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3D model of the maternal-fetal interface: challenges, recent advances and beyond.

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Running title: 3D model of the human implantation process

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Abstract:
Embryo implantation is a complex and highly coordinated process that involves an intricate network of factors establishing intimate contact at the maternal-fetal interface. Knowledge of the human implantation process is compromised by both ethical issues, which do not allow the study of this process *in vivo*, and by the accuracy and reproducibility of *in vitro* models of human endometrium. Effective and reliable embryo implantation models are, therefore, necessary to mimic the molecular event cascade that occurs *in vivo*. 3D models are considered a new step to foster precision medicine and an advanced tool for the study of endometrial biology, endometrium associated diseases and to understand the complex mechanisms surrounding endometrium-embryo crosstalk.
In this review we explore the various methods by which 3D cultures of endometrium and trophoblast can be created, exploring targets and applications of these i*n vitro* models.

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Introduction

LIST OF ABBREVIATIONS

2D: two dimensional
3D: three dimensional
ECM: extracellular matrix
EOs: Endometrial Organoids
CTB: cytotrophoblast
STB: syncytiotrophoblast
WOI: Window of Implantation
LH: Luteinizing Hormone
PCOS: polycistic ovary syndrome
ER: Estrogen Receptor
PR: Progesterone Receptor

INTRODUCTION

Embryo implantation has been defined as the "black box" of human reproduction. Most of the knowledge on mechanisms underlying this process derives from animal models, but they cannot always be translated to humans. Therefore, new technologies such as the 3D cell-culture model, human induced pluripotent stem cells, and gene editing, have been developed leading to new solutions for replacing, refining, and reducing animal models. Indeed, the development of an in vitro/ex vivo model recapitulating as closely and precisely as possible the key functional features of human endometrial tissue is foreseen (Luddi et al., 2020).

Fundamental is the relationship that is established between the endometrium and the placenta during pregnancy. Indeed, the success of pregnancy depends not only on migration and invasion of the trophoblast into the maternal decidua, but also on signals from the decidua playing a fundamental role in the implantation process and invasion of the trophoblast.

To initiate a pregnancy, a complex network of factors surrounds the mother and the embryo to establish intimate contact and to create a maternal-fetal interface that requires the coordination of three events: decidualization of the endometrium, development of the embryo, and formation of the placenta (Mannelli et al., 2015).

THE MATERNAL INTERFACE

1- Endometrium: a unique tissue

The endometrium is the inner epithelial layer, along with its mucous membrane, of the mammalian uterus and constitutes the maternal site for embryo implantation. The adult endometrium is a complex tissue that consists of stromal cells, luminal and glandular epithelial cells and endothelial and vascular smooth muscle cells, as well as a complex network of leukocytes populations (Sandra, 2016) (Figure 1).

During the menstrual cycle, the endometrium undergoes remodeling, shedding and regeneration, all of which are driven by substantial gene expression changes in the underlying cellular hierarchy. A healthy endometrium regenerates approximately 450 times in a woman's reproductive life without the formation of scar tissue (Chavez-MacGregor et al., 2008). The blastocyst can implant during a limited period between days 20 and 24 of a regular menstrual cycle (7 to 11 days after LH surge) (Figure 2). During this period, called the window of implantation (WOI) (Psychoyos, 1974), the human endometrium is primed for blastocyst attachment, once it has acquired the accurate morphological and functional state induced by ovarian steroid hormones (Finn and Martin, 1974).

The initial adhesion of the blastocyst to the uterine wall, called apposition, is unstable. Microvilli on the apical surface of syncytiotrophoblasts interdigitate with microprotrusions from the apical surface of the uterine epithelium, known as pinopodes (Norwitz et al., 2001). Despite its importance in human fertility and regenerative biology, our understanding of this unique type of tissue homeostasis remains rudimentary.

The endometrium is a fascinating and unique tissue that has tremendous plasticity as the biological interface dedicated to the interactions with the embryo and with the feto-placental unit; therefore, the endometrium represents a critical tissue for normal fertility and reproductive success.

Many identified molecular mediators, under the influence of ovarian hormones, have been postulated to be involved in this early feto–maternal interaction. These mediators embrace a large variety of inter-related molecules including adhesion molecules, cytokines, growth factors, lipids and others (Apparao et al., 2002; Luddi et al., 2020; Governini et al., 2021). Endometrial receptivity consists of the acquisition of adhesion ligands together with the loss of inhibitory components that may act as a barrier to an attaching embryo (Aplin, 2000).

Implantation failure remains an unsolved problem in reproductive medicine and is considered a major cause of infertility in healthy women. Indeed, the implantation rate in IVF is around 25% (de los Santos et al., 2003). Inadequate uterine receptivity is responsible for approximately two-thirds of implantation failures, whereas the embryo itself is responsible for only one-third of these failures (Lédée-Bataille et al., 2002).

The recent discovery of molecules crucial for successful embryo implantation has offered researchers precious insight into this field. Nevertheless, important questions regarding the molecular mechanisms governing this process remain to be deciphered. A better understanding of the mechanisms regulating embryo implantation may improve the ability of clinicians to treat infertility, prevent early pregnancy loss and develop new contraceptive approaches and discover the causes of recurrent implantation failure (Cakmak and Taylor, 2011). Knowledge of the human implantation process is compromised by both ethical issues, which do not allow the study of this process *in vivo*, and by the accuracy and reproducibility of *in vitro* models of human endometrium. Effective and reliable embryo implantation models are necessary to mimic the molecular event cascade that occurs *in vivo*; many steps have been taken towards a model that is reliable and reproducible. Human *in vitro* models have been successfully developed by many groups with the goal of obtaining effective tools to explore the complexity of this process (Teklenburg et al., 2010; Weimar et al., 2013; Stern-Tal et al., 2020; Rosner et al., 2021;).

Despite the enormous importance of *in vivo* models and the fact that they represent an invaluable asset in many aspects, these are laborious and expensive and do not yet address the human translational medicine issues represented by interspecies variability.

The goal of this review is to stress the importance of adequate and robust 3D endometrial and placental models able to recapitulate all the cascade of events that surrounds embryo implantation without the use of animal models.

2. Endometrial cell types and characterization

In the proliferative (or follicular) phase both the endometrial glands and stroma proliferate in response to the rising estrogen levels of ovarian follicle origin. The thickness of the endometrium increases, glands become increasingly tortuous and are lined by a tall, pseudostratified columnar epithelium. The cytologic appearance of proliferative glands is

very "active," and characterized by a moderately high nuclear/cytoplasmic ratio, abundant mitotic activity, and prominent nucleoli. The progesterone surge of ovulation ends the proliferative phase, and the endometrium moves into the secretory (or luteal) phase. Under normal conditions, the secretory phase is 14 days length, and the endometrium moves through an orderly sequence of morphologic changes. Under the influence of local autocrine factors, the secretory endometrium disintegrates and collapses. The resultant menstrual endometrium is characterized by cellular whorls of endometrial stroma exhibiting nuclear debris ("blue balls"), often with overlying, draping epithelium.

Endometrial epithelial cells proliferate in close relationship with the production of predominantly ovarian estrogens, complemented by conversion to estrogen from adrenal androgens, that occurs in significant proportions in women with adrenal hyperfunction, in some of those with polycystic ovarian syndrome (PCOS) (Rosenfield and Ehrmann, 2016), and in some obese women; in the latter, an endometrial development hyperproliferative and inadequate for embryonic implantation has been demonstrated (Lv et al., 2022).

To depict the sophisticated alterations of endometrial cells and the local microenvironment in thin endometrium, scRNA-seq was applied to analyze the discrepancy in gene transcription between normal and thin endometrium during the late proliferative phase. There are fifteen distinct cell types with their own unique characteristics of gene expression profiles in the endometrium (Figure 3). The number of stromal, proliferating stromal (pStrs), epithelial, natural killer (NK), and T cells are reduced in thin endometrium accompanied by increased cellular senescence in perivascular cells. An analysis of cell interactions revealed that signaling pathways related to cell growth were markedly interrupted in thin endometrium, especially in stromal niches (stromal cells, pStrs, and perivascular cells). These findings indicated a potential mechanism of thin endometrium pathogenesis and provide insight into improving fertility.

Moreover, estrogens also induce a complex neo-angiogenesis with the determination of an intricate network of endometrial microvessels destined cyclically to break down along with the rest of the endometrium (Mandalà, 2020). Endometrial stromal cells instead respond very poorly to estrogen stimulation. Progesterone during the ovarian cycle is mainly synthesized by estrogen-secreting granulosa cells of the ovary, concomitant with ovulation and in relation to LH surge (Niswender et al., 2000). Progesterone acts on the endometrium by an anti-proliferative estrogen antagonistic effect (Li et al., 2011) and an adequate preparation of the endometrium for embryo implantation through: a) specific effects on stromal cells (decidualization); b) structural and secretory modifications of luminal and glandular epithelial cells of the endometrium; c) structural and flow modification effects on the endometrial microcirculation; d) reduction of contractile activity on the endometrium, inhibitory action of maternal embryotoxicity toward the embryonic trophoblast (Herrler et al., 2003).

During its endometrial priming, progesterone produces a ticker endometrium by the proliferation of its epithelial cells, the increase in the volume of its stromal cells (from stellate to globular and round shape), the edematous imbibition of the extracellular matrix,

and the dramatic modification of endometrial microcirculation. This latter consists of an increase in the vessel calibre and the formation of true venous lakes, resulting in increased oozing of fluid on the surface of the uterine cavity and a predisposition to rapid, enveloping vascularization of the embryo immediately after its invasion process of the endometrium itself (Vinketova et al., 2016).

These structural changes are accompanied by a complex and intricate production of factors, predominantly protein or glycoprotein, produced by epithelial, stromal, and transudate-derived cells from the extracellular matrix that come to make up the natural culture medium that accommodates, in the lumen of the uterine cavity, the embryos for about three days prior to implantation, thereby conditioning their success and the onset of pregnancy.

3. 2D models of Endometrium

Two-dimensional (2D) cell culture has been used since 1985 to study early feto-maternal interactions. The most used cells type for endometrial models are stromal and epithelial cells, obtained from endometrial tissues digested into single-cell suspension by various methods such as filtration, cell adhesion, density gradient centrifugation, immunomagnetic selection and fluorescence-activated cell sorting (Liszczak et al., 1977; Kirk et al., 1978; Varma et al., 1982). Stromal and epithelial monolayers have been largely employed to study in a 2D environment the early feto-maternal interactions (Lindenberg et al., 1985), and trophoblast spheroids were added to mimic human blastocysts to investigate molecular events beyond the luminal epithelium-embryo attachment and endometrium dysfunction in reproductive failure (Weimar et al., 2013; Lee et al., 2015; Aplin and Ruane, 2017; Huang et al., 2017).

The main disadvantage of the 2D culture of primary endometrial cells is their reduced biological activities after several passages and diminished response towards sex hormones, which are not supportive to study their morphological and functional roles (Hannan et al., 2010; Fitzgerald et al., 2021). Considering the limited availability of primary human endometrial tissues, many researchers started to use endometrial adenocarcinoma and immortalized epithelial cell lines in 2D models to investigate endometrial epithelial functions. However, even though the cell line models are relatively easy to access and maintain, the potential genetic aberration associated with prolonged culture is an obstacle to investigating the physiological properties of the endometrium (Hannan et al., 2010). Another major aspect that needs to be considered in 2D models is that they cannot address questions about the complex environment related to the tridimensional (3D) architecture; indeed, cell-to-matrix interactions cannot take place. The endometrial cell-extracellular matrix (ECM), a 3D matrix scaffold surrounding the cells, provides biochemical and biophysical support to the endometrial cells and plays pivotal roles in the menstrual cycle and embryo implantation. A monolayer culture without ECM alters the functional activities of the endometrial epithelial cells; they lose their polarities and change their secretory functions (Aplin et al., 1988). Meanwhile, monolayers of multiple cell types are difficult to

co-culture, and thus are unable to reproduce the *in vivo* crosstalk between the endometrial stromal and epithelial cells.

All these critical issues have pushed the scientific community towards the search for alternative approaches. 3D cell culture has been shown to be more effective to model a cell in vivo while being cultured in vitro, also enduring specific methods of cell culture depending on the type of tissue to be mimicked (Jensen and Teng, 2020).

4. 3D models of the endometrium

A 3D cell culture model can be set up by using either a scaffold, cell supporting matrix, or non-scaffold-based culture method (Li et al., 2022). A variety of 3D culture methods have been used to mimic human endometrium, such as organoids, 3D hydrogels, 3D bioprinting, and organ-on-a-chip (OOAC) platforms (Figure 4). Some of these approaches will be described in depth in the following sections.

4.1- Organoids

Organoids are defined as "self-organizing, genetically stable 3D culture systems that replicate the tissue of origin and contain both progenitor and differentiated adult cells of the tissue of interest" (Cui et al., 2020; Li et al., 2022). The first mention of the endometrial organoid-like structures (EOs) was 34 years ago in 1988 by Rinehart *et al*. (Rinehart et al., 1988). In this study, the endometrial glands were isolated from the endometrial tissue biopsies and embedded in Matrigel at a ratio of 1:1 with a culture medium. The endometrial gland fragments initially spread in the Matrigel as monolayers of tiny colonies and eventually formed a gland-like structure. Similarly, some other studies also found the development of prominent glandular structures from endometrial epithelial cells when they were cultured on basement membrane extracts (BME) or Matrigel. These cells showed polarization, hormone responsiveness and secretory functions that recapitulate the endometrial glands *in vivo* (Negami and Tominaga, 1989; Schatz et al., 1990; White et al., 1990; Classen-Linke et al., 1996). Even though the gland-like structures were not well characterized, these studies set the basis for the development of the endometrial glandular organoid model.

EOs are considered an important tool for the study of female reproductive biology. Compared with conventional 2D and 3D culture models, organoids contain more cell types within the intact 3D structure, along with the presence of ECM that reproduce the physiological microenvironment. Unlike most 3D culture models of the endometrium, which can be cultured for up to 10 days, organoids are more stable and can be cultured for a much longer period, with high genetic stability. Therefore, the organoid culture model has been gradually applied for the study of embryonic development, diseases, and drug testing in regenerative medicine (Rossi et al., 2018) (Figure 5). EOs may derive from endometrial tissue from all stages of the menstrual cycle, as well as from decidua and atrophic endometrium (Nikolakopoulou and Turco, 2021). Only recently has the derivation of epithelial organoids from menstrual blood also been possible (Cindrova-Davies et al., 2021).

This new methodology, less invasive, can allow researchers to have a broad spectrum of samples considering the limited amount of “healthy” endometrial biopsies.

Currently, many protocols have been established for 3D endometrial epithelial organoids (EEOs) in a chemically defined medium to fully generate and analyze their functions (Boretto et al., 2017; Turco et al., 2017). In addition, these studies have shown that EEOs also exhibit physiological hormonal activity, demonstrating that they can maintain intrinsic disease properties and resemble the human endometrium, which undergoes extensive remodeling during the menstrual cycle, regulated mainly by ovarian estrogen and Progesterone (Boretto et al., 2017; Turco et al., 2017; Fitzgerald et al., 2019; Luddi, et al., 2020).

Recent progress has been achieved with the creation of organoids incorporating both epithelial and human stromal cells (also named assembloids) (Rawlings et al., 2021). Based on transcriptomic analysis, these assembloids contain different subpopulations of stromal and epithelial cells that secrete implantation factors. Therefore, the upgraded EOs represent a powerful tool for precision medicine in static conditions.

An improved version of the 3D endometrial model, composed of both epithelial and stromal cells, was established for examination of the impact of two contraceptive drugs, levonorgestrel and mifepristone, on the expression of endometrial receptivity markers (Lalitkumar et al., 2007). This model demonstrated not only that the epithelial and stromal cells expressed estrogen receptor (ER), and progesterone receptor (PR), but also that the treatment with estrogen and progesterone induced the expression of several endometrial receptivity markers including LIF, IL-1β, VEGF and cyclooxygenase-2 (COX2). Interestingly, mifepristone inhibited the expression of these markers like that observed *in vivo*, and inhibited blastocyst attachment, thus confirming EOs as suitable in vitro models to study endometrial receptivity. Most importantly, human embryos can be incorporated to interact with the established 3D endometrial model, enabling the use of the model to study embryo implantation as well (Lalitkumar et al., 2007).

A 3D culture model of the human endometrium may also combine the use of both primary and continuous cell lines. Arnold *et al*. (Arnold et al., 2002) cultured primary endometrial stromal cells in Matrigel and seeded directly on top of Matrigel the Ishikawa cells (a cancer cell line of endometrial glandular origin) expressing epithelial-like phenotype. This model demonstrated that the interaction between stromal and glandular cells induced the Ishikawa cells to produce glycodelin, a key glycoprotein produced by the endometrial glands in the secretory phase of the menstrual cycle (Seppälä et al., 2002; Lee et al., 2016). This confirms that stromal cells control in a paracrine way the proliferation and differentiation of the co-cultured endometrial epithelial cells. This study further showed that the absence of Matrigel reduces the regulatory functions of secretory factors derived by stromal cells on the Ishikawa. This suggests that in the 3D culture model, secretory factors from the Matrigel support the cells' function in a more physiological way. One limitation of the study was the use of Ishikawa cells (adenocarcinoma cell line) as epithelial cell surrogates instead of primary cells.

Another aspect of the 3D model culture is that ECM materials such as collagen and Matrigel, are easily degraded in culture, which reduces the length of the co-culture. Therefore, different inks, such as agarose and fibrin, have been used as ECM to set up more effective endometrium-like *in vitro* 3D models. Moreover, the use of several compounds, including calcium chloride, to stabilize the ECM was reported (Wang et al., 2012). In this model, the endometrial stromal cells (primary or immortalized cell lines) were cultured in the supplemented fibrin-agarose, and the epithelial cells (primary or Ishikawa cell line) were seeded on top of the gel. By this approach, the cells maintain an intact structure for at least 10 days, outperforming the previously established 3D endometrial models. These modifications make this model suitable for the integration of trophoblast spheroids, allowing a comprehensive study of the trophoblast invasion into the endometrium (see following sections).

4.2- 3D tissue-slice culture model

3D tissue-slice culture models are 3D tissue explants that can be cultured *ex vivo* for an extended period (Majorova et al., 2021).

In the beginning, the tissue slice culture model had major limitations, due to the inaccuracy of the razor blades used and the suboptimal incubation conditions; this resulted in a very rapid decrease in cell viability. These problems were solved by the advent of new tissue slicers, and through the improvement of incubation technologies. 3D tissue-slice culture models have the advantage of retaining not only the physiologic architecture of the in vivo tissue, but also maintaining some important tissue-specific functions, such as metabolism and immunologic functions. This makes 3D models an efficient alternative to animal models. Their use as in vitro models of human endometrium is so far very limited and recent. Muruganandan and colleagues (Muruganandan et al., 2020) reported the setup of a 3D tissue slide model obtained from full thickness biopsy of human endometrium embedded into a 3D matrix scaffold of type I collagen gel, incorporating an air-liquid interface, which allows sustained tissue viability over three weeks. Compared to the conventional cell-based models, which generally show diminished cell viability and hormone responsiveness in vitro approximately after 5 days, the endometrial tissue slice in this double-dish tissue-based model was viable after 21 days. Noteworthy, these 3D models retain the ability to respond to ovarian hormones, eliciting a correct gene expression profile and the typical changes in endometrial morphology, usually found in the in vivo tissue. This model further confirmed the importance of ECM in preserving the functionality of endometrium *in vitro*.

Although further studies are needed to optimize preparation protocols, these models have the undisputed advantage of including all cellular populations present in the endometrium.

4.3-Endometrium on a chip: dynamic models

While 3D organoid cultures represent a distinct improvement over monolayer cultures, they still fail to exactly mimic the architecture of tissues, which includes vascular and interstitial fluid flow.

Organ-on-a-chip (OOC), flexible devices representing the convergence of microfluidics and tissue engineering, are an emerging technology able to address this limitation.
In these devices, cells are cultured in micrometer chambers that are continuously perfused, thus creating a sheer force that provides nutrition and waste transport to mimic *in vivo* vascularized tissues.
Another approach is a dual reproductive organ-on-a-chip system that enables bidirectional communication between the ovaries and endometrium. This model reproduces the multicellular complexity of both tissues: the ovarian compartment contains granulosa and theca cells, while the endometrial compartment includes fibroblasts, vascular epithelial cells, immune cells, and endometrial stem cells (Park et al., 2020).
A new microengineered 3D device of vascularized endometrium on a chip was recently conceived (Ahn et al., 2021). This model faithfully reproduces the endometrial microenvironment, consisting of three distinct cell layers, epithelial cells, stromal fibroblasts, and endothelial cells, in a spatially and temporally 3D extracellular matrix. Two central channels are intended for 3D culture and morphogenesis of stromal fibroblasts and endothelial cells. In addition, the outermost channel is intended for the culture of additional endometrial stromal fibroblasts that secrete molecules to induce pro-angiogenic directional responses of endothelial cells (Ahn et al., 2021).
An important breakthrough in generating more sophisticated *in vitro* models has been the application of microfluidic technologies by Xiao *et al*. (Xiao et al., 2017). This Multi-Organ" EVATAR" microfluidic system simulates in vitro the 28-day human menstrual cycle by combining murine ovary, fallopian tube, uterus, cervix, and human liver explants with steroid hormones released from ovarian follicles.

THE EMBRYO INTERFACE

5. Implantation, early placental development, and human trophoblast lineage specification

The embryo can implant into the uterine wall one week after conception: at the blastocyst stage, a hollow sphere is formed externally by trophoblast cells, internally by the cells of the inner cell mass, and a fluid-filled cavity called the blastocoel (Carson et al., 2000). The blastocyst forms all embryonic and extraembryonic tissues, including the placenta (Rivron et al., 2018a). In particular, the inner cell mass will give rise to the embryo and the trophoblast will develop in the placenta, the extra-embryonic organ that surrounds the embryo during development and allows for nutrition, protection, and secretion of important molecules (Widmaier et al., 2011). Although dysfunctions of the placenta cause serious pregnancy disorders with immediate consequences for both mother and fetus, our knowledge of the human placenta is limited due to the lack of functional experimental models.
Embryo implantation is a three-stage process: apposition, adhesion, and invasion (Weimar et al., 2013). Implantation involves crosstalk between an active blastocyst and a receptive endometrium; therefore, the invasion of the trophoblast into the decidua, the receptive endometrium that forms during the early stages of pregnancy, is essential for proper

implantation of the blastocyst and a physiological pregnancy (Mier-Cabrera et al., 2009). During the stages of the implantation process, many molecules are involved in the initial interaction between mother and fetus, coordinated by ovarian steroid hormones.
These mediators include adhesion molecules, cytokines, growth factors, lipids and others (Zhao et al., 2013; Fitzgerald et al., 2016). During apposition, which occurs when the embryo stops moving freely within the uterine cavity, the embryo shows polarity marks (Ebner et al., 2012). The trophoblast cells adhere to the luminal epithelium of the receptive endometrium and proceed to anchor to the basal layer and extracellular matrix. At this point, when the blastocyst is properly positioned, adhesion occurs, characterized by a stable interaction between the trophectoderm and the endometrial epithelial cells. This interface is mediated by adhesion molecules (Cellular Adhesion Molecules-CAM), expressed on the apical surface, including integrins, cadherins, selectins and immunoglobulins (Lessey et al., 1995; Lessey and Young, 1997).
The trophoblast through the secretion of various proteolytic enzymes allows penetration into the endometrial epithelium and stroma.
After implantation, the trophectoderm of the blastocyst differentiates into an inner mononuclear layer called the cytotrophoblast (CTB) and an outer multinucleate layer called the syncytiotrophoblast (STB) (Turco et al., 2018). Cytotrophoblast cells differentiate into two main subpopulations: the syncytiotrophoblast, the multinucleated villus epithelium responsible for nutrient exchange and hormone production, and the extravillous trophoblast cells (EVT), which anchor the placenta to the maternal decidua and convert the maternal spiral arteries into low resistance vessels (Albrecht and Pepe, 2020). Therefore, the study and generation of long-term, genetically stable trophoblast organoid cultures that can differentiate into both syncytiotrophoblast and extravillous trophoblast is fundamental.
Studying placentation *in vivo* presents considerable ethical issues, which is why the study of the early stages of pregnancy mainly relies on *in vitro* cell cultures. Indeed, in vivo models are mainly developed in mice, which significantly differ from humans in term of placenta morphology, hormone production and migration/invasion processes (Stojanovska et al., 2022).

6. Traditional in vitro models of human trophoblast

Traditional models for studying human trophoblast differentiation are represented by placental explants, obtained from placental tissues of any gestation time (Horii et al., 2020; Bačenková et al., 2022). The presence of a mixed cell type doesn't allow manipulating gene expression in a cell type-specific manner, representing the main limitation of these models. Therefore, some researchers have turned to trophoblast isolation; however, ethical, and religious issues make placenta tissue and isolation of primary trophoblast cells not widely available in many countries.
Advances in the study and use of human pluripotent stem cells (hPSCs) have made it possible to differentiate into trophoblast cells in 2D monolayer culture, with fine control

over chemical factors, size, and density. However, cells cultured in 2D culture cannot faithfully recreate the complex structure of early placental villi (Cui et al., 2022). Conventional 2D in vitro models poorly reflect the in vivo microenvironment, mainly due to reduced cell-cell contact and the increased surface area directly exposed to gas and nutrients (Duval et al., 2017; Jensen and Teng, 2020).

As previously discussed for endometrium, 3D cell culture models more accurately reflect the complex physiology and morphology of the tissue than 2D cell monolayers (Barros et al., 2018; Souza et al., 2018). So, although they are extremely useful in understanding the biochemical aspects and behavior of each cell line, they cannot fully reproduce the complex cell-to-cell and cell-to-matrix interactions occurring in placentation. Over the years, several methods have been proposed to implement an *in vitro* model of the human blastocyst (Barros et al., 2018).

Kagawa and colleagues (Kagawa et al., 2022) reported the formation of blastoids generating blastocyst-stage analogues of the three founding lineages (trophectoderm, epiblast and primitive endoderm) according to the *in vivo* timing of blastocyst development. The epiblast of these *in vitro* modelled blastoids can induce the local maturation of the polar trophectoderm, thus assuring the blastocyst the ability to directionally attach and stimulate endometrial cells, as during implantation in vivo. Moreover, they have shown that human blastoids apposed on unstimulated endometrial organoids did not attach, while they did to the stimulated endometrial organoids. Furthermore, the use of the contraceptive levonorgestrel altered the attachment of the blastoids to the endometrial organoids, thus concluding that human blastoids can interact specifically with receptive endometrial cells (Kagawa et al., 2022). Therefore, here we propose a human blastoid as a faithful, scalable and ethical model for investigating human implantation and development (Rivron et al., 2018b; Clark et al., 2021).

7. 3D models of the human trophoblast

Experimental models rely on the use of human trophoblast and endometrial cell lines. Indeed, adhesion models involving the coculture of a monolayer of human endometrial epithelial cells (to mimic uterine epithelium) and trophoblast spheroids (to mimic blastocysts) are widely used (Ho et al., 2012).

Spheroids are obtained by various techniques that promote cell adhesion leading to cell-cell aggregation which spontaneously develops into three dimensions (Burdett et al., 2010; Hardelauf et al., 2010).

Some basic methods used are the following:

- suspension culture in bacterial-grade dishes, in which an untreated hydrophobic polystyrene plate is used for liquid suspension culture (Pettinato et al., 2015);
- methylcellulose semisolid media culture, where cells, seeded on semi-solid methylcellulose media, tend to remain single, isolated from the methylcellulose matrix, which will then develop into aggregates (Liu et al., 2006). Culture in methylcellulose allows the reproducible formation of spheroids from single cells.

- culture in hanging drops, the rounded bottom of a suspended drop allows the aggregation of cells. The number of cells can be controlled by varying the number of cells in the initial suspension thus, homogeneous spheroids can be reproducibly formed from a predetermined cell number.

In addition to the above three basic methods, others may be applied by using a round-bottomed 96-well plate and a conical tube (Kelm et al., 2003; Kurosawa, 2007).

The most widely used cell lines for the development of trophoblast spheroids are BeWo (Figure 6), HTR8/SVneo and JEG3 cells. The growth rates of the spheroids of the JEG3, BeWo and HTR8/SVneo cell lines are similar to each other and significantly larger than the spheroids from primary trophoblasts; in addition, all spheroids show migratory properties (Suman and Gupta, 2012; Stojanovska et al., 2022). Stojanovska *et al*. reported that all spheroids made from the three cell lines listed above show invasive capabilities, but only the HTR8/SVneo spheroids gave rise to specific branching. They also evaluated the production of human chorionic gonadotropin, which was maximal in JEG3 spheroids, whereas in BeWo spheroids it increased only when stimulated with cAMP and forskolin. Finally, this study confirmed that the gene expression profile of 3D trophoblast cell cultures, particularly the HTR8/SVneo spheroids, is comparable to that of primary placental tissue (Stojanovska et al., 2022).

Therefore, it is important to select the best 3D spheroid model of trophoblast according to the scientific question to be answered. The spheroid model was first used by radio biologists in 1970 (Sutherland et al., 1970). Only in recent years has it been useful in pathophysiology for the study of tumorigenesis and drug screening (Hirschhaeuser et al., 2010).

The spheroid has dividing cells in the periphery, which mirror the proliferation of CTB cells during chorionic villus formation and therefore proves to be an excellent model for studying the villous trophoblast and the formation of the primitive placenta specifically in villogenesis. As growth continues, the inner layers of the spheroid become quiescent and subsequently lose viability through apoptosis or necrosis due to hypoxia (Friedrich et al., 2007). As the size increases beyond 400-500µm in diameter, a necrotic core is generated mainly due to the accumulation of catabolites and toxins, as well as to the poor diffusion of oxygen and nutrients (Acker et al., 1987).

Normal placental development is dependent on the orchestrated differentiation of cytotrophoblast cells. First-trimester cytotrophoblast cells cultured as spheroids maintain their high proliferative and invasive phenotype and respond to different cytokines upon stimulation in a three-dimensional invasion assay. In contrast, third-trimester cytotrophoblast spheroids maintain their quiescent non-proliferating phenotype and invasion can only be induced by EGF (Korff et al., 2004). Haider *et al*. created long-term expanding organoid cultures from purified first-trimester cytotrophoblasts. Molecular analyses revealed that CTB organoids expressed markers of stemness, and trophoblast proliferation and they were highly like primary CTBs at the overall gene expression level (Haider et al., 2018). Interestingly, the results obtained by Indovina et al. show that hypoxia

increases both the adhesion of human osteosarcoma spheroids and their ability to spread in the surrounding fibroblast culture (Indovina et al., 2008). This is a significant finding for the in vitro study of blastocyst implantation in endometrial tissue, which physiologically occurs under a low oxygen environment (PO_2= 39.6 mmHg) that characterizes the entire first trimester (Rodesch et al., 1992).

8. Targets and Applications of in vitro models of embryo-maternal interface

The process of implantation is accomplished by the proliferation, differentiation, migration, and invasion of the trophoblasts from the blastocyst, together with the regulation of the endometrial decidua. To better understand the detailed process of embryo implantation, the incorporation of the blastocyst or blastocyst surrogates with the *in vitro* 3D endometrial models are widely studied (Figure 7), and their applications in association with the endometrial models will be discussed.

A widely used *in vitro* model of trophoblast invasion involves the use of Matrigel, added on top of an endometrial stromal cell monolayer mimicking the extracellular matrix. The trophoblast cell spheroid is used to mimic the trophectoderm and it is transferred to the top of the Matrigel. The trophoblast cells are only able to invade the Matrigel when the stromal cell monolayer is present underneath. Matrigel alone does not provide the necessary signals to promote trophoblast migration and invasion. Studies by You *et al*. also claim that trophoblast cells fail to invade the Matrigel when endometrial epithelial cells are present beneath the Matrigel (You et al., 2019).

Nevertheless, the interaction between the trophoectoderm and the epithelium is a critical step in the implantation process and failure to interact would lead to infertility. Therefore, it is useful to establish models as close to reality as possible. The use of endometrial epithelial cell lines together with endometrial stromal cells and blastocyst-like spheroids can help us in the study of the implantation process.

Here, we propose a schematic embryo implantation model to study endometrium-embryo interaction composed of the three main different cell types of the endometrium: stromal cells and epithelial organoids above the endothelial cells and divided with a matrix from trophoblast spheroids and blastoids (Figure 8).

To study trophoblast invasion Transwell-co culture approaches were employed, seeding endometrial epithelial cells on top of the Matrigel-coated insert before adding trophoblast cells and stromal cells (Arnold et al., 2001; Pierro et al., 2001; Gellersen et al., 2010). However, these models lack the glandular structures necessary to maximally reproduce the physiological environment surrounding the implantation.

Foreseeable applications of organoids and 3D models involve endometrial diseases such as endometriosis, endometrial fibromatosis, adenomyosis, chronic endometritis, and birth defects which negatively affect the integrity and receptivity of the endometrium, as well as blastocyst implantation (Galliano, 2015; McQueen et al., 2015; Bouet et al., 2016; Luddi et al., 2019; Luddi, et al., 2020). Reproductive Medicine needs robust models, recurrent

implantation failure is the main limiting factor in *in vitro* fertilization success rates, despite clinical and technological advances in recent years to improve assisted reproductive technology (Bashiri et al., 2018). For a long time, the reason for this failure has been attributed to the low quality of gametes and embryos *in vitro*, so the focus of researchers has been on improving the quality of the oocytes achieved by ovarian hyperstimulation and selecting the best embryo for transfer. Nevertheless, the causes of infertility may originate from diverse factors, and embryo implantation still remains the "black box" of reproductive medicine (Quenby et al., 2007; Lucas et al., 2020).

Hence the enormous interest in studies related to embryo implantation and characterization of the role of the endometrium in this important relationship. Various animal and in vitro models have been obtained to shed light on the bidirectional crosstalk between the endometrium and the embryo, which is necessary up to the time of implantation. The entire phenomenon is ascribed to paracrine signaling by extracellular vesicles and molecular pathways presented by both the embryo and the endometrium, with two main objectives, to find an optimal implantation site (Saravelos et al., 2016), and to reprogram the immune system in a manner that induces immunological tolerance.

Since several endometrial pathologies exist, the use of human organoids may provide insight to advance human therapies while not being subject to ethical constraints.

These advances in endometrial organoid technology are providing models for prenatal development, tissue maintenance and pathologies, which are otherwise intractable processes to study in humans. Below is figure of the biomedical applications of organoids, including their use for disease modeling, drug screening, toxicology studies and regenerative medicine.

CONCLUSIONS

Much effort has been made to develop *in vitro* models for implantation studies, since animal models do not resemble human physiology and for ethical concerns. As for the trophoblast model, for ethical reasons, human studies can only be performed on human placenta tissue or cells obtained after birth or elective termination of pregnancy. Although they do not fully reflect the *in vivo* situation, *in vitro* models can give us insight into the effect of chemicals and the various interactions that come into play at the maternal-fetal interface. Therefore, *in vitro* models are an important tool for studying factors that may interfere with physiological processes and pregnancy outcome.

In vitro 2D to 3D models of endometrium and placenta are good tools for understanding the molecular mechanisms behind embryo implantation and early pregnancy in humans. The new organoid concept, including the endometrial glandular organoids, endometrial assembloids, trophoblast organoids and blastoid model, are a step forward in *in vitro* 3D culture and can better mimic the physiological trophoblast-endometrium interaction for investigating the pathophysiology of implantation failure or pregnancy complications such as recurrent pregnancy loss. The outcome of the investigations on patient-derived

endometrial organoids/assembloids would enable the detection of potential biomarkers and causative factors for early diagnosis and the development of novel personalized treatment strategies. Although the integration of these models needs to be optimized, they set the basis for ideal modeling of the endometrium, which would eventually benefit fertility treatment.

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Figure legend

Figure 1: Schematic representation of Endometrial tissue layers and composition.

Figure 2: Schematic representation of the female reproductive system and uterine phases.

Figure 3: UMAP of cells with the associated cell types in samples of normal endometrium (n=3). Macro: macrophage; Lymph: lymphatic endothelial cell; Endo: endothelial cell; Peri: perivascular cell; Str: stromal cell (Lv et al., 2022)

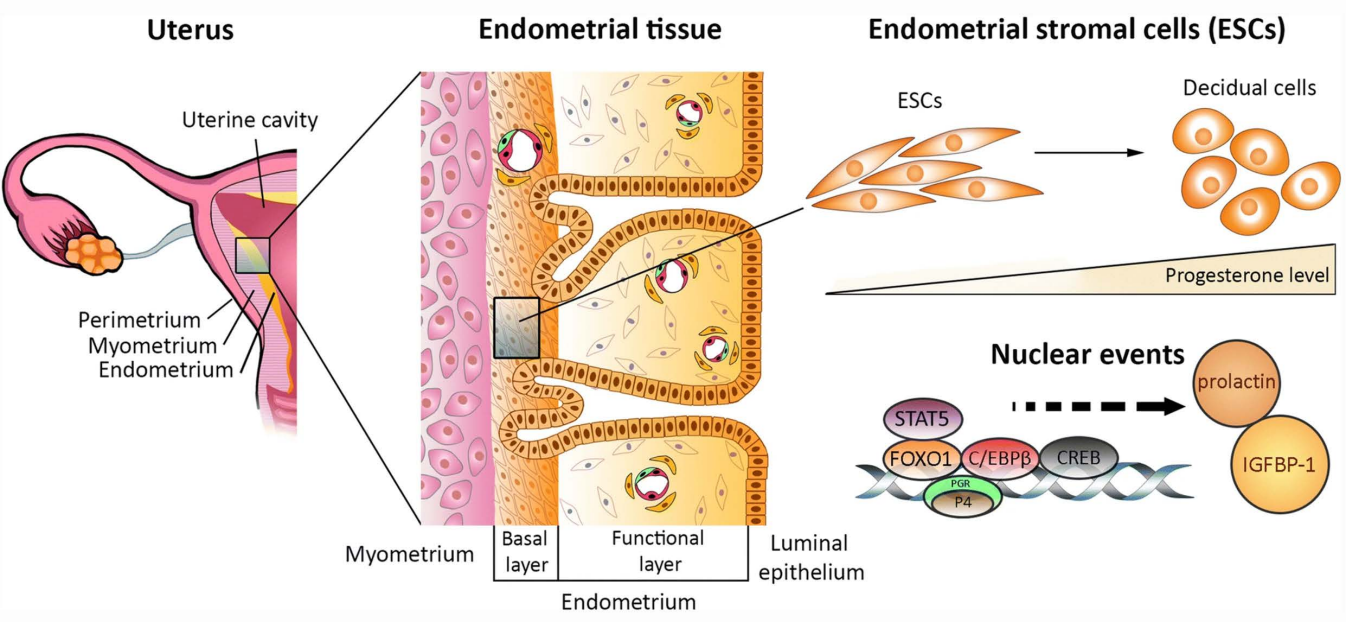
Figure 4: Schematic of the comparison between 2D and the 3D *in vitro* models.

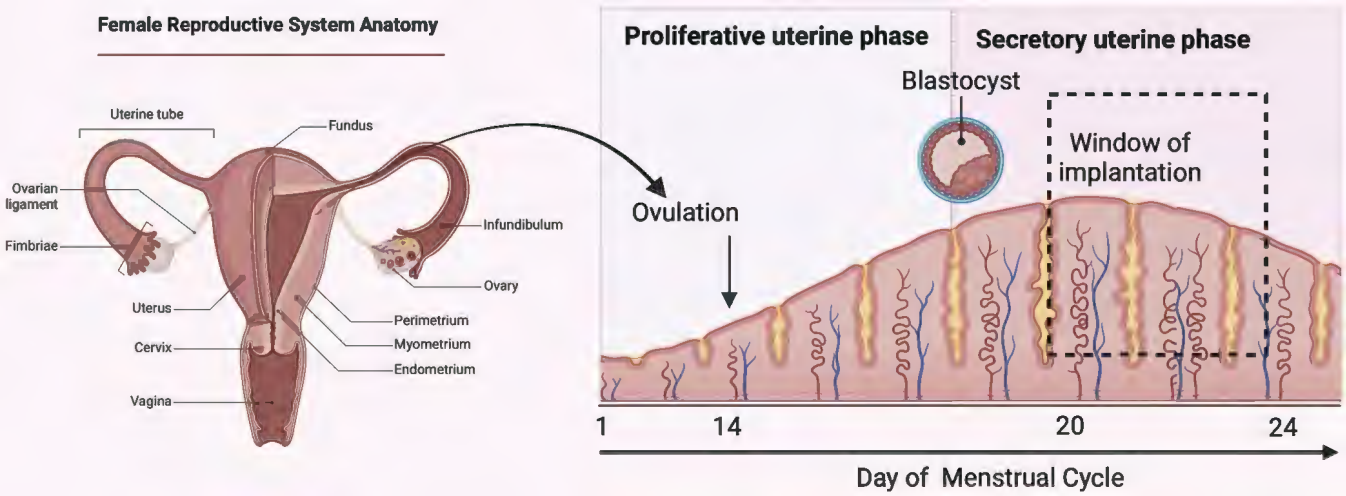
Figure 5: Endometrial Organoid derived from Menstrual Blood cells (unpublished data from our laboratory). Scale bar 25 µm.

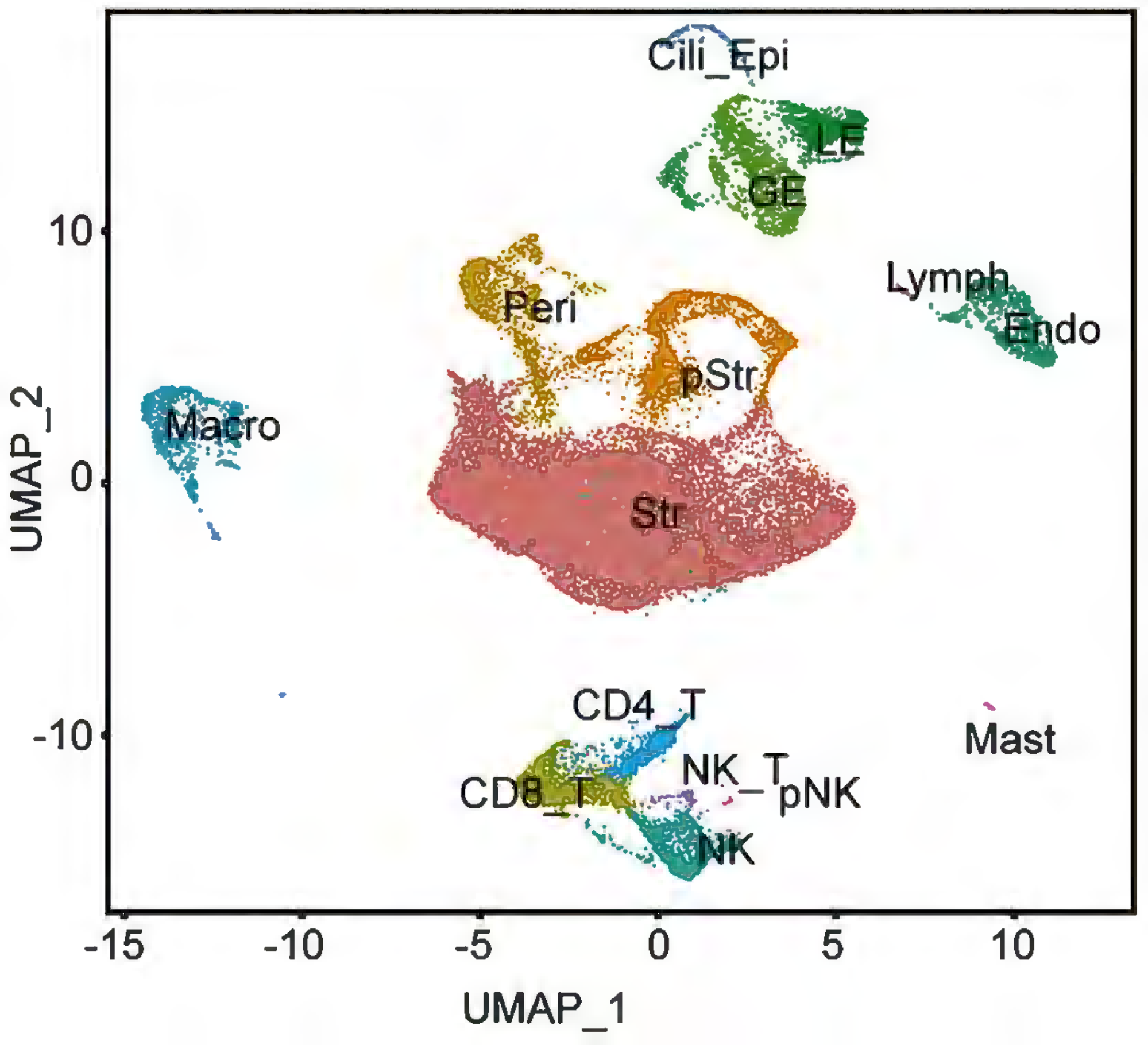
Figure 6: Trophoblast spheroid made with Bewo cells using a round-bottomed 96-well plate (unpublished data from our laboratory). Scale bar 100 µm

Figure 7: Schematic of potential application of 3D models (organoids)

Figure 8: Schematic of embryo implantation model proposed by the authors.

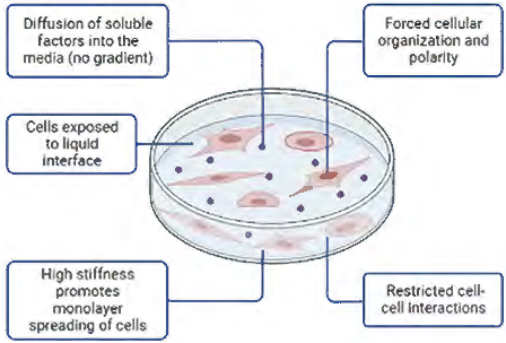

Uterus
Endometrial tissue
Endometrial stromal cells (ESCs)
Uterine cavity
Perimetrium
Myometrium
Endometrium
ESCs
Decidual cells
Progesterone level
Nuclear events
prolactin
IGFBP-1
STAT5
FOXO1
C/EBPβ
CREB
PGR
P4
Myometrium
Basal layer
Functional layer
Luminal epithelium
Endometrium


Female Reproductive System Anatomy
Uterine tube
Fundus
Ovarian ligament
Fimbriae
Infundibulum
Ovary
Uterus
Perimetrium
Cervix
Myometrium
Endometrium
Vagina
Proliferative uterine phase
Secretory uterine phase
Blastocyst
Window of implantation
Ovulation
1
14
20
24
Day of Menstrual Cycle

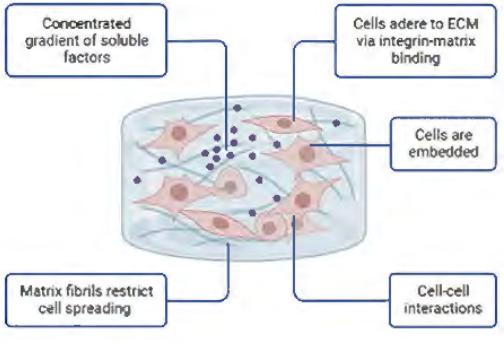

Cili_Epi
LE
GE
Peri
pStr
Lymph
Endo
Macro
Str
CD4_T
NK_T
pNK
CD8_T
NK
Mast
10
0
-10
UMAP_2
-15
-10
-5
0
5
10
UMAP_1

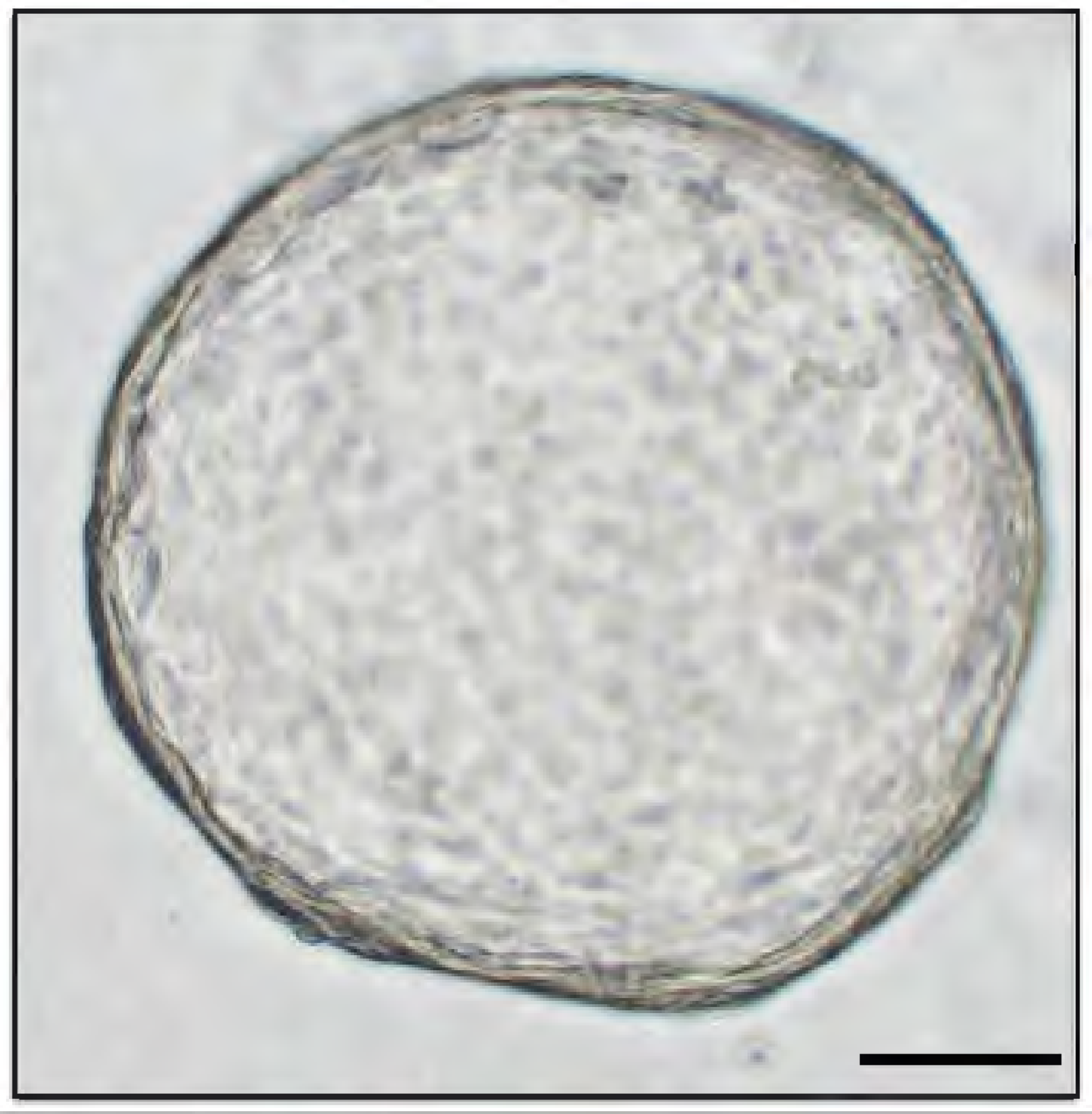
Comparison of 2D vs. 3D Cell Culture

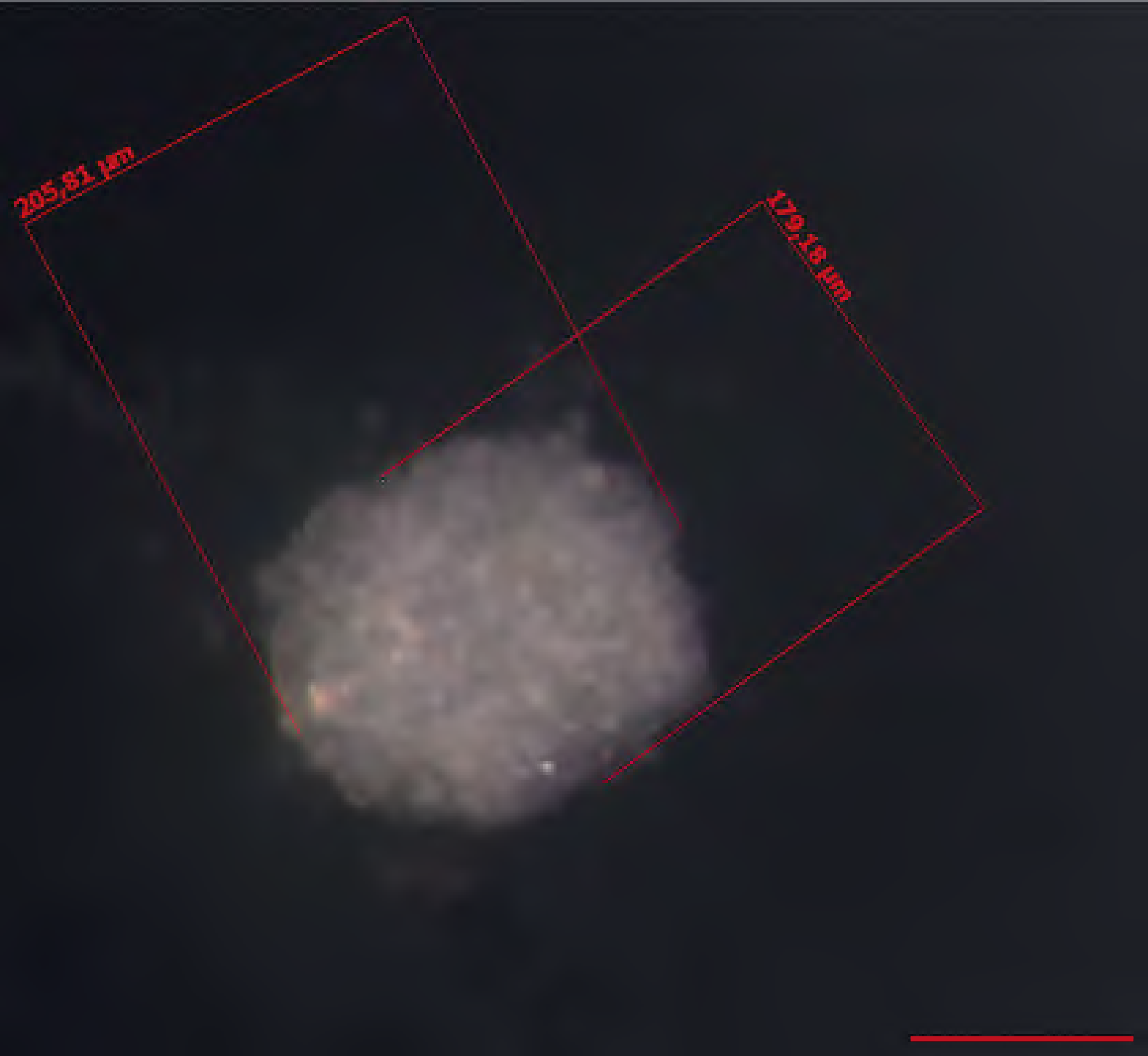
Cells growing on a 2D dish

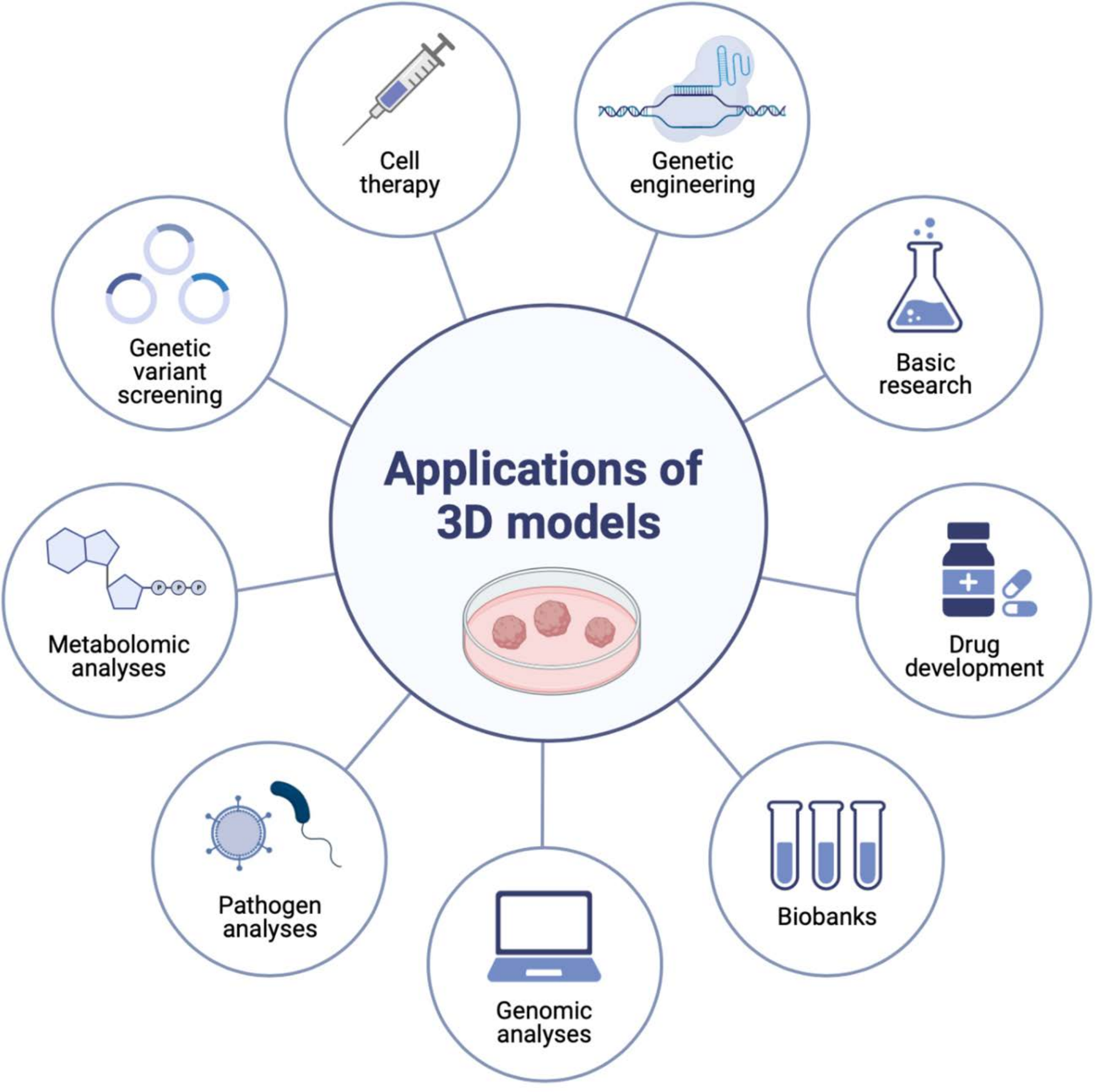


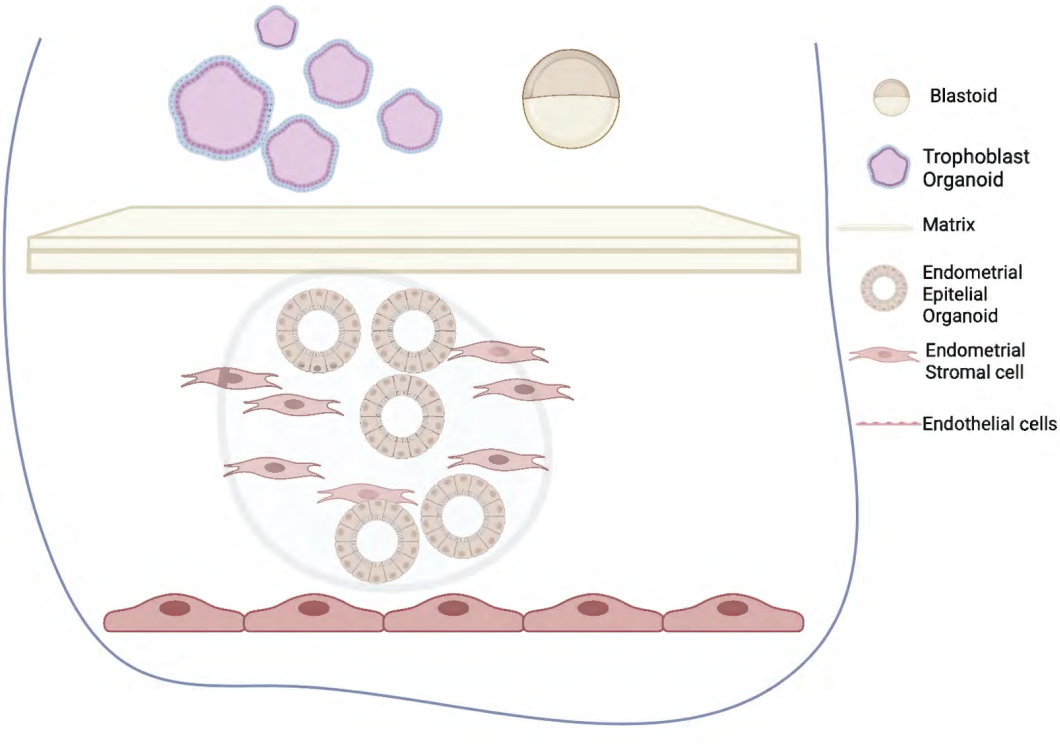
Cells growing on a 3D ECM






205,81 µm
179,18 µm


Applications of 3D models
Cell therapy
Genetic engineering
Genetic variant screening
Basic research
Metabolomic analyses
Drug development
Pathogen analyses
Biobanks
Genomic analyses


Blastoid
Trophoblast Organoid
Matrix
Endometrial Epitelial Organoid
Endometrial Stromal cell
Endothelial cells