Next-generation of *in vitro* 3D human tissue models for preclinical applications in life sciences: application to reproductive biology

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Summary

In recent years *in vitro* research models have been shifting from 2D cell culture to more complex and predictive 3D systems to better mimic the morphological and physiological feature of human body. It has been proved in fact that 3D structure guarantees more physiological cell-to-cell and cell-to-matrix interactions and, therefore, an improved predictability of the model.
Currently used and next generation 3D models for pre-clinical studies and research are described in this review, with a focus on the models for the investigation on human reproductive biology, considering the increasing interest about pre-clinical applications in this field.

KEY WORDS: endometrium, spheroids, in vitro *testing*

3D *vs* 2D cell culture

Traditionally, cell culture methods are based on bi-dimensional culture of mammalian cells which are grown on flat surface forming a monolayer. In this system, the cells present a sheet-like flat shape and they are submerged and directly exposed to culture medium with a maximum exchange of nutrients and catabolites. Hence, the proliferation rate in 2D *in vitro* systems is usually higher and the necrosis lower compared to the tissue (1). Furthermore, the bi-dimensional disposition greatly limits the cell-to cell contact, influencing the cell behavior and physiology and the extra cellular matrix (ECM) production and composition (2, 3).
When cells are instead cultured in 3D systems and once the 3D model is formed, the cell proliferation rate is lower and the cells form a multilayered structure (e.g. in air lift culture) or aggregate in spheroids within a matrix (scaffold), on a matrix, or in a suspension medium, better recapitulating the tissue or organ of origin. The spatial tridimensional disposition greatly modifies the contact cell-to-cell and cell-to-matrix ratio and therefore the physiology of the model. In fact, in 3D models gradients of nutrient, growth factors and catabolites are generated from the center to the model to the surface and vice versa. Consequently, the reconstructed spheroids contain proliferating, quiescent, hypoxic and necrotic cells at the same time. The increased cell-to-cell interaction affects a range of cellular functions (4), such as cell proliferation, differentiation, morphology, gene and protein expression, and cellular responses to external stimuli. All together these properties give 3D spheroids a higher biological relevance and potential applications in pre-clinical investigations (Figure 1).

A 3D spheroid aims to mimic the tissue or organ of the human body

The production of a spheroidal microtissue is based on the aggregative capability of cells which self-assemble in a tridimensional structure whose shape can vary to round to grape-like or stellate in a cell-type dependent manner (5-7). The aggregative process can be due only

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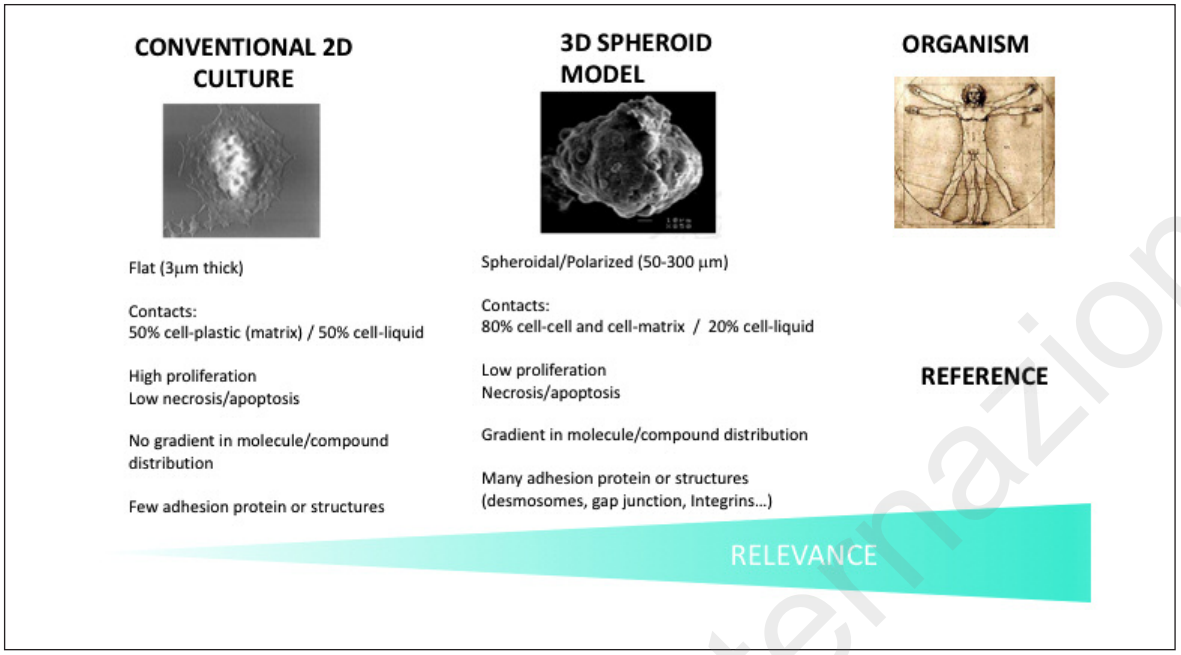

Figure 1 - Schematic representation of relevance in 2D culture *versus* 3D spheroidal model, compared to reference organism. Image for courtesy of InSphero AG.

to cell auto-assembly features in scaffold-free systems or can be mediated by biopolymers [e.g. commercially available products such as basement membrane matrix (Matrigel, BD Sciences)], basement membrane extract (Cultrex BME; Trevigen), and hyaluronic or synthetic scaffolds [Polyethylene glycol (PEG), polyvinyl alcohol (PVA)] (8, 9).

The production of scaffold-free spheroids can also be achieved using hanging drop technology in which small volumes (20-30 μL) of a cell suspension are distributed in specific plates (GravityPLUS system, InSphero AG). Gravity causes the cells to settle and concentrate at the bottom of the drop, and a single spheroid is formed. Spheroids can be formed starting from different primary cells or cell lines, moreover heterotypic complex spheroids can be formed co-culturing different cell types. Spheroid size and cellular composition is usually controlled by adjusting the cell density in each drop (10, 11).

The advantages of scaffold-free system rely on the autogenous and physiological production of ECM by cells and the absence of scaffold interference with downstream applications (traditional histology, cell-based assays, enzyme-linked immunosorbent assays, proteomics, transcriptomics, and high-content imaging).

3D cell cultures have been characterized, upgraded and engineered (following a proto-typing to up-scaled process) to mimic the more complex integrated system of organs in the body, becoming a powerful tool for the research in many fields of biology, among them cancer biology.

In the study of tumor formation and evolution, the application of scaffold-free spheroidal models can elucidate mechanisms of growth, differentiation, proliferation and apoptosis depending on the distribution of nutrients, oxygen and catabolites, mimicking the behavior of avascular tumors (12). Moreover they can be used in high through-put high-content pre-clinical studies in order to predict the responsiveness to therapy or to elucidate the mechanisms of drug resistance (13).

The use of scaffold-free spheroids as models for *in vitro* test can be applied in many other fields of biological and medical research.

An example of heterotypic complex 3D model to predict liver toxicity is Microliver (InSphero AG) which has been developed as an alternative to animal testing for drug discovery and hepatic toxicity. It is very difficult to maintain a liver-specific functionality *in vitro* because hepatocytes should retain their polarized 3D structure to maintain liver-specific functionality. 2D co-

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culture of hepatic cells are the current gold standard. However 3D spheroids formed by hepatocytes and non-parenchymal cells in hanging drops result more similar, from a morphological and physiological point of view, to situation, presenting cell-specific markers (CK8, CD68, CD61), and the presence of bile canaliculi into which hepatocytes secrete their metabolized toxic products. Moreover, in comparison with 2D culture, microliver can be maintained in culture up to 5 weeks allowing long-term studies with repeated dosing, to evaluate chronic hepatotoxic effects (14).

In the field of skin biology, many efforts have been done to understand the function and the physiology of sub-dermal compartment and skin appendages. In order to investigate the role of sub-cutaneous fat in skin maintenance and renewal, Turner et al. (15) showed that 3T3-L1 adipocyte spheroid model exhibits a more differentiated phenotype compared to 2D monolayer analogues, based on triglyceride accumulation, CD36 and CD40 protein expression and PPAR-g and adiponectin mRNA expression.

The model has shown reduced metabolic functions and increased lipolysis in the presence of elevated fatty acid levels followed by acute exposure to TNF-a (15). De Servi et al. (16) produced and characterized a human microadipe model in which the maturation of pre-adipocytes towards lipid -producing cells has been characterized by a multi-parametric approach. In particular, the presence of lipid droplets and perilipin has been demonstrated by histo-morphological analysis, a gene expression profile very similar has been discovered and also the responsiveness to forskolin, a well-known inhibitor of lipid formation in adipocytes has been verified (16).

In the field of skin care and cosmetics, a great attention has been paid to dermal papilla cells and their natural spheroidal disposition in the hair bulb. Since there is no availability of tridimensional *in vitro* models of hair bulb, in our laboratory a model of proto hair-follicle has been produced. Higgins at al. demonstrated that the extraction of dermal papilla fibroblasts and their cultivation in 2D systems alters their transcriptional profile but a partial restoring of gene expression can be achieved once the cells have been cultured in 3D (17). On this basis, we obtained a multi-layered spheroid composed by human hair follicle keratinocytes and derma papilla fibroblasts which can be cultured up to 7 days, mimicking the hair bulb, presenting morphological features similar to an active (anagen-like) dermal papilla and a potential regression starting from day 7 (18).

3D Models applicable to reproductive biology: investigation on fertility

Endometrial receptivity models: the "implantation window"

In the field of reproductive biology and its clinical applications, the study of pathologies affecting the reproductive system, and fertility and pregnancy, has reached an increasing importance. For this reason, the interest in developing predictive and versatile models is high. However, obtaining representative material for the study of embryo implantation and embryo-endometrium interaction remains a challenge and rises many ethical issues since an endometrial tissue biopsy cannot be explanted during the implantation window without disrupting the process of implantation (19).

A first fundamental aspect in fertility and conception is the capability of endometrial tissue to be receptive for the implantation of embryo. In fact, appropriate endometrial maturation and receptivity are required for the rising of molecular cross-talk between uterus and embryo and, therefore, for a successful implantation. Endometrium is known to become receptive only for short periods, less than 48 h between 5 and 10 days after ovulation. This period is called the 'window of implantation' during which the uterine environment is conducive to blastocyst adhesion and invasion. The "dialogue" for the synchronization among ovulation and cell modifications of endometrial epithelium for the implantation is directed by series of hormonal and biochemical messengers. During the "implantation window", the endometrial epithelium expresses various molecules including cytokines, adhesion molecules and growth factors and a specific morphology is present (e.g. cellular protrusions named pinopodes are relievable) (20, 21).

The uterine secretion, in terms of cytokine and chemokine composition, seems to be determinant in the implantation process. In fact, the presence of pro-inflammatory cytokines (IL-10, TGF-β, IFN-γ, IL-6, IL-8 and IL-17), secreted

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principally by stromal compartment, and the consequent morphological alteration of endometrial tissue seems to have beneficial effects on the process of implantation in fertile women (22).

Many approaches to assessing endometrial maturation and receptivity have been described: some based on histological criteria (therefore with low reproducibility) (23) and, more recently, based on gene expression, which has allowed to identify key-mediators regulating endometrial receptivity, and the subsequent embryo-endometrium molecular cross-talk (24). Moreover, also the possibility to evaluate the secretory profile and the paracrine cross-talk between uterine stroma and epithelium has a critical importance in the development of *in vitro* models. Different endometrial models have been developed both in 2D and in 3D systems, as single cell type or co-culture, to study endometrium maturation and receptivity window. The more diffuse mono-cultures are endometrial epithelial cells (eEC) or endometrial stromal fibroblasts (eSF) cultured in monolayer to evaluate effects of steroid hormones, hormone receptor modulators and agents that could enter the uterine lumen (25, 26).

However, the mono-culture presents strong limitation due to absence of paracrine interactions that normally influence cell functions and behavior. Moreover, one of the fundamental properties of the endometrial epithelium is its polarity, with well-defined basolateral compartments (27) which guarantees the endometrium differentiation and proper secretion of cytokines during the menstrual cycle (28). Of course, in monotype-monolayer culture the cellular polarization is not well developed and no compartmentalization can be achieved. In order to improve model morphology, cell endometrial co-culture models have been developed despite the known difficulty of growing normal human endometrial epithelial cells in long-term culture. Many studies have demonstrated that the interaction between mesenchymal cell of the uterine stroma and epithelial cells is essential for the morphogenesis and physiology of epithelial compartment underlying the importance of paracrine cross-talk (29, 30).

Chen et al., used a two-chamber co-culture system in which the endometrial epithelial cells (eEC) and endometrial stromal fibroblast (eST) were maintained without direct cell-cell contact to analyse separately the secreted products in the apical or basolateral chambers. In this model, a functionally polarized epithelium has been obtained with an evident apical/basolateral secretion. The transcriptome of eEC and eSF has shown features consistent with those of the corresponding pure FACS-isolated cell populations from human endometrium. Moreover, it has presented a structurally competent tight epithelium displaying functional polarity with apical/basolateral secretion. Moreover, some clear indications of paracrine interactions between the cell compartments have been demonstrated, as evidenced by differential gene expression and secretory activity in co-cultured cells compared to their mono-cultured counterparts (31).

In our laboratory, a spheroidal micro-endometrium model by using the hanging drop technology, is currently under development. The cells are two established lines of endometrial epithelial and stromal cells to produce multi-layered spheroid in which a core of stromal cells is surrounded by a stratum of epithelial cells to mimic the endometrial structure.

In this model, the epithelium-mesenchyma interaction is guaranteed by the optimized co-culture allowing the "miniaturisation" of the system with a low use of cells and a consequent formation of a high throughput analysis system. The produced micro-endometrium has been characterised during the culture verifying the behaviour of single cell types in the hanging drop system and their capability to aggregate in co-culture in a spatial well-defined structure, expressing cell-specific histochemical markers (E-cadherin, F-actin and vimentin, cytokeratin-7, integrin beta-1). The stability and viability of the model has been assessed in a time course experiment up to 10 days in culture.

Embryo adhesion: models of implantation

In parallel to investigations on endometrial receptivity, also the study of implantation process requires adequate *in vitro* models to elucidate the molecular mechanisms and the morphological modifications both in blastocyst and in endometrial tissue.

The implantation is a strictly driven-by, complex sequence of signals which are crucial for setting up pregnancy; it is supposed that a great number of molecular mediators under the influence of ovarian signals are involved in the embryo-endometrial interaction. These mediators

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comprise a wide range of molecules like hormones, cytokines, growth factors, lipids, adhesion molecules (32). The molecular dialogue between the conceived and the endometrium implies interactions among cells, and between cells and biochemical factors: these mechanisms, if suitably expressed or inhibited, help determine the receptivity or non-receptivity state of the endometrium *versus* the embryo.
The investigation of this complex and delicate process, implies the availability of representative *in vitro* models to evaluate early implantation. Early *in vitro* models for human implantation used simple mono-layer culture of epithelial cells and trophoblast cell lines grown as multicellular spheroids to mimic the spherical structure of the blastocyst during the early attachment phase and early invasion into the endometrium (33-35). In these studies, the investigated mechanism was the trophoblast attachment to endometrium but not the invasive process itself.
In a recent study, Buck et al. (36) started from *ex vivo* (37) and observations (38) showing that early implantation in humans is accompanied by adhesion and invasion of the endometrial epithelium by blastocyst. They created and validated a model for invasion of reconstructed endometrium by a trophoblast. For this purpose, a gland-like 3D model of endometrium in a Matrigel system has been produced and the trophoblast attachment and penetration have been mimicked by a trophoblast cell line spheroid. In particular, different endometrial epithelial cell lines have been cultured in Matrigel and evaluated by their aggregative capabilities, their ability to form lumen and their susceptibility to be invaded by trophoblast. This complex and dynamic *in vitro* co-culture system would help to establish whether the altered distribution of maternal epithelial junctions influences the penetration and invasion of trophoblast cells.

New challenges and technologies for in vitro *3D models development*

The evolution of technology and bioengineering applications are a driving force to improve the complexity in the structure and functions of *in vitro* models toward the reproduction of tissue, single organ or multi-system. Microfluidic has been applied to reconstructed tissue to re-create fluid movement in the body, increasing nutrient supply, catabolites elimination, paracrine cross-talk and exerting a mechanical action.
Some of the most interesting examples are: lung-on-a-chip to evaluate pulmonary thrombosis and liver-on-a-chip to predict liver toxicity (Emulate and Horsham), human body-on-a-chip integrating ten human "organs" to study complex human physiology *in vitro* (Harvard University), heart-on-a-chip incorporating human heart tissue derived from adult stem cells for drug safety screening (University of California, Berkeley), human gut-on-a-chip that mimics the mechanical, structural, absorptive, transport, and pathophysiological properties of the human gut along with its crucial microbial symbionts (Wyss Institute).
Considering the interest in studying the complexity of the human reproduction, the human placenta represents a major experimental challenge. Many placenta-derived cells have been isolated and cultured by traditional culture systems, but the developed *in vitro* models present many limitations in recapitulating structure and key physiological functions of the placenta. Lee et al. (39) demonstrated that it is possible to conjugate microfluidic and microfabrication technologies to develop a model that replicates the architecture and function of the placenta. This "Placenta-on-a-Chip" micro device provides possibilities to simulate a verisimilar structure and analyze critical physiological responses of the placental barrier.

Conclusions

In vitro approaches on 3D human models can be considered as the reference for new millennium toxicological investigation and are taking an increasing importance in preclinical studies. In the field of human reproductive biology this phenomenon acquires a critical importance considering all the ethical and practical implications. For this reason, in the last decade a hi-tech assisted exponential evolution of *in vitro* models occurred to better mimic the reproductive system, its morphology, its physiology as well as to recreate the first stages of conception and pregnancy.
The morpho-functional complexity of the developed models is increasing day by day, from basic bi-dimensional co-culture model to the development of three-dimensional multi-type tissue (or mini tissue) connected in multi-organ

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systems to obtain more and more reproducible, reliable and predictive models.

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