polymeric sponge coated with 3D endometrial cells (PE), and collagen sponge coated with 3D endometrial cells (CE). All the experimental groups were evaluated at 48^{th} and 96^{th} hours. SEM, IF Staining, confocal microscopic imaging, and qPCR techniques were used to evaluate the implantation potential within the groups.

2.1. Immunofluorescent Staining Analysis

Routine, polymeric sponge (P) and collagen sponge (C) groups are stained with a variety of antibodies to reveal the differences regarding the implantation parameters. In comparison with the qPCR results, IF staining has sometimes showed variations, which may be attributed to the fact that compared to gene expression analysis number samples for this stainings were fewer and there may be also a difference that is caused by the difference in expression profiler versus formation of the related molecular product, which needs to be a further confirmation via Western blotting, as well. The results of IF stainings are outlined in Figures 19-21.

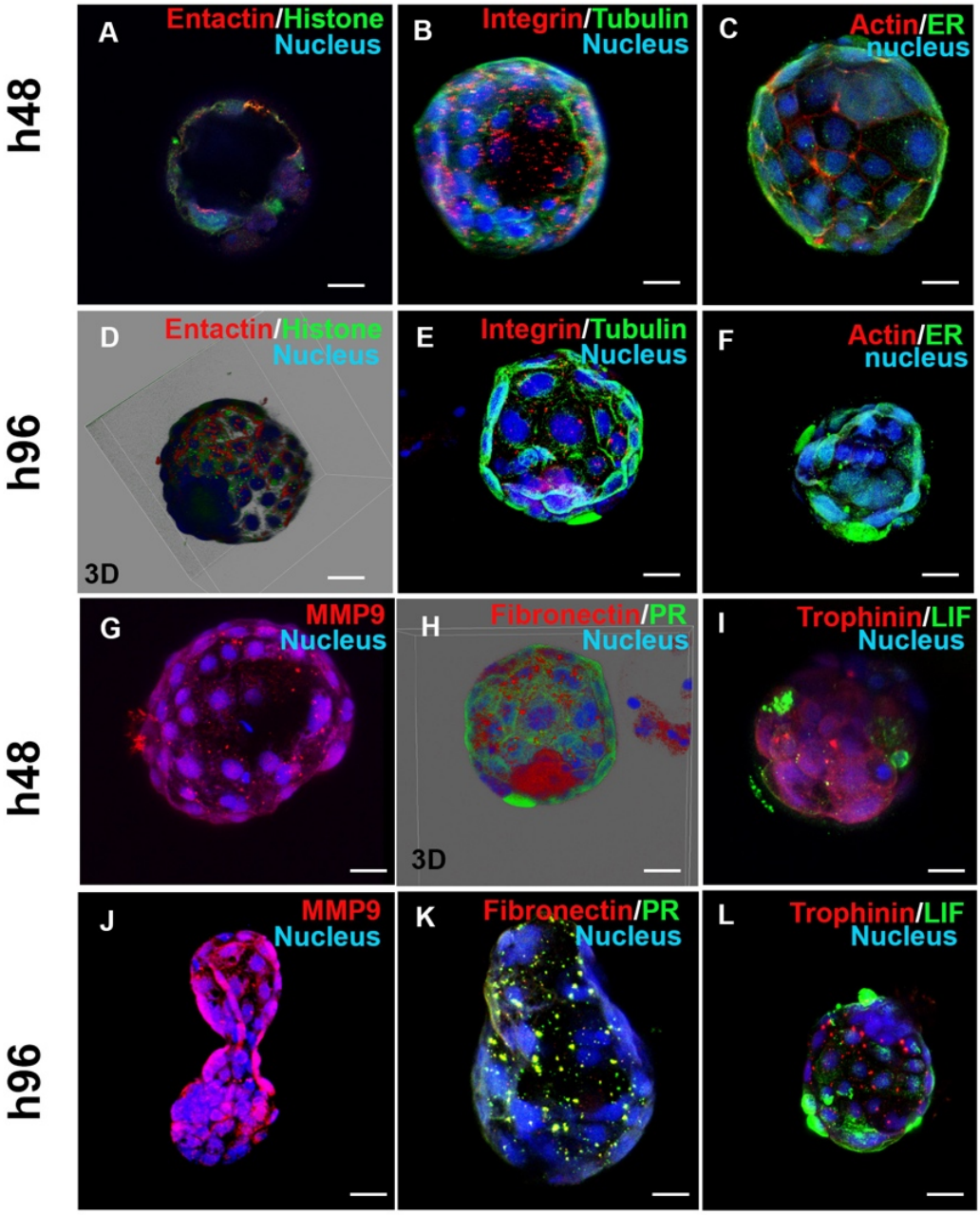

Figure 19*. IF stainings of routine IVF group*. From hour 48h to 96h an increase in ER, entactin, MMP9 and trophinin was observed, while fibronectin and integrin have decreased. The images are formed by maximum projection or 3D construction of 20 consecutive z-stacks. Scale bars=25 µm.

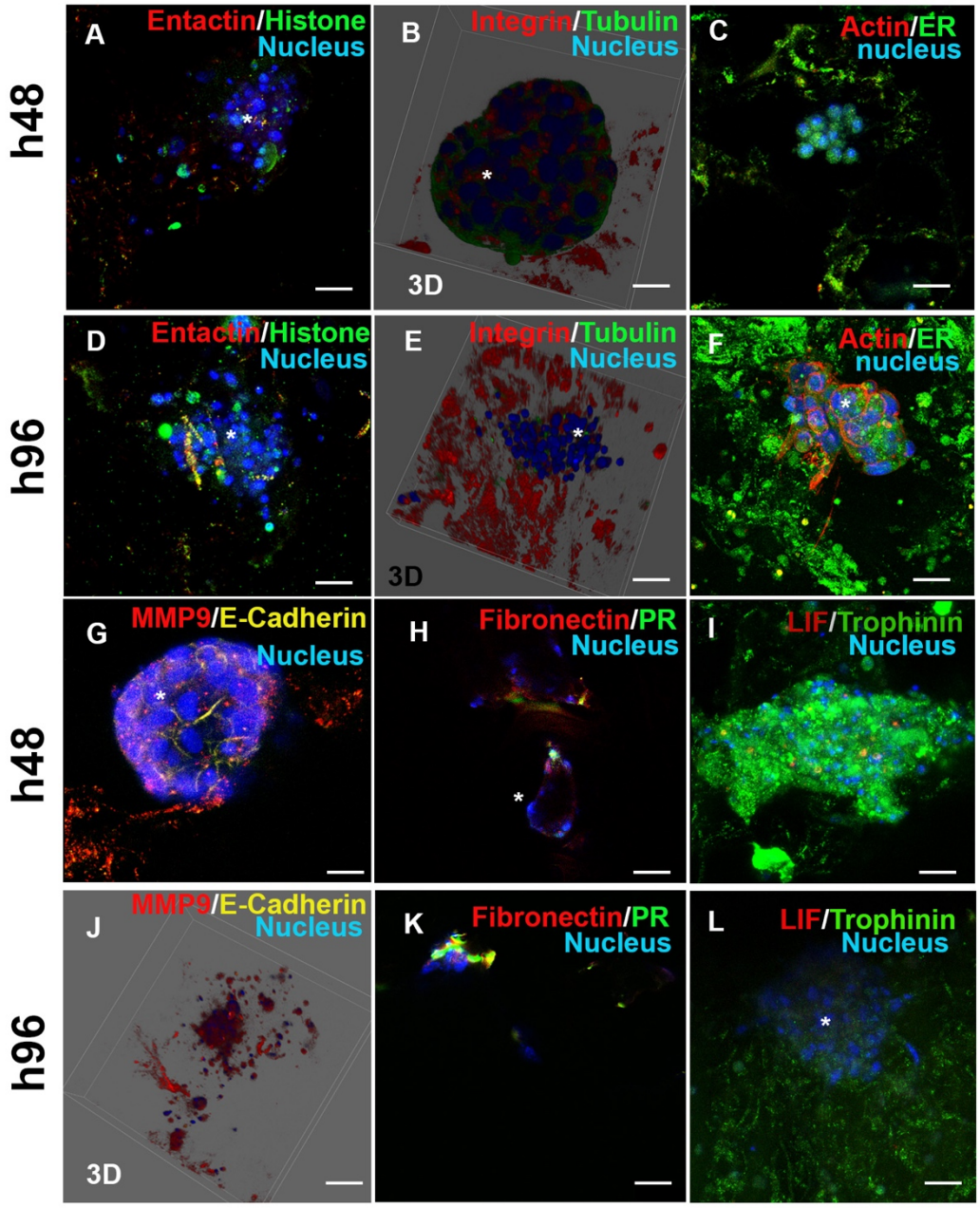

Figure 20. ***IF stainings of polymeric sponge (P) group***. From hour 48h to 96h an increase in ER and a decrease in LIF, trophinin, fibronectin was observed. Integrin decreased at the embryonic site but increased in the trophoblastic area. E-Cadherin has also decreased. Since this material tends to give auto fluorescence some staining results are not clear enough. The images are formed by maximum projection or 3D construction of 20 consecutive z-stacks. Asterisk: Blastocyst. Scale bars=25 µm.

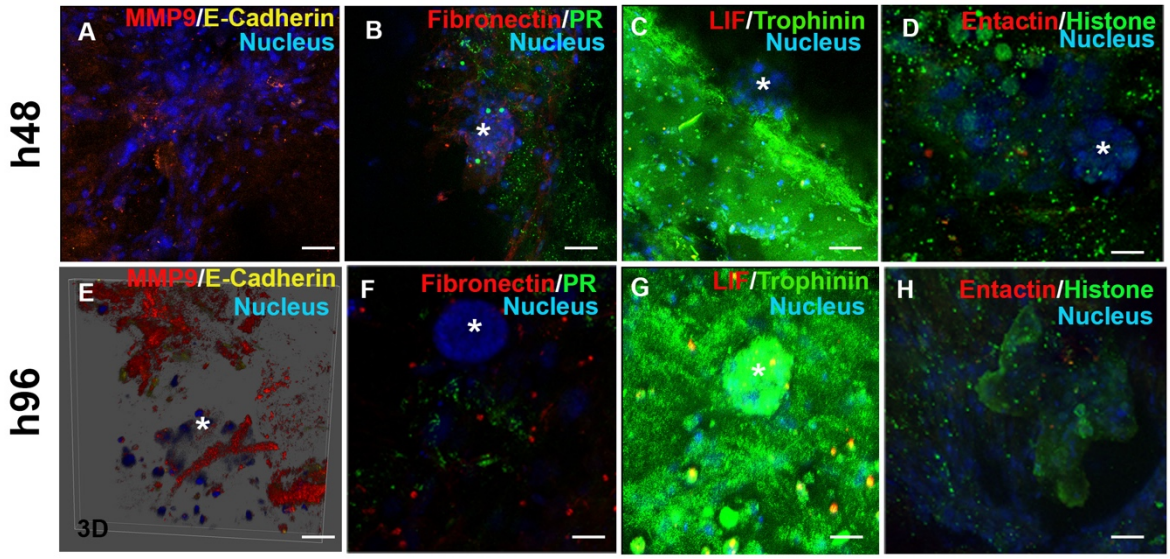

Figure 21. ***IF stainings of collagen sponge (C) group***. From hour 48h to 96h an increase in, trophinin, and MMP9 was observed. Fibronectin has decreased. Since this material tends to give auto fluorescence some staining results are not clear enough. The images are formed by maximum projection or 3D construction of 20 consecutive z-stacks. Asterisk: Blastocyst. Scale bars=25 µm.

2.2. SEM Analysis

The analysis of Scanning Electron Microscope (SEM) was also evaluated as another methodology to observe information about surface composition of the sponges and co – culture systems. Besides the sponge groups, routine IVF group was also observed to understand the structures of the blastocyst (Figure 22). In the same figure light microscopic images can also be seen.

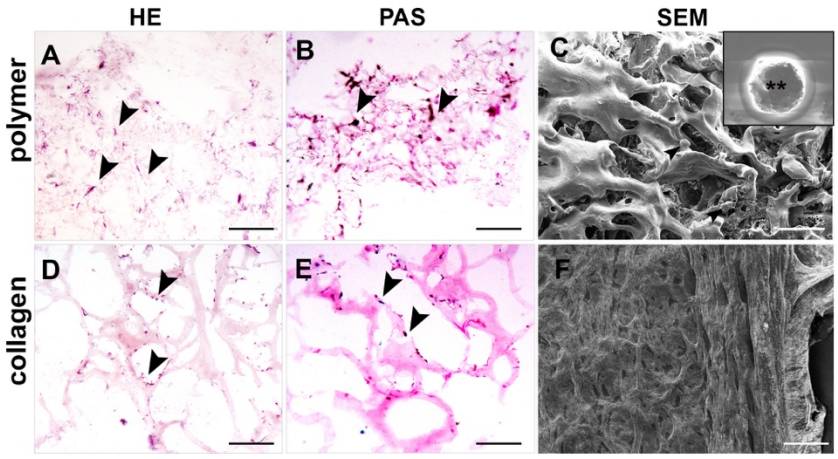

Figure 22*. SEM and light microscopic analysis.* Cryosections of polymer and collagen sponges revealed the cellular structures aligned along the pores (black arrowheads). Pas staining showed the typical glycogen deposition in these cells. SEM analysis shows the cells on the polymeric sponge, the ones on the collagen sponge did not preserve well. The inlet (C, asterisk) shows the blastocyst in the routine IVF group.

2.3. Comparative Analysis of Experimental Groups

The comparative data was obtained depending on the expression levels of the experimental groups as Routine IVF (R), POL (P), COL (C), POL + ECC (PE), COL + ECC (CE). To be able to see all the biological and technical replicates, heatmap representation was preferred as a visualization method for the data. Also, boxplot graphics were constructed based on the data of groups with blastocyst and without the blastocysts.

The statistical analysis of the data which comes from the cycles of the gene expressions were done on R Lab Statistics Platform. The purpose was to find the expression level differences from 48^{th} to 96^{th} hour of implantation related interested markers; the data were used as mean values of -ΔΔCT values in heatmap.

23 genes were selected to evaluate the experimental groups. LIF, ErbB1, ErbB2, HBEGF, ER and PR were categorized into functional, MMP9 and TIMP3 were selected as invasion; Hoxa10, Hoxa11, Wnt4, and IHH were grouped into developmental and E – Cadherin, Fibronectin, Laminin, Entactin, L -Selectin, Trophinin, Integrin and Collagen I, III, IV, V) were selected as adhesion related genes. For each gene, 3 biological and 2 technical replicates were performed.

Among 23 implantation markers, Trophinin showed a severe increment in COL + ECC group. Collagen V and L – selectin showed a decrease in POL and CE groups. Besides, L – Selectin also decreased

in PE group. E – Cadherin, Hoxa10, HBEGF and Fibronectin showed an increase especially in POL and PE groups. Integrin, ErbB1, ErbB2, TIMP3, ER, PR, IHH, Entactin, Wnt4 and Laminin also increased in POL group whereas they showed a decrement in CE group. MMP9 showed a slight increase in PE group. In COL group, Collagen I, Hoxa11 and Collagen III were decreased. Collagen V showed an increase especially in first replicate of POL group. For routine group, constant decrements were observed for the genes as Collagen I, E – Cadherin, ErbB2, PR, Entactin, Wnt4, Fibronectin and Laminin. Collagen IV decrease was evident in CE group. ER, Collagen I, and L – Selectin expression was decreased when CE group was evaluated in comparison to other groups.

In POL group, Hoxa11, Wnt4, Hoxa10, and IHH, as developmental genes, showed increment in all biological replicates. When COL group was evaluated, Hoxa11 was decreased whereas Wnt4 and IHH decrement was occurred in second biological replicate. On the other hand, Hoxa10 was increased in COL group opposite to other developmental genes. From adhesion genes, L – selectin, Integrin, Fibronectin, Laminin, E – Cadherin, Entactin, and Collagen IV increased especially in second replicates of POL and COL groups. Besides, Collagen I and Collagen III showed a steady decrement in all replicates of POL and COL groups. In first replicate of POL group, the decrement of Collagen IV expression was evident. Collagen V marked an overexpression in first replicate of POL group. Among all adhesion genes, especially Laminin, E – Cadherin and Entactin was decreased in routine group. ErbB1 and PR as functional genes, decreased in routine group. ErbB1, ErbB2, HBEGF and PR increased in POL groups when compared to COL and routine (Figure 23).

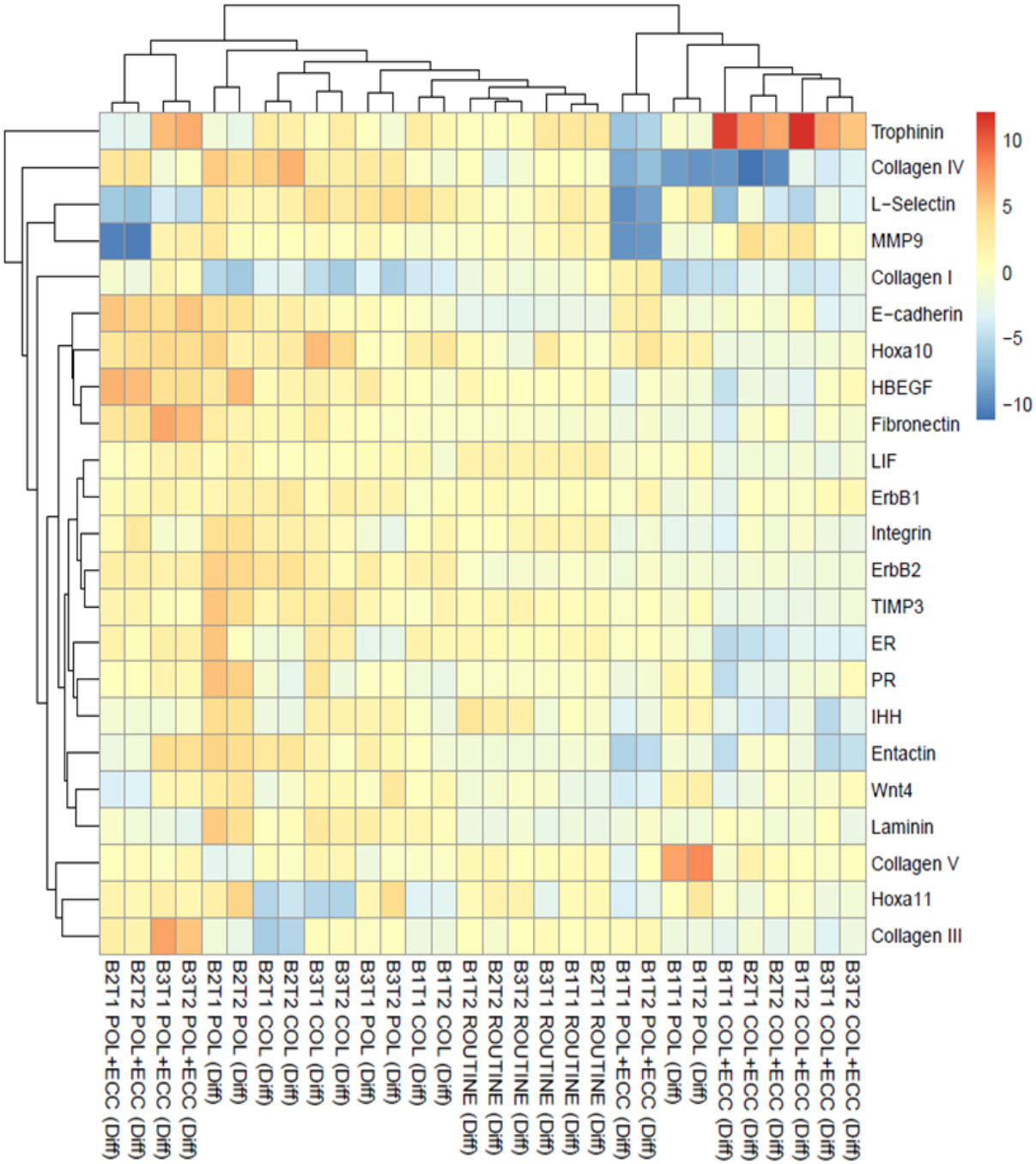

Figure 23. ***Expression level differences of 23 genes within all groups from 48th to 96th hour by heatmap representation.*** According to RT – PCR replicates, blue color represents downregulation whereas red color shows upregulation depending on expression level changes from 48th to 96th hours of genes. Dendrogram representation shows the hierarchical relationship between the experimental results. Color codes in the heatmap were represented by the scale bar which shows the mean values of -ΔΔCT values. Routine = Routine IVF, POL = Blastocyst culture on ECC coated polymeric sponge, COL = Blastocyst culture on ECC coated Collagen sponge, POL + ECC = ECC coated polymeric sponge, COL + ECC = ECC coated collagen sponge.

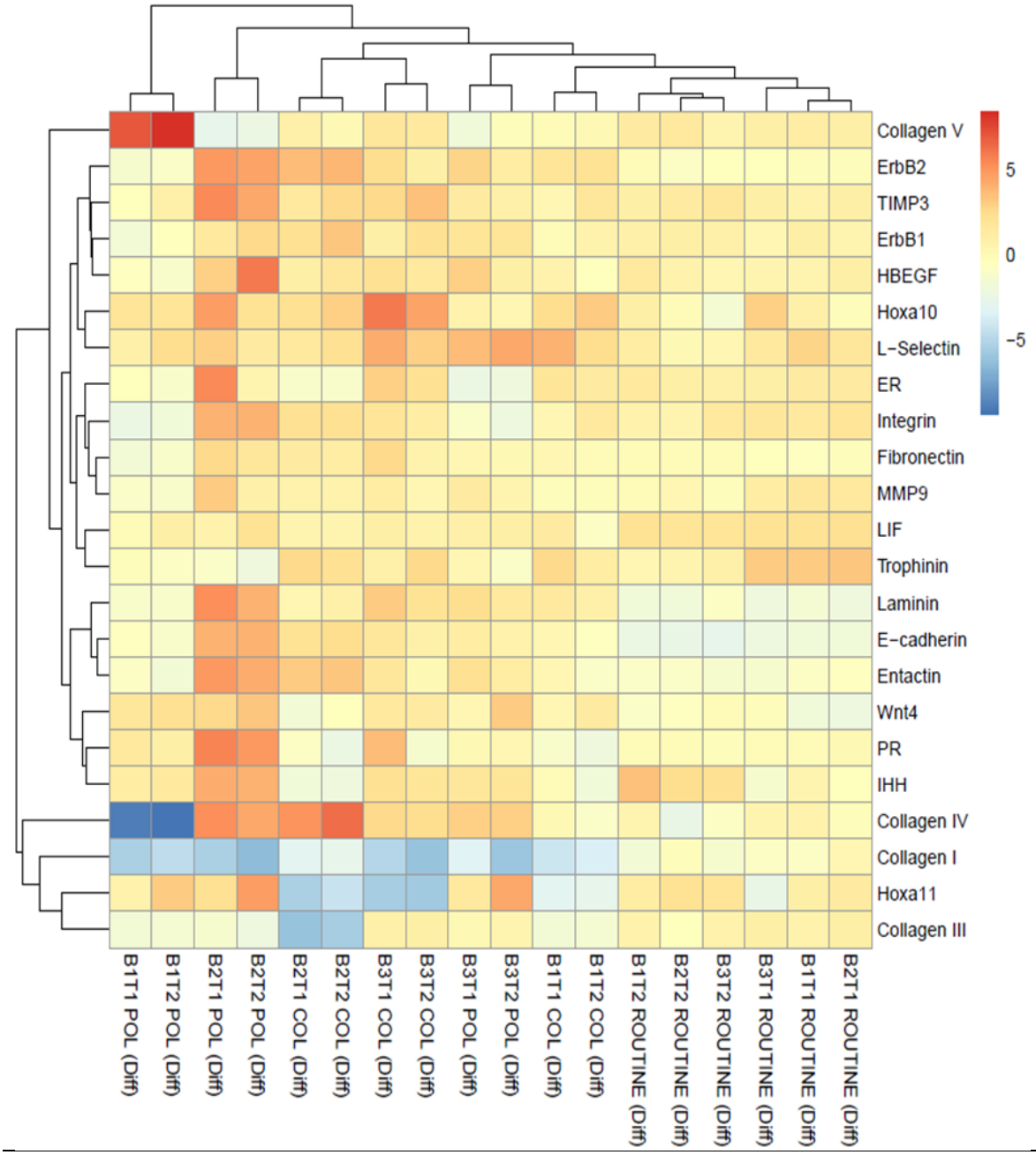

Figure 24. ***Expression level differences of 23 genes within groups with blastocyst from 48th to 96th hour by heatmap representation.*** According to RT – PCR replicates, blue color represents downregulation whereas red color shows upregulation depending on expression level changes from 48th to 96th hours of genes. Dendrogram representation shows the hierarchical relationship between the experimental results. Color codes in the heatmap were represented by the scale bar which shows the mean values of -ΔΔCT values. Routine = Routine IVF, POL = Blastocyst culture on ECC coated polymeric sponge, COL = Blastocyst culture on ECC coated Collagen sponge.

Trophinin expression showed a significant downregulation in POL group when compared to both routine IVF and COL. In the CE, Trophinin was upregulated among all other experimental groups and it has been found significant for both routine and COL groups. The expression levels between routine IVF and COL groups were almost same and there was not any significance. Trophinin decreased approximately six – fold in PE which was non – significant.

Entactin, which is a member of adhesion genes, increased approximately two-fold in COL group. The increase was significant when compared to routine IVF. Similarly, decrement in routine IVF was found to be significant to COL group. For PE and CE groups, the expression of Entactin is decreased from 48^{th} to 96^{th} hour and decrement in CE group was significant according to COL. Although there was an increment in POL group, it has found statistically non – significant.

Fibronectin showed a decrease in routine IVF group significantly for COL. The increase in COL group was found to be significant when compared to routine IVF group. In POL group, the expression of Fibronectin was found upregulated whereas it was non – significant. There was a decrement in CE group and this downregulation was found to be significant according to COL group.

E – Cadherin expression was downregulated in routine IVF approximately four – fold from 48^{th} to 96^{th} hour and it has been found to be significant when compared to COL group. Both POL and COL groups upregulated E – Cadherin expression and they were found to be significant when compared to routine IVF. It has been found similar increments in both POL and COL groups and they were also significant when compared routine IVF. In PE group, the expression of E – Cadherin upregulated approximately twenty – seven-fold and it was significant for both COL and routine IVF. Downregulation in CE group was significant when compared to COL group.

Integrin decreased approximately three – fold in CE group and it was significant when compared to COL and routine IVF. Also, there was a downregulation in PE, but it was not statistically significant. In COL group, there was an upregulation, but it was not significant for both routine IVF and POL groups.

L – Selectin showed sixty – four – fold downregulation in PE group from 48^{th} to 96^{th} hour and it has been to be significant when compared both to routine IVF and COL groups. Similarly, the expression in CE group was also decreased significantly for routine IVF and COL. In COL group, six– fold significant downregulation was shown when compared to routine IVF group.

Laminin expression was significantly decreased four – fold in routine IVF group according to COL group. For POL and COL groups, there were also upregulated expressions, and they were both significant

when compared to routine IVF group. In the experimental groups of PE and CE, Laminin expression also decreased which were significant compared to COL. Also, the downregulation in CE group was statistically significant according to routine IVF.

Among Collagens, Collagen I showed a slight upregulation in PE group and it has been found to be statistically significant when compared to COL. In the other groups as routine IVF, POL, COL, and CE, expression level of collagen I was decreased from 48th hour to 96th hour. The decrement in expression of Collagen I in these groups were significant according to routine IVF. The expression was downregulated approximately two – fold in routine IVF with a statistical significance according to COL.

Collagen III expression increased two – fold in PE group and it was significant when compared to both routine IVF and COL. In routine IVF, there was almost a stable expression with a slight upregulation. From 48th to 96th hour, Collagen III expression in POL was decreased approximately four – fold with a statistical significance according to routine IVF group. Similarly, almost six - fold downregulation was prominent in CE group which was significant in comparison to routine IVF.

Collagen IV expression showed the only significance in the CE group when compared to both routine IVF and COL groups. For POL and COL groups, the expressions of Collagen IV were increased approximately eight – folds and found to be statistically non – significant. The gene expression level in routine IVF group was evaluated stable from 48th to 96th hour.

Among all experimental groups, Collagen V was not shown any significance. Expression levels in COL, PE, and CE groups were almost stable and approximately same as one another. There was a slight increment in all experimental groups except POL group. It has been shown almost two – fold non – significant decrement.

ER expression slightly increased except for POL and CE groups. Only significance was found in CE group according to both routine IVF and COL group with a downregulation of approximately eight – fold. From 48th to 96th hour, routine IVF showed a non – significant three – fold change whereas PE and COL groups showed four – fold upregulation.

Another receptor, PR, increased in POL group which was significant according to both routine IVF and COL. PR expression in routine IVF were remained stable from 48th to 96th hour. For COL and CE groups, the expression of PR almost downregulated two – fold, which was found to be non – significant.

Although ErbB1 showed an upregulation in POL and COL groups, it was not found statistically significant. In routine IVF, the expression of ErbB1 increased almost two – fold whereas the expressions in POL and COL were increased four – fold from 48th to 96th hour. None of the expression levels within all experimental groups was found to be statistically significant.

ErbB2 increased in POL and COL groups. Approximately five – fold increment in COL group was statistically significant when compared to routine IVF. In routine IVF, there was a slight increment, and it was found to be significant when compared to COL group. Although the expression change in POL group was almost four – fold from 48th hour to 96th hour, it was not significant. CE group showed a decrease which was significant for both routine IVF and COL.

HBEGF expression showed an increase in both POL and COL groups when compared to routine IVF whereas they were statistically non – significant. Only significance was observed in CE group according to routine IVF and COL with a four – fold downregulation. There was a severe upregulation with sixteen – fold increase in PE group.

LIF increased approximately two – fold in both COL and POL groups and found to be significant when compared to routine IVF groups. Besides, almost two – fold downregulation was observed in CE group which was also statistically significant according to for both routine IVF and COL groups. There was an upregulation in POL group but there was not any significance according to any other groups.

Among invasion genes, MMP9 showed almost the same and stable expression level differences within routine, POL and COL groups. The upregulation was found to be two – fold from 48th to 96th hour. There was a slight downregulation in PE group which was not statistically significant.

The decrease in TIMP3 expression was significant in CE group in comparison to both routine IVF and COL groups. The routine IVF and POL group showed almost the same non – significant expression level differences. In COL group, the expression was upregulated six – fold from 48th to 96th hour without any statistical significance when compared to other groups.

Hoxa10, as a development related marker, increased four – fold in POL group which was significant when compared to COL group. In COL, nearly eight – fold increment was also found to be significant when compared to routine IVF group. PE group showed an increase which was significant to routine IVF. There was a severe downregulation in CE group with a significance according to routine IVF and COL groups.

Similarly, Hoxa11 showed a significant six – fold increase in POL group according to COL from 48th to 96th hour. In COL group, the expression was downregulated sixteen – fold which was significant when compared to routine IVF. For both CE and PE groups, Hoxa11 expression increased significantly in comparison to COL group.

IHH expression was increased in POL group but there was not any significance when compared to routine IVF. There were downregulations in both PE and CE groups from 48th to 96th hours which were significant to routine IVF. The decrement in IHH group was also significant when compared to COL group.

Wnt4, as a member of developmental genes, showed a severe increment as eight – fold upregulation in POL group which was significant for both routine IVF and COL. Although there was a severe downregulation which was almost sixteen – fold in PE group, it was not significant according to any other groups. The expression from 48th to 96th hours, Wnt4 remained almost the same in CE group. Wnt4 decreased in routine IVF whereas it showed an increase in COL group.

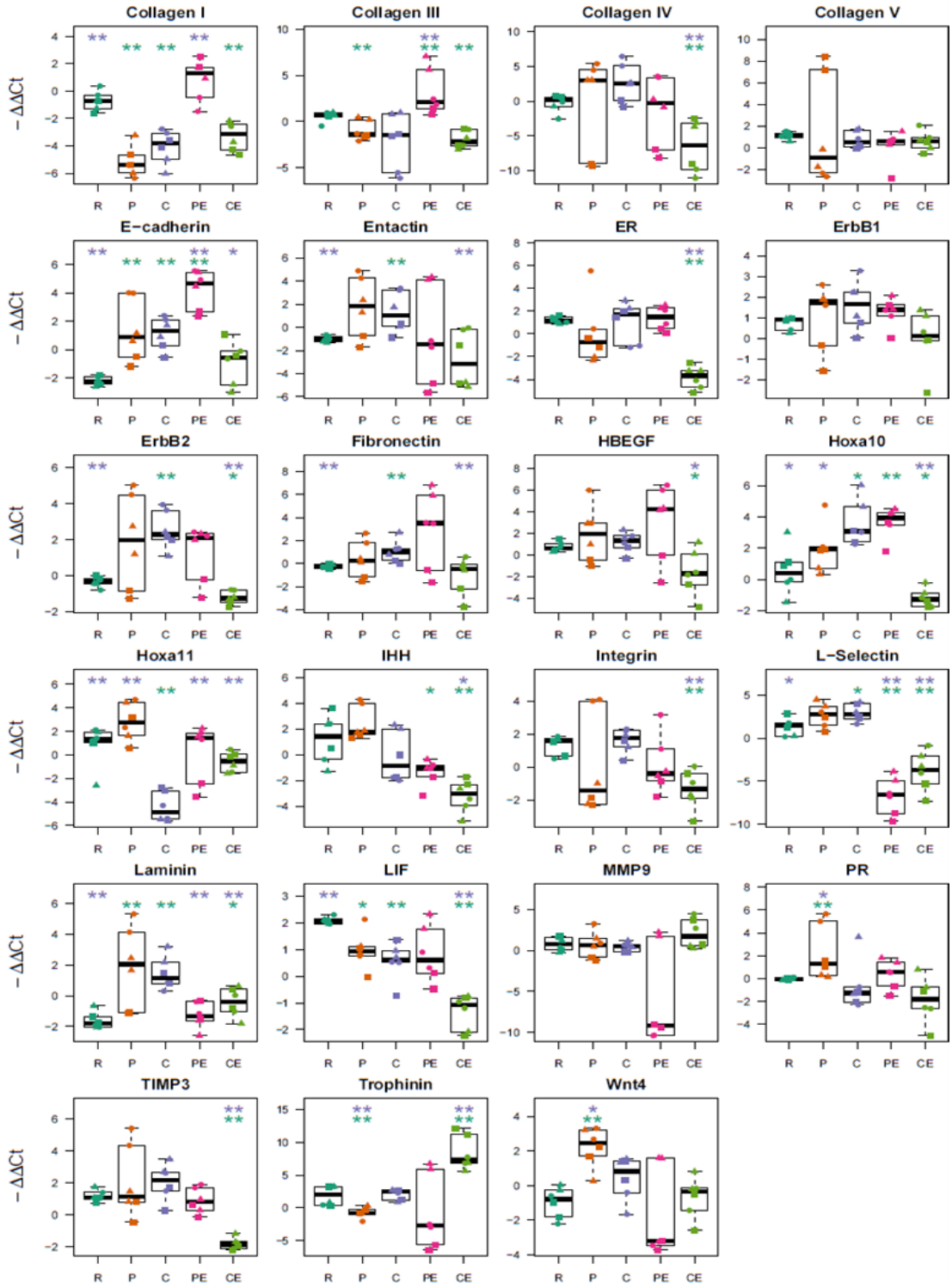

Figure 25. ***Expression level differences of 23 genes within all groups from 48th to 96th hour by boxplot representation.*** In each boxplot, the median levels of -ΔΔCT values were represented. Green stars are statistically significant compared to the R group and purple stars show statistical significance compared to COL group. Routine(R)= Routine IVF, POL(P) = Blastocyst culture on ECC coated polymeric sponge, COL(C) = Blastocyst culture on ECC coated Collagen sponge, POL + ECC (PE)= ECC coated polymeric sponge, COL + ECC (CE)= ECC coated collagen sponge. (**: $p < 0.01$, *: $p < 0.05$)

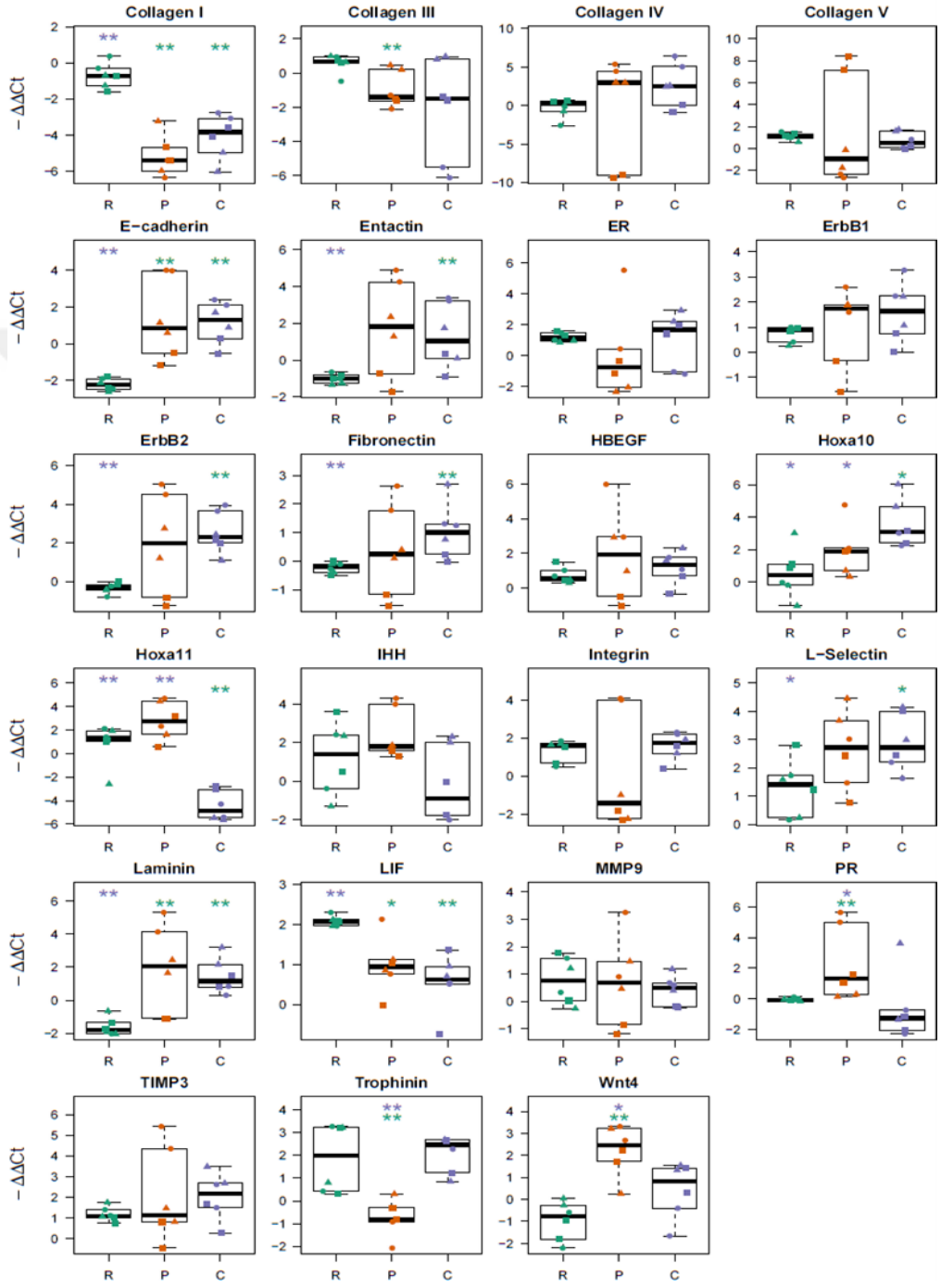

Figure 26. ***Expression level differences of 23 genes within all groups from 48th to 96th hour by boxplot representation.*** In each boxplot, the median levels of -ΔΔCT values were represented. Green stars are statistically significant compared to the R group and purple stars show statistical significance compared to COL group. Routine (R)= Routine IVF, POL (P)= Blastocyst culture on ECC coated polymeric sponge, COL (C)= Blastocyst culture on ECC coated Collagen sponge (**: $p < 0.01$, *: $p < 0.05$)

2.4. Cell Vitality Evaluation

Since the biomaterials are considered as non – toxic for cells, the cell viability assay was performed to observe the vitality of the blastocyst. Cell viability assay was performed with live / dead cell kit to observe vitality change from 48th to 96th hour. Live cells gave green fluorescence whereas red cells gave red fluorescence.

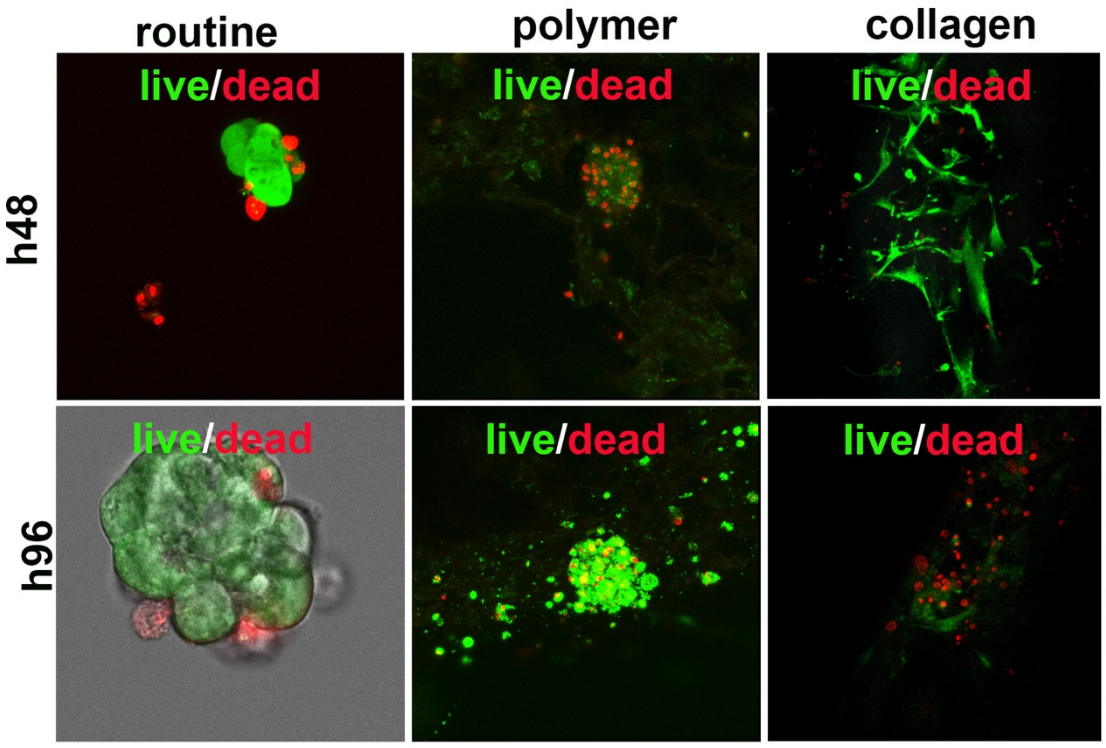

Figure 27. ***Cell Viability Assay.***

The experiment was repeated as two biological replicates and the percentage of live and dead cells were plotted. Graphs show that the most viability was seen in POL group so that it can be concluded that co – cultured polymeric sponges mostly supported the vitality of the blastocyst.

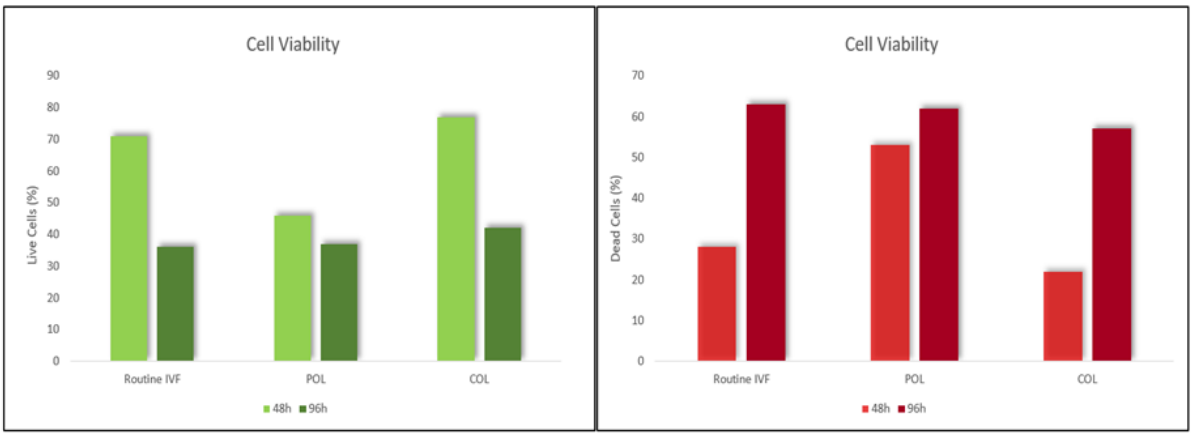

Figure28.Quantitative evaluation of the vitality of blastocysts from 48th to 96th hours. Cell viability assay was performed to evaluate the vitality of the blastocysts. Green bars show the live, red bars show the dead cell percentages.

CHAPTER 4

Discussion

As the implantation process is still fulfilled with question marks, it is necessary to establish new approaches to enhance the success rates of ART and infertility. Idiopathic infertility is one of the major restrictions in front of the ART because of the complex molecular interactions and unclarified conceptions related to the implantation.

During clinical applications of ART, the rate of successful conception is still low even if the best qualified embryos are chosen for the transfer. According to previous results from applied IVF cycles, it has been found that the implantation rate is 26% per blastocysts, whereas the implantation rate is only 18% for eight-cell embryos on day 3 even though the embryos were good quality. [94] The pregnancy rate may be induced when transferred embryo number is increased but also this situation may lead to multiple pregnancy and its complications such as low birth weight or prematurity. The results among the clinical trials and approaches show that it is necessary to achieve the usage of different culture models and media conditions to enhance the blastulation and augmented the implantation rates.

In the past decade, there were many experimental trials such as *in vivo* culturing models to understand the implantation process the way better, but due to the ethical limitations and restrictions about the usage of human embryos, it is fundamental to search the process deeply. The uterus receptivity and adhesion potential were the object of interest and there were several researches which examine the adhesion potential of the blastocyst, cultured on an endometrial segment which was obtained from a pregnant mice. [95]

In this project, we aimed to take a step forward to understand the development, invasion, adhesion, and functional progress of embryos with a 3D *in vitro* culture model. In accordance with this purpose, usage of biomaterials has been found appropriate to construct the endometrial environment. Biomaterials are widely used in the fields of cancer therapies, nanotechnological achievements, and tissue engineering applications due to their biocompatibility, non – toxicity and biodegradability characteristics. Poly (L – Lactic acid – co – D, L – Lactic Acid) (PLDLLA) and Poly (D, L – Lactic acid – co – Glycolic Acid) (PLGA) polymers were mixed to obtained polymeric artificial sponges whereas collagen was used to obtain the natural sponges. Collagen as an extracellular matrix protein has been preferred for coating the polymeric artificial sponges to enhance the adhesion potential of endometrial cells to the surface of the

sponges. Matrigel which is an ECM mimicking gel formed material also rich in laminin, fibronectin and entactin, also used with both natural and artificial sponge cultures to imitate the 3D environment rather than 2D.

Routine IVF group was also constructed to examine the expression differences in routine culturing conditions such as regular IVF mediums and culture methods on plastic plates used in clinical trials. Endometrial stromal cells were coated within Matrigel and endometrial epithelial cells were grown on the top of the gel formed Matrigel to assimilate multi-layered cell structure of endometrium. Besides, the same constructed sponge groups were also evaluated without blastocyst to examine whether the gene expression difference is coming from the blastocyst itself or not. To see the expression changes within groups, the experiments were evaluated at 48^{th} and 96^{th} hours.

Implantation is depending on endometrial interactions alongside the uterus receptivity and the blastocyst activity. According to literature, HOX genes are responsible for embryonic development as well as development of reproductive tracts. In a recent study, it has been shown that Hoxa10 and Hoxa11 gene – knockout mice showed a defective decidualization and the implantation results. [96] In our data, Hoxa10 expression increased in COL group significantly when compared to Routine IVF group. Also, POL group increased the expression significantly even though the increment in COL group was further. These results may lead to the inducement of the blastocyst development in a 3D models which comes from the co – cultured cells. Also, there was a severe significant increment in PE group in comparison to routine IVF. Decidualization process which functions by the HOX genes in the endometrial cells may give rise to this increase. Alongside the Hoxa10, Hoxa11 which is functioning the development, increased its expression in POL group significantly for both COL and Routine IVF. The data shows that the underlying supporting endometrial cells are supporting for development of the embryo, decidualization and receptivity of the uterus. Although there was no significance about IHH in between the routine and sponge groups, there was a slight increase in POL group among the others. As IHH is a P4 regulated gene [54], and there was a significant severe expression of Progesterone Receptor in POL group, the increment of IHH in POL group was meaningful. On the other hand, it is also suspicious that the data shows increment in CE and PE groups which were significant for both routine IVF and COL groups because it is expected that IHH supports the crosstalk between endometrial epithelial and stromal cells to provide receptivity during WOI.[97] This decrease may link to increase in the groups with blastocyst as a successful implantation depends on both uterus receptivity and the presence of an active blastocyst. Wnt4 is also another member of developmental genes regarding the embryo implantation, showed an increase in POL group significantly

for COL and routine IVF. Wnt4 signalling pathways are functioning endometrial regulation, proliferation and survival of stromal cells, and support for the developing embryo under the effect of progesterone. So that the progesterone receptor was also increased significantly in POL group in comparison to other groups, increase of Wnt4 shows that crosstalk between the endometrial cells and the development of the embryo was supported by the underlying system. The data indicates that, 3D co – cultured environment with sponges supports the embryo development and uterine receptivity when compared to routine IVF cultures.

As mentioned in Chapter 1, Progesterone is an important hormone functions the implantation, decidualization of stromal bed and the glandular development. [79] Progesterone receptor (PR) increased its expression in POL group, significantly for both routine IVF and COL groups. This also supported the upregulated expression of developmental genes which are dependent on progesterone hormone. Oestrogen is also necessary in proliferative phase of the cycle by linking to the oestrogen receptors (ER). In mice, on the day 4 of the pregnancy, it is expected to see a small surge of oestrogen to initiate the implantation. This spike gives rise to positioning and adhesion of the blastocyst followed by the morphological and structural changes which refers decidualization. Different from humans, in mice, oestrogen and its receptor ER decreases its expression whereas progesterone and its receptor increases throughout the WOI. [10] Among all experimental groups, POL group showed a slight decrement and COL approximately remained similar to routine IVF. Even though this increment was not significant, it might be caused by this secretion timing difference of oestrogen between humans and mice. In PE group, the expression was increased, and this indicates that co – culture system was establishing its receptivity for the coming embryo whereas the significant decrease in CE group may evoke that collagen sponge may not support the oestrogen related receptivity as polymer group.

As a progesterone hormone receptor ligand, HBEGF increased its expression more in POL and COL groups when compared to routine IVF. The increment in POL group was also correlated with the PR increase, although it was not significant. The only significant decrease was found in CE group which has no blastocyst. HBEGF is responsible from the signaling between the uterus and implanting embryo, so that the significant decrease regarding to COL and routine IVF in the collagen group without blastocyst may linked to the absence of the embryo.

ErbB1 and ErbB2 are the receptors for HBEGF ligands and expected to give correlated expressions with HBEGF. ErbB1 showed an upregulation for both POL and COL groups even though they were non – significant. When ErbB2 was examined, expression in COL increased significantly according to routine IVF

which supports that the endometrial lining 3D system of collagen sponge induces the expression of ErbB2. Similarly, ErbB2 decreased significantly according to routine IVF and COL groups, like HBEGF in CE group. This indicates that the expression is linked to the presence of blastocyst in addition to endometrial cells requirement.

LIF regulates important roles as decidualization, and immune response during the implantation.[98] Among all experimental groups, both POL and COL groups significantly increased their expression. However, the increment in routine IVF group was approximately four – fold whereas the POL and COL groups were increased the expressions nearly two – fold. The expression of LIF in routine IVF group at 48th hour may be lower than the ones in sponge systems, thereby any inconsiderable change might result much more increase in routine IVF. Endometrial cells supported the LIF expression both in POL and COL groups but for CE group, there was a significant downregulation. According to literature, LIF also supports to increase the rate of preimplantation embryo development in mouse [99] and this decrease in the collagen group without blastocyst may be linked to the absence of the embryo.

In a very recent study, it has been shown that E – Cadherin expression needs to be increased especially during the proper attachment of an embryo to the surface epithelium of the endometrium which was supported by the idea of E – Cadherin absence is resulting in recurrent implantation failures fatefully.[100] Depending on our data, E – Cadherin expression decrement in routine IVF may be due to the looseness of the cells of a blastocyst while implanting. When the groups with sponges were evaluated, both collagen and polymeric sponges gave significant upregulations in terms of E – Cadherin expression. Besides the attachment of blastocyst, this result may also come from the epithelial cell proliferation of non – implanting areas. On the other hand, it is also suspicious that the endometrial cells need to be loosen their integrity to make room for implanting blastocyst under the effect of MMPs. [101] Due to this, E – Cadherin expression may also be expected to be decreased in the presence of an implanting embryo. Polymeric sponge without blastocyst also significantly increased the E – Cadherin expression, so that underlying co – culture system supports the epithelial layer formation and cell proliferation.

In humans, it has been shown that L – Selectin initiates the initial adhesion of the blastocyst to the uterine epithelium especially for human embryos.[102] The function and expression of L – Selectin in mouse models still requires questioning. It has been also proved that L – Selectin on the blastocyst and its oligosaccharide ligand on the maternal surface interacts with each other. [56, 103] Both for POL and COL groups, the endometrial co – cultured systems supported the expression of blastocyst-based L – Selectin. Even the routine IVF group increased the expression, COL group was significantly supported the L –

Selectin expression. Groups without the blastocyst downregulated the L- Selectin expression due to the absence of the embryo. This may be attributed to the production of the L-selectin by the embryo and the 3D endometrial cell coated sponge modelling supported this expression.

Trophinin, is expressed by the cells which are involved in the implantation process in humans. Ovarian hormone, especially oestrogen triggers the expression of trophinin from the endometrial cells. According to a previous study, there has been found no difference between the expression levels of trophinin transcripts in normal or pseudo pregnant mouse uterus or the absence of blastocyst. [104] Also, depending on another study, it has been mentioned that the expression of trophinin is not absolutely necessary for early implantation steps. [103] Depending on our data, trophinin expression did not give any significant difference between COL and routine IVF groups. But for CE groups, which has no blastocyst, significantly supported the expression of Trophinin both for routine IVF and COL. As mentioned above, endometrial cells induce upregulation of trophinin in the presence of oestrogen hormone whether there is a blastocyst in the environment or not. As the culturing medium includes oestrogen hormone, the supplementation might be triggered the expression of the trophinin from the underlying system in collagen sponge modelling.

Fibronectin, which is an extracellular matrix protein, responsible from the formation of fibrillar network. The expression in routine IVF almost remained stable from 48th to 96th hour, which also supports the idea of fibronectin is an ECM component and requires the presence of endometrial cells. For polymeric sponge groups, the expression differences were found to be non – significant even for the groups with blastocyst or not. COL group increased the expression of fibronectin significantly when compared to routine IVF group, which is suggesting that the requirement of the endometrial epithelial and stromal cells. Also, when CE group without the blastocyst was examined, there was a severe significant decrease in the expression which leads that the proper attachment requires an active blastocyst and properly prepared endometrial surface. As Integrin binds to the Fibronectin, it was expected to give correlated results with the expression of fibronectin. Even though it is not statistically significant in COL group, data shows an increment which is similar as the fibronectin expression. Whether the POL group increased the fibronectin expression, it was decreased in terms of integrin, but these results were statistically non – significant. For CE group, Integrin was also decreased significantly both for routine IVF and COL groups, as linked with fibronectin expression. So that, the fibronectin – integrin expressions were integrated with the presence of blastocyst and this attachment might be triggered by the co - culture models.

Laminin is an ECM molecule which supports the integrity of the basal membrane almost in all tissues. According to our data, both COL and POL groups gave significant increases when compared to routine IVF group. It was expected a decrease in routine IVF since there was not any underlying endometrial system and the data showed a decrement significantly as expected. According to previous studies, it has been shown that trophoblast invasion upregulates the laminin expression from the decidualized stromal bed. [105, 106] Correspondingly, there were significant decreases since there was not any implanting blastocyst in CE and PE groups. The endometrial epithelial and stromal cells based co – cultured models required an implanting blastocyst to enhance their laminin expressions to remodel the endometrial characteristics.

A basement membrane glycoprotein, Entactin, is responsible from the assembly of ECM and attachment of trophoblast cells. In routine IVF group, there was a slight significant increase when compared to COL group since there was not any supporting underlying cells. Polymeric sponge group expression differences were not found to be statistically significant but, COL group increased its expression in comparison to routine IVF. Since the entactin expected to be co – localized in all extraembryonic matrices in the presence of an hatched blastocyst[107], the data was found to be coherent. Also, the significant decrease in CE group according to COL group might be linked to its role in trophoblast outgrowth[108], so that groups without blastocyst decreased their expression in the absence of an implanting blastocyst.

Among collagens, Collagen Type I is fibrillar collagen which is responsible from fibril formation. According to literature, fibril-forming Collagen I and III are needs to be reduced in the sites of the implantation when compared to the non-implantation sites in the uterus of rats, because they disappear from the primary and secondary decidual zones.[109] In our data, routine IVF decreased its expression significantly according to COL group depending on the absence of endometrial underlying cells. When POL and COL groups are observed, they both showed significant decrements in comparison to routine IVF. As collagen needs to be degraded to home the implanting blastocyst, it was an expected result that collagen fibrils are getting degraded under the implantation site. Also, Collagen III is a type of reticular fiber, so that it needs to be disappear from the implantation site like Collagen Type I [110, 111]. Depending on the data, even the COL was non – significant, it was decreased when compared to routine IVF. POL group decreased the Collagen III expression regarding the degradation of fibers at the implantation site. Both for Collagen I and III, PE group increased the expression depending on the absence of blastocyst and shows that sponge supports the formation of endometrial surface by the coated cells. Collagen IV is a member

of basement membrane components and underlies epithelial and endothelial cells. Previous studies showed that Collagen IV expression during implantation is still unclear but, alpha chains upregulated their expression during menstrual cycle and decidualization.[66] On the other hand, it also shown that the primary extra villous trophoblast cells express collagen receptors and collagen IV is secreted in vivo and *in vitro.*[66] Collagen IV expression increased in COL and POL groups, but they were not found significant. In CE group, the expression was decreased significantly according to COL group. Since Collagen IV upregulated in the presence of blastocyst, it can be linked that the co – cultured systems need to be in contact with an embryo to support the regulation of the Collagen IV.

MMP9 is the major enzyme which functions the endometrial remodeling in terms of invasive characteristics. In all experimental groups, there was not any statistical significance. Both POL and COL groups increased their expressions, but they remained almost the same as routine IVF group. As they are the key enzymes for a successful cytotrophoblast invasion and degradation of basement membrane molecule[112], it was expected to be decreased in the groups without blastocyst. Although the difference is not significant, PE group decreased its expression visibly due to the absence of the blastocyst in the environment. TIMP3, as a major inhibitor of MMP9, expected to be correlated with MMP9 expressions. To regulate a successful trophoblast invasion, TIMP3 in the stroma surrounding the embryo and MMP9 expression needs to be in a fine balance.[113] Like MMP9, the experimental groups except CE, did not give any statistical significance. Only significance was seen on CE group with a slight decrease which can be related with the requirement of the embryo. Since there was no embryo in the group, there was not any MMP activity to trigger the TIMP activity which is also a suspicious case that only increment of MMP9 was seen in CE group although it was nonsignificant.

Some discordances between qPCR results and IF stainings are thought to be lack of replicates in IF and as IF in this state cannot be evaluated as a quantitative approach.

CONCLUSION

In conclusion, it was found that 3D co – culture systems constructed with artificial and natural sponges gave more effective results rather than routine IVF culturing systems. The expression of Entactin, Fibronectin, L- Selectin, and Laminin form adhesion, ErbB2 from functional and Hoxa10 from developmental genes was significantly supported by collagen sponge-based system whereas artificial polymeric sponge-based system significantly promoted the expression of developmental genes such as Hoxa11 and Wnt4. Depending on the degradability and malformation of natural collagen sponge, finding the blastocyst for imaging was difficult. Therewithal, the opacity of artificial polymeric sponge restricted the manipulation of the blastocyst. Consequently, usage of 3D endometrial co – culture coated sponge enhanced the implantation by upregulating some important markers when compared to routine IVF. A more comprehensive study that includes an in vivo experimental approach would be the next goal to reveal the effectivity of polymeric carriers for implantation process.

VITA

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After she graduated from university, she has continued her education in Reproductive Biology Master Program at Koç University Graduate School of Health Sciences (GSHS). She has worked as teaching and research assistant during her master program. Her research has mainly focused on implantation biology and has been working on three-dimensional mouse models.

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