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REVIEW ARTICLE

3D *In Vitro* Models of Early Pregnancy: How to Choose the Right Scaffolding Material?

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Abstract: Following fertilization, the blastocyst has to complete two distinct steps to assure further development of pregnancy. After apposition it establishes a firm connection with the luminal epithelium of the endometrium (attachment) and subsequently enters the decidualizing stroma (invasion). If this step is not achieved successfully, fertility problems arise. Development of the placenta ensures an adequate supply of nutrients and gas between the mother and the fetus. Preeclampsia is a prevalent disorder arising from defects in the process of placentation. It is associated with an increase of maternal morbidity and mortality. Numerous attempts have been made in order to elucidate the etiology of the syndrome and identify women at risk.

The lack of reliable animal models has turned the attention to the development of *in vitro* assays, which could provide a better insight into the individual processes that will later trigger preeclampsia symptoms. In particular, 3D *in vitro* models more closely resemble the complexity of the extracellular environment. The choice of the scaffolding material should be done carefully as cell-matrix interactions are very often as important as cell-cell interactions for the correct attachment, proliferation and differentiation of cells.

The following review is aimed to provide a general overview of the scaffolds available for the *in vitro* modeling of these complicated systems as well as to discuss the importance surrounding the choice of the scaffolding material and its influence on the results obtained.

Keywords: 3D *in vitro* models, early pregnancy, scaffolds, biomaterials.

INTRODUCTION

Pregnancy success requires multiple adaptations on the host. This includes changes in the endocrine and immune systems, as well as anatomical adaptations. The placenta plays an essential role in this process. During its limited life, the placenta works as a multitalented organ, performing vital tasks that are later taken over by lungs, liver, kidney and the endocrine system of the newborn [1]. This multifunctionality relays on the role of different cell types that control the attachment and invasion of the placenta villi, the remodeling of the vasculature, the hormonal production and gas-nutrient exchange [2].

Extravillous trophoblasts (EVTs) are the main players that attach the embryo to the uterine wall and modify the maternal vessels to provide the maternal blood flow by the end of the first trimester. EVTs migration starts at the tips of the villi and continues to the first third of the myometrium [3]. Trophoblasts also secret several angiogenic factors that are important to the vascular development in a process where leukocytes also take an active part [4]. The success in the establishment of the uteroplacental circulation depends highly on the remodeling of the spiral arteries and failure in the modification of the uterine vasculature is thought to be causative of pregnancy-related disturbances [5,6]. Preeclampsia and IUGR (Intrauterine growth restriction), for instance, are characterized by a shallow placental invasion [7]. Poor placentation, even resulting in an apparent normal pregnancy outcome, has consequences in the health of the born adult, including brain and vascular disorders [8-10]. Therefore, much interest has been put on the processes that might disturb the physiological vascular changes that need to occur during pregnancy.

The trophoblast invasion is by itself a strongly regulated process which involves hormones, cytokines, oxygen and other autocrine and paracrine factors [11]. This complex process involves an array of different cell types present at the fetal-maternal interface, such as decidual stromal cells (DSCs), mesenchymal stem cells (MSCs), endothelial cells and lymphocytes [12-15]. In addition, trophoblast-matrix interactions regulate the initial adhesion to and invasion of the endometrial stroma, for which an integrin switch is required [16]. Indeed, in conditions such as preeclampsia it has been shown that the invasive interstitial trophoblast fails to upregulate the expression of the α1β2 heterodimer responsible for their binding to collagen and laminin [17].

As the main events that guarantee a proper placentation occur during the first trimester of pregnancy and clinical manifestations appear only towards the third trimester of pregnancy, much focus has been put on investigating the first events of pregnancy establishment. In this regard, animal models have been helpful to elucidate the mechanisms that affect trophoblast invasion. However, there are considerations to be carefully evaluated when working with animal models. Several differences in the rodent placentation limit the extrapolation of the data to the human counterpart [18]:

a) human placenta anchoring is achieved by deep invasion, which does not occur in mice. As stated previously, a shallow placenta invasion is associated to pregnancy disturbances. Deep invasion, however, also occurs in other rodents (e.g. rat, guinea pig, and hamster). This deep invasion, at the same time, represents an extra immunological challenge to the host.

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b) human placenta is hemodichorial during the first trimester and hemomonochorial at term. In mice, however, the hemochorial placenta consists of three layers of trophoblasts.

c) Humans have villous placentas, while rodents develop a blood vessel labyrinth.

d) Humans and mice possess differences in terms of MHC alloantigens expression in trophoblasts.

e) HLA-G role in the fetal-maternal interface has been well characterized in humans, while no equivalent has been consensually found in mice.

f) NK cells, of preponderant presence in the feto-maternal interface, have remarkable differences concerning their activation pathways [19].

The advantages and limitations of using rodents as animal models to study placentation have been discussed frequently [20-22]. The rat represents a more suitable model to study placentation than the mouse. However, certain rat strains differ prominently in the quality of placentation. As an example, in the Brown Norway strain the uterus responsiveness to progesterone is diminished, leading to a deficient decidualization and placental development with shallow trophoblast invasion. On the other hand, rats possess multiple implantation sites, which represent an advantage compared to humans at the time of ensuring some offspring upon environmental challenge, by limiting the number of implanting embryos [21].

Taking into account the above mentioned differences, several *in vitro* models have been proposed to facilitate the study of the first events of human implantation. Indeed, human placentation research has relied on the use of 2D and 3D *in vitro* models in an attempt to further improve our understanding concerning the biological processes that direct the implantation of specialized placental stem cells during health and disease. Cell growth over 3D matrices provides several advantages over 2D ones, such as the possibility to obtain higher cell counts and the ability to evaluate cell interactions in a three-dimensional environment, an environment which more closely resembles the actual cell niche. Given the complexity of the human endometrium, commercially available 3D cell culture systems very often need to be customized for each specific application.

2D *in vitro* models often overlook the role of the scaffolding material and will not be contemplated in this review. Simpler in nature, the data obtained from these experiments cannot be underestimated and might serve as the building blocks for the design of more complex 3D systems. In this sense, a well-structured description of 2D models (as well as 3D models) that encompasses the study of different stages of pregnancy was reviewed by Weimar *et al*. [23].

Traditionally, 3D invasion studies are performed with basement membrane preparations as the scaffold to be invaded. Very often cells react to the composition and strength of the matrix they are faced with and react upon. The complexity of the human endometrium is such that more information is needed regarding the role of the matrix composition in trophoblast invasion and differentiation. The present review is aimed to provide a better understanding of this role in 3D experiments, taking into account the selected scaffold composition and overall design of the experiment.

1. *IN VIVO* COMPONENTS

1.1. A Glimpse into the Physiology of Implantation

Following fertilization, the blastocyst must successfully complete two distinct milestones to assure the beginning of pregnancy (see Fig. **1**). After apposition, it must be able to establish a firm connection with the luminal epithelium of the endometrium (attachment) and subsequently enter the decidualizing stroma (invasion). These steps are highly dependent on the quality of the blastocyst, on the receptive capacity of the endometrium and on the endocrinal dialogue between both. If this step is not achieved successfully, the consequences impact on fertility. Placentation continues

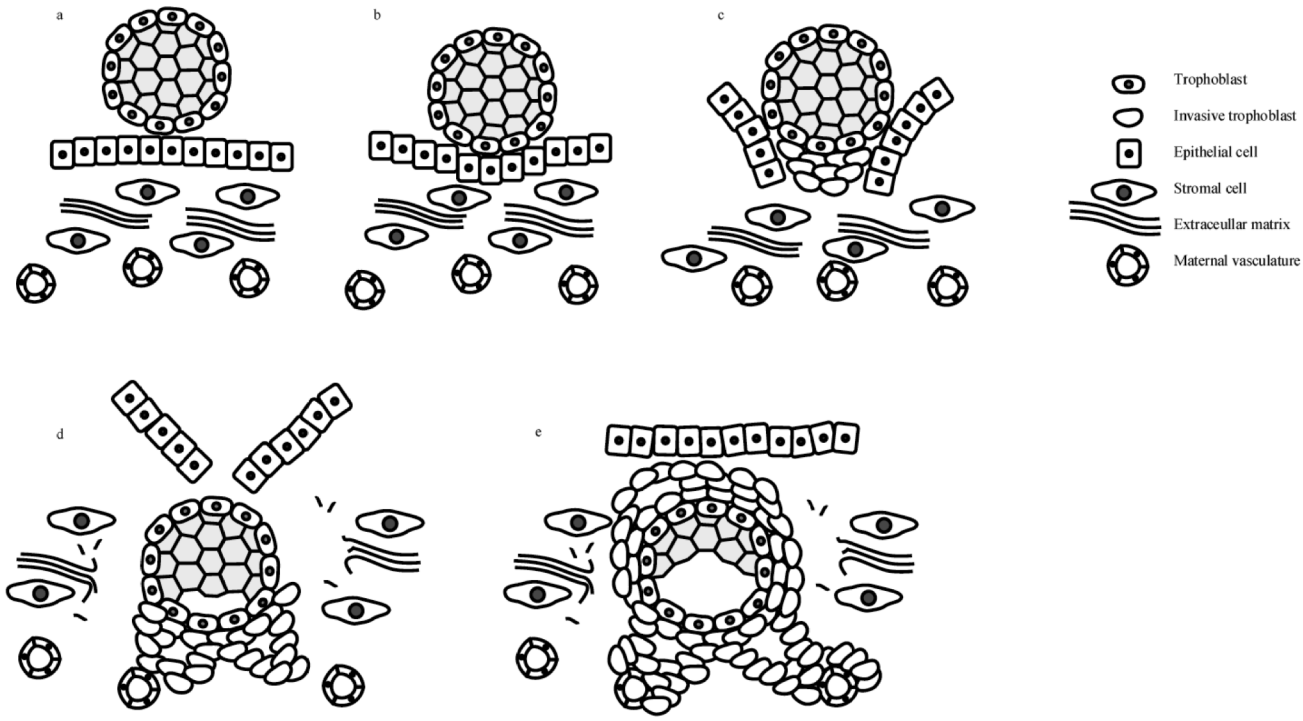

Fig. (1). Schematic representation of the implantation steps of a human blastocyst. (a) The fertilized blastocyt interacts with the endometrial epithelia to establish a weak contact (apposition) and afterwards (b) a firm contact (attachment). Thereafter (c), the blastocyst enters the endometrial wall as the invasive trophoblast modifies the stroma (d). A deep invasion and maternal arteries modifications take place to enable the maternal blood flow into the foetal circulation (e).

with the invasion of the trophoblast into the decidua and the modification of the maternal blood vessels to secure the exchange of gas and nutrients between the mother and the fetus [24,25]. Although this step occurs during the first trimester of pregnancy, its malfunction may have consequences with symptoms appearing towards the end of pregnancy or even during the adult life of the offspring [26-29].

It is indubitable that *in vitro* models to study the establishment of pregnancy should ideally mimic as close as possible the cellular milieu that is being assessed, which ultimately depends on the question to be evaluated. Therefore, *in vitro* models of placentation vary according to the interest of the investigator and may require the inclusion of epithelial cells, decidual stromal cells, mesenchymal stem cells and lymphocytes including Dendritic Cells, NK cells, T cells and Innate Lymphoid Cells. In addition to the cellular components involved, it may be required to contemplate the cell-extracellular matrix interactions [30]. If the interest is put on the attachment and later invasion of the blastocyst, an epithelial layer of cells over a stromal cell scaffold is a well-accepted approach [31, 32]. Here, the blastocyst may be represented by spheroids of trophoblasts [23,33-36].

The study of deep trophoblast invasion and subsequent modification of blood vessels, requires the presence of the vascular counterparts, which can be represented by the direct use of spiral arteries obtained from biopsies [37] or CD133+ endothelial precursor cells and human umbilical vein endothelial cells (HUVEC) [38].

1.2. The Three-Dimensional Approach

Cells react not only to surface chemistry but also to surface topography [39], thus selecting a scaffold requires careful evaluation. Ideally the material should be able to provide cell support and enable cell proliferation and cell function, mimicking in this way the natural environment of the cells also known as the extracellular matrix (ECM). The extracellular matrix not only provides support but also clues that regulate cell growth and differentiation. Such an active role is certainly difficult to emulate and the fact that its composition rarely remains static is a factor that should be taken into consideration. Very often collagen and fibrin preparations are chosen for the design of *in vitro* models. However, even though the derived scaffolds result biocompatible, results may be influenced by reactions arousing from cell-matrix interactions.

Indeed, the *in vivo* actual distribution of the biomaterial chosen for the *in vitro* model might play an important role if we consider the differences in regional distribution of the different types of collagen within the endometrium. In the case of collagen, Iwahashi and collaborators carried on research to determine alterations in the distribution of the extracellular matrix during decidualization of the human endometrium. They demonstrated that interstitial collagens, such as type I, III and VI, were diffusely present throughout the menstrual cycle in the endometrium as well as in the decidua. On the other hand, type IV collagen and laminin appeared exclusively in the basement membrane of the endometrial glands and blood vessels during the proliferative and secretory phases. Additionally, type IV collagen was recognized in the pericellular region surrounding the stromal cells of the decidua [40]. Another study focusing on the ultrastructure of collagen during the first trimester in the decidua basalis and parietalis found substantial differences between the thickness and organization of the fibrils within the decidua. In the basalis, trophoblastic and decidual cells were surrounded by thick disrupted collagen fibrils, contrasting the ordered arrangement of thin fibrils seen in parietalis. The widely distributed disruption of fibrils observed was attributed to metalloproteinases and proteases secreted by the invasive trophoblasts, capable of degrading the rich collagen network [41].

Lastly, fibrin, which is not as widely distributed as collagen, is normally present as a patchy firm yellow plaque or Langhans layer found beneath the chorionic plate of the placenta, normally attributed to maternal blood stasis [42], at the Rohr layer, at the level of the basal plate, and deeper into the decidua basalis, where the fibrinoid deposits form the Nitabuch layer.

The choice among fibrin and the various types of collagen available in the market might condition the cell response and influence the results obtained, as their *in vivo* distribution might be physiologically relevant. In the following sections, an overview of the different materials is provided along with the *in vitro* models in which they have been employed.

2. *IN VITRO* COMPONENTS

2.1. Scaffolding Materials and Cell Constructs: The Known and the Unknown

2.1.1. Fibrin

The fibrin monomer is originated by activation of fibrinogen, one of the principal components of the coagulation cascade, by the enzyme thrombin in the presence of calcium. Soluble fibrin has a tendency to associate into an insoluble form, which is further crosslinked by the action of a transglutaminase. Both fibrin and fibrinogen play important roles in blood clotting, cell-matrix interactions, the inflammatory response and wound healing, among others. For research and clinical purposes fibrin networks are assembled from commercially purified allogeneic fibrinogen and purified thrombin [43,44].

When used as cell scaffolds, fibrin provides adhesion directly via integrin receptors on tripeptide amino acid sequences or indirectly via its capacity to bind proteins such as fibronectin and vitronectin. Additionally, the ability of fibrin to modulate the cell response derives from the variable number of bioactive molecules it is able to bind such as growth factors, enzymes and proenzymes [45].

The interaction of trophoblastic cells with fibrin has been studied *in vivo* as well as *in vitro* by Nelson *et al*. *In vivo*, they used morphometric, immunocytochemical and electron microscopy techniques to study fibrin deposits associated with villi of 14 normal term placentas. Placental villi stained for cytokeratin intermediate filaments showed fibrin was frequently attached to the villous core at sites were the syncytial epithelium appeared disrupted. Additionally, trophoblastic cells were frequently associated with sites of fibrin attachment to the villous core. The results suggested that damage to the syncytial trophoblasts caused exposure of the trophoblastic basement membrane, triggering the activation of the coagulation cascade with thrombolytic cleavage of fibrinogen and fibrin deposition. *In vitro*, cells isolated from term villi were seeded on top of fibrin gels at a density of 10^6 cells per 35 mm petri dish and grown in a humidified 5% CO_2 atmosphere at 37°C. After 72 hours, cell aggregates formed which reproduced the typical histologic pattern given by the trophoblastic layer on term villi, consisting of a syncytial layer overlying a discontinuous layer of mononucleated cellular trophoblasts. Based on their results, the authors suggested fibrin deposits appear at sites of villous repair and that cell-fibrin interactions might modulate as well trophoblastic differentiation [46].

In parallel, it was hypothesized that placenta growth and basal plate formation (Nitabuch´s deposits) originate fibrin deposition in decidual veins as trophoblast cells invade. To prove this, tissue was obtained from elective abortions at 5 to 12 weeks of gestation and placental bed tissue from a term pregnancy was used as a control. Results showed that deposition of fibrinoid material into the walls of decidual veins occurs at sites of trophoblast invasion. Additionally, intravenous clots and fibrin material suggest that trophoblasts may be able to overcome local anticoagulant mechanisms and might later incorporate the fibrin into the basal plate matrix to expand the basal layer [47].

Because of the rich environment it provides, the use of fibrin as a scaffold for tissue regeneration has been extensively reviewed [43,48-50]. Normally derived from pooled human plasma it promotes efficiently cell adhesion and proliferation. On the other hand, the use of fibrin as a scaffold for *in vitro* studies might pose a risk as fibrin preparations have an undefined and variable composition of contaminant plasma proteins which might affect the obtained results.

2.1.2. Reconstituted Basement Membrane Preparations

Solubilized basement membrane preparations comprise a mixture of laminin, type IV collagen, heparan sulfate, entactin and nidogen, as well as growth factors such as TGF-beta, epidermal growth factor, insulin-like growth factor, fibroblast growth factor and tissue plasminogen activator, all of which occur naturally in the Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, from where they are obtained.

Commercially known as Matrigel, such preparations have found numerous 2D and 3D cell culture applications, from maintenance of cell lines to *in vivo* tissue regeneration. Because of the rich environment offered by basement membrane preparations, cells are expected to improve their attachment and behavior when compared to preparations containing collagen alone [51]. However, a recent study aimed to compare matrices reconstituted from collagen type I and Matrigel as representative barriers for ovarian cancer cell invasion showed that, given the matrix metalloproteinase-dependent mechanism of invasion of the cells, polymerized collagen type I provides a good model to mimic the function of stromal matrices. On the other hand, Matrigel, which is frequently used *in vitro* for invasion studies, fails to reflect the barrier function of basement membranes, as cancer cells are able to penetrate the matrix in an MMP-independent way, which does not occur *in vivo*. The differences observed were attributed to the complex nature of Matrigel preparations, which differ in the concentrations of each of the components in it and to the strength of the network between collagen type IV and laminin, usually lower than the one found *in vivo* [52]. Whether these results can be extrapolated to the behavior of trophoblasts in collagen type I versus basement membrane preparations remains unclear, though the tensile strength often related to the concentration in protein content should be taken into account and ideally be the same in order to obtain comparable results when evaluating the ability of cells to degrade the proposed scaffold.

Alternatively, Lei and collaborators studied the influence of the extracellular matrix on the differentiation and invasiveness of trophoblast stem cells (TS). Cells were cultured on Matrigel and on plastic with basal media and with fibroblast conditioned media to suppress TS differentiation (TS-M and TS+M respectively). Interestingly, Matrigel appears to promote TS differentiation, even in culture conditions (TS+M) that favor proliferation when grown on plastic. The expression of MMP-9 and its tissue inhibitor TIMP-3 was also increased for the cells cultured in Matrigel, even when exposed to preconditioned media (TS+M) in agreement with the highly invasive characteristics shown by TS cells on this matrix. Because of Matrigel´s rich composition, the presence of laminin and several growth factors may affect the fate of trophoblastic cells and might be a good model to study the regulation of trophoblast-ECM interactions, their differentiation and invasion *in vitro* [53]. On the other hand, the effect of the preconditioned media on cells cultured over Matrigel scaffolds appears to be masked by the presence of the rich environment, thus suggesting that eventually it would be more difficult to study the effect of a treatment on cells cultured over complex systems. As an example of this, the culture of explants of human villous tissue on Matrigel may not be ideal for the study of the mechanisms of oxygen regulation of trophoblast differentiation. Newby and colleagues, who performed cultures under standard oxygen conditions (18% O_2) and low concentrations (2% O_2), failed to evidence oxygen-concentration related changes on the migration capacity of the explants when using Matrigel as a scaffold [54].

On the other hand, Matrigel has been shown to favor as well the attachment and differentiation of extravillous cytotrophoblasts (EVCTs) extracted from the chorionic villi of first trimester placentas [55]. These cells require a matrix to invade and it has been previously demonstrated that contact with an extracellular matrix preparation, either collagen type I or Matrigel, stimulates the production of type IV and interstitial collagenolytic activities, necessaries for their invasive phenotype [56,57].

While the use of basement membrane preparations might make difficult to observe differences between treatments, it is still the first choice for the evaluation of trophoblast invasion. Type I collagen, which will be discussed below, still lags behind and is less frequently employed in invasion assays.

2.1.3. Type I Collagen

Type I collagen is typically extracted from tendons and skin from animal sources and is present in the extracellular matrix from different tissues in variable amounts. In the particular case of tendon, it constitutes 65-80% of its dry mass, and after acid solubilization and subsequent purification the purity of the extract can reach 97%. For the obtention of scaffolds acid soluble collagen is neutralized inducing the *in vitro* assembly of collagen fibrils, the mechanical resistance of which can be easily adjusted by varying the concentration of collagen in the resulting gel [58].

Less is known about the interaction between collagen type I and trophoblastic cells, as most works concentrate on the interaction or effect of basement membrane preparations on trophoblastic cells. On the other hand, there is an intense focus on the study of the expression of the different MMPs that are expressed during normal pregnancies and their disorders. In particular, MMP-1 is mainly responsible for the degradation of fibrillar collagen and is normally expressed by a variety of cells at the maternal-fetal interface, such as decidual cells, Syncytiotrophoblast, cytotrophoblasts, endothelial and extravillous trophoblasts (EVTs). In a work by Vettraino and collaborators, Collagenase-1 or matrix metalloproteinase type I was detected in cells of the amnion, decidua, and chorionic villi at all stages of pregnancy [59].

Indeed, recently, a study showed reduced expression of matrix metalloproteinase type I in cases of preeclampsia and fetal growth restriction. Lian *et al.*, performed gene expression microarray analysis on decidua basalis tissue from eclamptic and fetal growth restriction complicated pregnancies as well as from normal pregnancies. They found that several genes involved in trophoblast invasion and differentiation were downregulated, among them the collagenase MMP-1 and the stromelysins MMP-7 and MMP-12. MMP-1 presented the highest fold change among the different genes under study and was selected for further investigation. Subsequently, they confirmed reduced MMP-1 mRNA in decidual tissue and diminished protein expression in EVTs when compared to controls. Based on their findings, MMP-1 might be involved in the pathologic trophoblast invasion observed in both fetal growth restriction and preeclampsia [60].

Another aspect of type I collagen matrices is collagen density and fiber organization. As stated above, the ultrastructure of collagen fibers in decidua basalis and parietalis shows remarkable differences. Whether the fibril thickness and organization could play a role in matrix metalloproteinases pattern of expression was analyzed by Fraley and collaborators [61]. They performed an integrated, quantitative study on the effects of pore size, crosslink density, structural organization, and stiffness of self-assembled collagen matrices on HT-1080 human fibrosarcoma cell motility. Collagen density was found to affect cell motility in a biphasic fashion. Briefly, at low collagen concentrations (1 $mg.mL^{-1}$) cells moved rapidly and persistently with a sustained high rate of protrusions orientating along the axis of movement of the cell. Interestingly, at

intermediate concentrations of 2-2.5 mg.mL^{-1} cells migrated more slowly whereas at higher concentrations of 4-6 mg.mL^{-1} cells speed increased again. Collagen extraction and gelation conditions are known to affect collagen density, porosity, fiber organization and overall stiffness of the gel. Hence they tried to explain the differences in migration by analyzing the resulting density, porosity and mechanical strength of the gels at the conditions assayed. They found that increasing collagen density resulted in a decrease followed by an increase in both pore size and fiber alignment, which correlated significantly with cell motility. Additionally, by means of transglutaminase II crosslinking, they altered the microstructure independently of density and found that cell motility is mostly predicted by fiber alignment. Regarding the expression of MT1-MMP, they found that expression of this metalloproteinase provided no additional benefits to cells on low density collagen gels (1 mg.mL^{-1}) but did indeed benefit cells on high densities collagen matrices, in agreement to what is found in literature [62-64].

As it can be seen, there are a high number of variables affecting cell response all related to the final characteristics of the collagen matrix employed. While low collagen concentrations give rise to higher cell motilities, higher collagen densities might represent more closely the cellular milieu, though tuning the mechanical properties of the gel to match those conditions observed *in vivo* might represent a major challenge in the design of the experiment. In this sense, even though when compared to basement membrane preparations and fibrin scaffolds, type I collagen appears as a less rich matrix with easily tunable mechanical properties, more studies are needed to determine the ideal conditions for each specific application.

2.1.4. Non-Physiological Materials as Extracellular Matrix Substitutes

For the study of the implantation process, scaffolds are constructed taking into account the before mentioned physiological materials, which are commonly found in the extracellular matrix. For this purpose 3D constructs are assembled varying the concentration, composition and spatial distribution of either fibrin or the different types of collagens and basement membrane preparations. The available designs including their advantages and limitations will be discussed in the following section.

In the field of biomaterials, non-physiological scaffolds from natural sources, such as alginate [65, 66], synthetic scaffolds, such as polyethylene terephthalate (PET) or natural-synthetic hybrids, such as gelatin methacrylate [67], are under constant revision. Though many remain unexplored in the field of reproductive medicine, neutral materials such as chitosan and alginate, which are not normal constituents of the extracellular matrix, have been shown to be useful for the long-term culturing of mouse embryonic stem cells [68] and human blastocysts [69]. Even PET non-woven fibrous matrices have been used successfully to study the effect on the long term development of ED27 trophoblastic cells [70].

As previously stated, non-physiological materials have not been exploited as scaffolds for the study of the process of placentation, though they could provide a more neutral and controllable environment, along with a stable composition and the potential to manipulate the tensile strength and porosity of the material.

2.2. Spheroids: A Living Scaffold

The implanting blastocyst can be modeled through the use of trophoblastic cells spheroids of 50 to 200 μm in diameter. Trophoblasts from explants can be used in combination with methylcellulose to generate these spheroids [38,71]. However, explants still deal with difficulties in terms of availability and reproducibility between explants. Furthermore, while trophoblast cells can be easily obtained from primary tissue, their *in vitro* proliferation is very limited (about a week) and the cell numbers achieved are often not enough for meaningful studies. To overcome these difficulties, different trophoblastic cell lines are offered [31,35,72-74], which are often derived from choriocarcinomas, like BeWo, JEG-3 and Jar, and from trophoblasts explants, like SGHPL-4, SW.71 and HTR8/SVneo. The choice of each cell line must be done considering their biological properties, as reviewed by Hannan *et al*. [75].

In particular, the use of the BeWo line is very extended. BeWo displays most of the characteristics of extra villous trophoblasts and enables studies of syncytialization, while other cell lines do not. As a drawback, BeWo is a long existence cell line presenting different strains which vary significantly in their fusion rates. In addition, studies evaluating their interaction with the extra cellular matrix should be taken into consideration, as they have shown to impact on BeWo functionality in terms of hCG secretion [76]. The other cell lines are all suitable for migration and invasion assays, while HTR8/SVneo is suitable for adhesion assays as well. When evaluating invasion of HTR8/SVneo cells on Matrigel, it should be taken into consideration that Matrigel induces on HTR8/SVneo an endothelial phenotype, including increased VE-cadherin expression and the formation of endothelial-like tubes. On the other hand, invasiveness markers, like MMP2 and ITGA1, are reduced as a consequence of the culture con Matrigel [77]. Nevertheless, in general terms, Jar cells spheroids represent an interesting tool to study the first steps of blastocyst implantation (see Table **1**).

The use of cell lines also permits the study of genetic determinants of preeclampsia, as in the case of STOX1 in JEG-3 and SGHPL cell lines. However, most of the candidate genes that after Genome-wide association studies (GWAS) are considered to confer susceptibility to preeclampsia, are implied in the coagulation system or take part of the renin-angiotensin system [78], which exceed the technical limitations of the methods mentioned in this review.

To induce the formation of spheroids, the available approaches vary in time and complexity. Spontaneous spheroid formation can be obtained utilizing the first trimester trophoblast cell line Sw.71 on low attachment 6-well plates [35]. The use of additional material and equipment to induce spheroid formation is very common as well. Korff and colleagues utilized methylcellulose and cytotrophoblast cell suspensions in 96-well plates to obtain a single spheroid per well. A similar approach including endothelial precursor cells and villous stromal cells can be used to study a more integrated structure closer to morphologically placental villi [38]. In addition, Wang and collaborators formed spheroids using Jar cells over agarose-coated petri dishes [31]. Finally, the utilization of equipment varies from shakers [73,79-85] to NASA-engineered bioreactors [72].

The composition and method used for the obtention of spheroids will later impact on several of their properties, including their size, structure and biological behavior. This may difficult the extrapolation of the data among experiments involving different methods. A very simple approach that adjusts the size of the spheroid to the implanting human blastocyst and at the same time improves reproducibility was described by Ho *et al*., [73]. In their work, they use a two-step filtration through sieves of 100 and 70 μm, a size that resembles the implanting blastocyst. The method described by Wang *et al*., generates spheroids between 150 and 200 μm [31]. Using Sw.71 cells, Holmberg and colleagues designed an approach to generate spheroids that resembled human blastocysts in terms of morphology and interactions with stromal and epithelial cells [35]. Very interestingly, they also observed a polarized migration of trophoblast cells toward stromal cells in culture, including a cavity formation on the opposite pole, similar to the lacunae formation after blastocyst implantation.

The use of endometrial spheroids is much less extended. Buck and colleagues used endometrial epithelial cells spheroids to form gland-like polarized structures to evaluate the impact of endothelial cell polarization on EVT invasiveness. Compared to the culture over monolayers of endothelial cells, the glandular nature of this 3D

Table 1. Commonly used strategies for blastocyst spheroid formation.

Cells	Complemetary material/equipment	Application	Reference
Jar	Shaker	Attachment	[79,82-85]
BeWo, Jeg-3, Jar	Shaker	Attachment	[80]
BeWo	Shaker	Attachment	[73]
Jar	Agarose-coated petri dishes	Attachment over 3D epithelial + stromal cells layers	[31]
Sw.71	Low-attachment 6-well plates	Attachment, invasion	[35]
Trophoblast explants	Methylcellulose	Invasion, vascular development	[38,71]
SGHPL-4	Microcarrier beads, NASA-engineered bioreactor	Differentiation, invasion	[72]

model offers a more accurate situation to what occurs at a later stage of implantation [86]. Helige and collaborators utilized small mid-secretory endometrial fragments in glass spinner flasks. After 3 to 6 days of stirring, 800 μm spheroidal structures were obtained. These included proliferating $CD56^+$ NK cells. The authors set these spheroids in contact with JEG-3 or BeWo choriocarcinoma cell spheroids to study trophoblast invasion into endometrial spheroids. To enable the confrontation and guarantee a proper nutrient supply, they used small silicon funnels. Between 2 and 6 days of culture, different stages of invasion of the choriocarcinoma cell lines could be observed. A remarkable advantage of this method is the possibility to observe endometrial lymphocytes in their natural environment [87].

2.3. Current Designs: Advantages and Limitations

A general overview of some of the 3D designs available for the study of early pregnancy events is depicted in Fig. **2**. Different approaches have been made towards the design of *in vitro* pregnancy models. The effect of the scaffold on the overall design is often underseen, and will be discussed below, followed by the introduction of some of the 2D models available.

2.3.1. Effect of the Scaffolding Material on Cell Differentiation and Invasion

The study of the influence on cell differentiation of different scaffolding materials in comparison to simple tissue culture plates is the first step in the design of 3D models of health and disease. Studies evaluating the effect of the extracellular matrix on trophoblast differentiation have been undertaken in the past involving primary culture of villous explants [88], TS cell lines [53] and BeWo choriocarcinoma spheroids [89].

Genbacev and collaborators started with the culture of villous tissue from 17 human placentae aged 5-7, 8-10, 15 and 20 weeks of gestation on different substrates. To determine the best cell culture conditions to support EVT differentiation and migration they placed the villous tissue on top of either type IV collagen or laminin precoated dishes or embedded them in different scaffolds containing either Matrigel or type I rat tail collagen alone or supplemented with either laminin, laminin and type IV collagen or 10% decidual extract. From their results, they concluded that very complex matrices like Matrigel and type I collagen supplemented with 10% decidual extract were among the best scaffolds to support extravillous trophoblast differentiation and migration. They added that it was probably the presence of growth factors in both conditions what rendered them suitable [88]. Similar results were obtained with the TS cell line by Lei and collaborators, as described before in the basement membrane preparations section [53]. In their work, TS cells were placed on top of Matrigel and compared with cells growing in tissue culture plates. The TS cell line was found to form colonies on top of Matrigel instead of the monolayer observed in tissue culture plates. Additionally, cells grown on the ECM substitute showed an invasive phenotype.

It is long known that many cell types form multilayered aggregates in matrix gels in contrast to rigid matrix substrates. However, whether the effect of the scaffold on cell differentiation was caused by increased cell to cell contact or cell to matrix contact remained unknown. Consequently, Hohn and collaborators evaluated the expression of differentiation markers by choriocarcinoma cells (BeWo) spheroids cultured in suspension and on Matrigel and poly-HEMA coated plastic, a treatment that decreases overall cell adhesion to the material. Either cells, those cultured on poly-HEMA coated plastic and Matrigel, formed spheroid-like aggregates over both substrates in contrast to BeWo cells monolayers observed in cell adhesive tissue culture plates. Chorionic gonadotropin as a differentiation marker was stimulated significantly in aggregates bound to a substrate in contrast to spheroids in suspension. However, the expression of additional differentiation markers (placental lactogen, placenta-type alkaline phosphatase and pregnancy specific glycoprotein β1), matrix components (collagen type I, type IV, fibronectin and laminin) and different integrin subunits were randomly distributed showing no preferential expression for attached cells or cells in suspension. Overall, results showed that cell-cell communication plays a subordinate role in the cytodifferentiation of cell aggregates when placed on matrix gels, so that substrate anchorage and physical properties of the substrate may be the decisive factors [89]. However, they found that, even though anchorage to a substrate seems to be essential for differentiation, in multilayered aggregates not every cell is in direct contact with the matrix and differentiation markers are expressed indistinctively, suggesting that cell shape and a balanced cellular tensegrity might be the principal regulators of cell differentiation in BeWo cells.

As direct contact to the substrate does not seem to promote the differentiation of BeWo spheroids on top of acellular matrix gels, experiments aiming to study the sole effect of a treatment on cell spheroids might be done in suspension. However, *in vivo* the endometrium matrix is loaded with different cells and other factors that are known for determining the blastocyst fate, so that *in vitro* models should eventually involve coculture as will be discussed below.

2.3.2. Endometrial and Blastocyst Models

During the mid-luteal phase decidualization occurs and with it the transformation of endometrial stromal fibroblasts into secretory ephitelioid decidual cells. Spontaneously occurring in the cycling human endometrium, the decidualization process is induced by the mother to be, regardless of the event of fertilization [90]. Once the blastocyst arrives to the uterine cavity, around 5 to 6 days after ovulation, its inner cell mass orientates towards the endometrial lining and hatching occurs. Blastocyst attachment *in vivo* is an intricate process involving the interaction of different types of cells such as trophoblasts, endometrial cells and stromal cells, immune

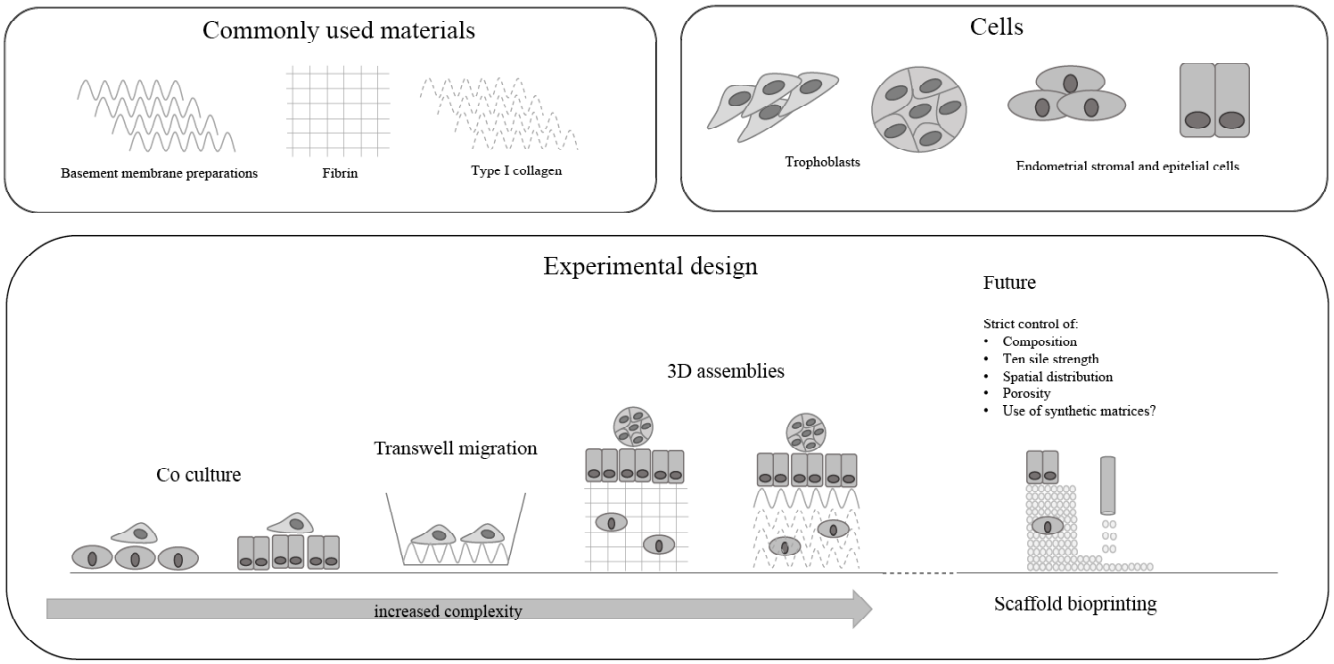

Fig. (2). Overview of some of the approaches made towards the design of 3D models of early pregnancy disorders.

cells and endothelial cells [91]. For the *in vitro* study of early human implantation and trophoblast invasion endometrial and blastocyst models provide researchers with the possibility to study cell-cell and cell-matrix interactions in a 3D environment.

As stated by Bentin-Ley *et al*., for *in vitro* purposes, an endometrial cell culture system should mimic the architecture of the normal human endometrium. For such purpose, they developed a model that mimics the secretory endometrium, comprising both primary endometrial epithelial cells growing over an artificial basement membrane (Matrigel) and stromal cells underneath, embedded in a type I collagen matrix. They observed that the epithelial compartment, derived from glandular and epithelial cells, appeared polarized and after a few days deposition of basement membrane was evidenced through transmission electron microscopy, suggesting a dynamic environment. From a morphological point of view, the model proposed by Bentin-Ley and collaborators was suitable as a 3D model for implantation, however, with time, considerable shrinking of the scaffold was observed due to matrix remodeling [92]. The shrinking observed can be avoided as demonstrated by Dolznig and collaborators [93]. Briefly, if the gel is assembled on top of a nylon mesh, the mesh prevents shrinkage of the gel, which could otherwise not only interfere with microscopic techniques but also lead to structural changes altering the results.

Alternatively, endometrial stromal cells were embedded in a fibrin matrix and epithelial endometrial cells were seeded on top of the fibrin gel, forming a tight cell monolayer on top of the scaffold. The model was assembled with either primary epithelial and stromal cells (EECs and HECs respectively) or human endometrial adenocarcinoma cell lines (HEC)-1A or Ishikawa cells on top of immortalized human endometrial stromal cells (HESCs). Through histological analysis a clear boundary was observed between the two layers and gland like-structures appeared when primary cells were employed, probably due to the presence of differentiating stem cells in the stromal fraction or contaminating epithelial cells. Additionally, Jar spheroids were generated to mimic the blastocyst structure and deposited on top of the epithelial layer of either the 3D models containing cells lines or primary cell cultures and on top of epithelial monolayers of the cells. Results for attachment confirmed differential attachment rates to the epithelial cells employed, favoring the attachment of Jar spheroids on top of Ishikawa cells. Additionally, trophoblastic spheroids showed a preference for the attachment on top of a 3D structure, indicating that the interaction between stromal and epithelial cells in the scaffold might promote trophoblast attachment [31].

2.3.3. 3D Models of Trophoblast Invasion

Trophoblast invasion in the first trimester is strictly regulated by signals arising from the same invading trophoblasts and also by endometrial stromal cells and natural killer (NK) cells, leading to the synthesis and activation of different metalloproteinases and their inhibitors at the maternal-fetal interface, which appears to be critical for the correct implantation and placentation. The role of matrix metalloproteinases regulation during implantation and placentation has been extensively reviewed [94]. Studies suggest temporal and regional expression of metalloproteinases take place during pregnancy, with MMP-2 and MMP-9 localized strongly at the placental bed in early pregnancy with the aim to regulate trophoblast invasion. [95]. As pregnancy progresses, different MMPs are activated such as MMP-9, MMP-10 and MMP-19 among others. In the case of pregnancy disorders, such as preeclampsia, matrix metalloproteinases regulation was shown to be altered [96,97]. There are numerous studies which evaluate the expression of collagenases, gelatinases and stromelysins in trophoblast cells in response to different stimuli [55,98]. Very often the invasive phenotype is characterized in a transwell migration assay by seeding the cells on top of basement membrane preparations or Matrigel. Indeed, *in vitro* cell migration and invasion assays have been thoroughly reviewed by Kramer and collaborators [99].

Other interesting options for the evaluation of invasion include the use of a NASA engineered rotating wall vessel bioreactor, which was used to grow CTB, which formed 3D aggregates with a tissue like structure. Invasiveness of 3D structures was compared to that of cells grown in monolayers after culturing them in fibrin gels. It was shown that cells cultured three-dimensionally differentiated into an aggressively invasive cell population, characterized by increased levels of metalloproteinases and urokinase-type plasminogen activator secretion and activation [72]. Based on this method, McConkey *et al*. added collagen-coated Cytodex beads to form spheroids with different trophoblast cell lines (BeWo, HTR8, Jar,

JEG-3) and human brain microvascular endothelial cells (HBMECs) under rotation in the bioreactor. Interestingly, trophoblast cell lines did not attach directly to Cytodex beads. However, when the beads were pre-coated during 3-5 days with HBMECs, the addition of JEG-3 induced the dissociation of HBMECs from the beads. Finally, after 21 days of culture, JEG-3 formed a single layer that coated the beads [100].

A more recent work includes the development of a 3D printed, bioingeneered placenta model (BPM) to evaluate the role of trophoblast migration in preeclampsia. Through the use of bioprinting and shear wave elastography the authors developed a BPM composed of gelatin methacrylate, which proved useful for the study of trophoblast migration in response to an epidermal growth factor gradient. Taking into account that the stiffness of the matrix is known to influence cell migration as discussed before, they analyzed the mechanical properties of *ex vivo* term placentas and based on these results they designed a construct that mimicked closely the biophysical properties of the target tissue. They even attempted to print spiral arteries in a contrasting color, though the utility of the design still needs to be proven [66].

As a drawback, the models mentioned before, and even simple transwell migration assays, fail to mimic entirely the process of trophoblastic invasion as decidualized cells are suspected for playing an active role in the process, with some contradicting results regarding their involvement. An extensive work by Sharma and collaborators provides an overview of the function of decidualized stromal cells and their potential involvement in trophoblast invasion [101]. The use of a scaffolding material could shed some light into the processes of cell interaction, through the coculture of stromal and trophoblast cells as well as trophoblast cells and others in a 3D environment.

2.3.4. 3D Models of Spiral Artery Remodeling and Early Placentation

Following trophoblast interstitial invasion, spiral artery remodeling involves the modification of maternal vessels and their transformation into low-flow, low-resistance arteries capable of meeting the demands of the developing fetus. The process involves the interaction between immune cells, EVT and vascular cells leading to the replacement of the endothelial cells with endovascular EVT and deposition of extracellular fibrinoid matter among them. Starting as early as 8 weeks, endovascular EVT invasion continues until the inner third or the myometrium is reached around the half of the second trimester [102].

In order to study the process of spiral artery invasion, Cartwright *et al.* developed a model using spiral artery explants obtained from uterine biopsies at caesarean sections. To study interstitial invasion, fluorescently labeled trophoblasts were seeded on top of fibrin embedded spiral arteries and for the study of endovascular invasion the isolated artery was perfused with the trophoblasts and transferred to a fibrin gel with cell culture media on top [103]. Their *in vitro* model provides an insight on how the trophoblasts interact with spiral arteries of third trimester placentas. However, the authors recognize that there might be differences between the structure of spiral arteries of first and third trimester placentas and support the idea that these should be taken into account, given the early stage of occurrence of spiral artery remodeling.

In a study carried on by Korff and collaborators spheroids of HUVEC cells were generated and embedded in collagen type I gels to evaluate the angiogenic sprouting in response to different cytokines as well as heparin treated plasma of patients with preeclampsia or heparin-plasma of patients with normal pregnancies. However, experiments with cytokines showed no different results among CTB from normal and third trimester preeclamptic pregnancies, indicating that either the responsiveness to cytokines as well as the invasive capacity of late stage preeclamptic CTBs is comparable to normal or the microenvironment plays a major role in the process [71].

Kalkunte and colleagues further characterized differences between first and third trimester trophoblasts in terms of their vascular remodeling capacity. Using a co-culture assay over Matrigel, they showed that first trimester trophoblast cell lines (HTR8 and Swan71) interacted with endothelial cells (HUVEC and HUtEC) leading to the formation of tube-like structures. Primary term trophoblasts or the third trimester trophoblast cell line TCL1, on the contrary, hamper endothelial tube formation. Authors attribute this differences to a possible intrinsic programming towards late pregnancy and found differential expression of VEGF receptors and E-cadherin between first and third trimester trophoblasts, that may explain the results observed [104].

During vascularization of human placenta, early sites of blood vessel development are formed within the chorionic villi after infiltration of stromal cells. Placental vasculature development relays on the interaction of the villous stromal cells, trophoblasts and endothelial/hematopoietic progenitor cells. Baal and Widmer-Teske successfully established a three component spheroidal model to morphologically mimic placental villi interactions, by mixing umbilical cord derived epithelial progenitor cells, first trimester trophoblast cells and villous stromal cells from early placental tissue with methyl cellulose [38]. The spheroids were further tested in low (1 and 8%) and normal (21%) oxygen concentrations, with notably higher invasive capacity of CTB cells under low oxygen concentrations. Though the system showed several morphological similarities to placental tissue, the difficulty to dissociate the cellular components from the spheroid hinders the quantification of antigen expression and further immunophenotyping analyses. However, it represents a valuable tool to evaluate the effect of toxins or microorganisms on the trophoblast invasion and vascular development of the early placenta.

3. CONCLUSION AND FUTURE PERSPECTIVES

To date, several *in vitro* models for the study of the process of implantation and placentation have been developed. The scarce availability and strong regulations associated to the use of human embryos or first trimester human placental tissue make necessary the search for new *in vitro* models of disease.

3D models mimic more closely the environmental niche of the cells than 2D ones. However, the use of complex and rich matrices such as fibrin and growth factor containing basement membrane preparations rises doubts as the matrix might buffer cell-cell interactions and thus important cell events might result overseen, while uncontrolled cell-matrix interactions take over proliferation and differentiation. As it has been mentioned along the text, cells react distinctly to 3D scaffolds, as they sense not only their composition, but also their tensile strength and porosity, all of which contribute to the cell response observed *in vitro*. If we add cell lines along with their limitations, then the extrapolation of observations to *in vivo* situations could be difficulted.

As an alternative, a more neutral scaffold with tuned mechanical properties and available cell adhesion sites would enable cell-cell interactions to occur in the absence of unwanted external stimuli. Indeed, to date, modified plastic tissue culture plates have proved very useful in the research and development fields and continue to be used extensively for cell growth and maintenance in a 2D environment. For 3D cultures, tissue culture plate inserts are readily available for purchase such as Alvetex® and 3D Biotek 3D Insert™, both made from polystyrene.

However, for the study of invasion assays the presence of a degradable extracellular matrix is mandatory and in these cases more information is needed on the effect of the quality, the tensile strength and the composition of the material selected to play this role. Additionally, few *in vitro* models are compared morphologi-

cally and functionally to the tissue found *in vivo*, decreasing the reliability of the proposed models.

ABBREVIATIONS

EVT	=	Extravillous trophoblasts
TS	=	Trophoblast stem cells
DSCs	=	Decidual stromal cells
MSCs	=	Mesenchymal stem cells
NK	=	Natural killer cells
HUVEC	=	Human umbilical vein endothelial cells
CTB	=	Cytotrophoblast
IUGR	=	Intrauterine growth restriction
MHC	=	Molecular histocompatibility complex
ECM	=	Extracellular matrix
MMPs	=	Matrix metalloproteinases
hCG	=	Human chorionic gonadotropin
NASA	=	National Aeronautics and Space Administration
PET	=	Polyethylene terephthalate
Poly-HEMA	=	Polyhydroxyethylmethacrylate

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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