Breast Cancer Research and Treatment **85:** 281–291, 2004.

Review

Three-dimensional *in vitro* tissue culture models of breast cancer – a review

Jong Bin Kim, Robert Stein, and Mike J. O'Hare
Department of Surgery, Royal Free and University College London Medical School, Ludwig Institute for Cancer Research/University College London, Charles Bell House, London, UK

Key words: breast cancer, extracellular matrix, heterologous co-cultures, *in vitro*, stroma, three-dimensional, tumour microenvironment, tumour spheroids

Summary

Three-dimensional (3D) *in vitro* breast tumour models have an invaluable role in tumour biology today providing some very important insights into breast cancer. As well as increasing our understanding of homeostasis, cellular differentiation and tissue organization they provide a well defined environment for cancer research in contrast to the complex host environment of an *in vivo* model. With the recent availability of relevant stromal elements together with the vast array of extracellular matrix constituents available, *in vivo* like microenvironments can be recreated. These tissue like structures more realistically model the structural architecture and differentiated function of breast cancer than a cellular monolayer providing *in vivo* like responses to therapeutic agents. Three dimensional *in vitro* models allow the study of cell–cell and cell–extracellular matrix interactions, in addition to the influence of the microenvironment on cellular differentiation, proliferation, apoptosis and gene expression. Due to their enormous potential 3D cultures are currently being exploited by many other branches of biomedical science with therapeutically orientated studies becoming the major focus of research. In return great progress in 3D culture techniques have been made, largely due to this greater interaction. At present they are being used in studies ranging from investigating the role of adhesion molecules (e.g., E-cadherin) in invasion/metastasis; VEGF and angiogenesis, to tissue modelling and remodelling. Progress in the development of complex 3D culture systems is more productive than ever, however further research is vital.

Introduction

Recent statistical reports show that breast cancer is the most common cancer among women in the western world and that approximately 1 out of every 8 women will be diagnosed with breast cancer if she lives long enough [1, 2]. The time course of breast cancer evolution can be very long, taking anywhere between 5 and 30 years to develop. To be able to follow the development of breast carcinoma *in situ* and its progression to invasive carcinoma, epidemiological studies in excess of 10–15 years are required. Conducting such studies is neither feasible nor easily reproducible. Thus there is a need to develop appropriate and functionally relevant model systems to use in studying the molecular basis of breast cancer progression.

The behaviour of invasive carcinomas in humans can be very varied, in that they can metastasize rapidly in one case or take many years to become invasive in another [3]. These observations emphasize the dynamic and progressive nature of breast cancer in humans. When designing model systems for breast cancer the inherent complexities and nature of the disease must be borne in mind.

The growth and maintenance of any normal tissue depends on a continuous series of cellular interactions in a microenvironment composed of various growth factors, hormones, and adhesion molecules as well as a complex extracellular molecular matrix. To successfully investigate the pathobiology of human breast cancer, it is necessary to maintain or recreate the characteristic three-dimensional (3D) architecture of the

tissue in culture. More specifically, 3D heterologous *in vitro* co-cultures involving tumour cell interaction with stromal cells; endothelial cells, fibroblasts and immunocompetent cells. In previous years the study of normal human breast physiology and breast cancer has been significantly impaired by limitations inherent to available model systems, specifically two-dimensional models (2D).

3D multicellular spheroids versus 2D monolayer cultures

Three mandatory requirements are necessary to develop human tissue:

1. Co-localization of different cell types with cell–cell interactions and the exchange of growth factors and other biological effectors.
2. Manufacture of an extracellular matrix (ECM) to provide a 3D scaffold for mechanical stability and to regulate cell function.
3. Synthesis of interstitial fluid containing the necessary nutrients and biological effectors required for tissue differentiation and maturation. *In vitro* co-culture models satisfy these three broad requirements and so produce credible and reliable preclinical tumour models.

Limitations of conventional 2D monolayers and organ cultures

A major limitation of 2D monolayers is their lack of stroma, this being important as stroma of the mammary gland accounts for greater than 80% of the resting breast volume [4]. They lack structural architecture and so are faced with transport limitations. Another key limitation is that normal epithelial cells when grown in monolayers are highly plastic and express many characteristics displayed by tumour cells *in vivo* [5, 6]. In addition, not all normal epithelial cell types are able to adhere and grow well on artificial substrata.

Previously 2D monolayers together with organ cultures have been the most popular *in vitro* models for breast cancer research. Monolayer culture models are easy and convenient to set up with good viability of cells in culture but they lack the 3D microenvironment of intact tissue. However in organ cultures the microenvironment of the tumour remains intact. This is of vital importance as cell–cell, cell–matrix interactions and interstitial fluid affect differentiated cell function within the 3D environment. However organ culture is not without its own problems; with difficulty in obtaining specimens and poor viability of the tissues in culture being major obstacles.

3D multicellular spheroids-cells and extracellular matrix

Multicellular tumour spheroids are very small spheroids consisting of mixed cells (tumour and stromal cells) organized in a 3D arrangement. Three-dimensional spheroids contain an extensive ECM that differs in the relative amount and assembly from the corresponding monolayer cultures. The complex 3D network of cell–cell and cell–matrix interactions is not only relevant in the penetration and action of drugs but it also affects the distribution and function of physiologically occurring factors. These biological effectors such as hormones and growth factors fundamentally determine the regulatory mechanisms of cell growth, differentiation and death. For example, human mammary epithelial cells, isolated from reduction mammoplasty, grown in culture on reconstituted basement membrane form polarized acinus type structures capable of gland specific function such as milk production [7, 8]. These results were also reproduced with primary mouse epithelial cells isolated from pregnant mice. However the same cells grown in a different substrate, Type 1 collagen, show altered integrins, abnormal cellular polarity and disorganization emphasising the importance of matching cell type with appropriate substrata [9]. With the microenvironment of normal tissue and tumours being of crucial importance in the control of growth and development it is vital to clarify the origin of the ECM present within *in vitro* and *in vivo* model systems. The ECM produced and present in the majority of 3D *in vitro* tumour model systems is pre-dominantly of tumour cell origin. In heterologous (tumour–stromal) model systems incorporating stromal cells such as fibroblasts, ECM is also produced by these stromal elements. However the situation present *in vivo* with animal models is very different. In xenograft and transgenic models it is the host cells that produce the ECM; this being significant as it is of rodent non-epithelial tumour tissue origin. This raises serious implications and issues regarding tumour–stromal cell interactions as well as cell signalling.

The value of experimental culture models using epithelial cells often depends on the degree of polarization as well as other critical features observed in nat-

ural tissues, including the formation of tight junctions, desmosomes and membrane interdigitations. The control of epithelial cell polarity is one of the important functions of the stroma. Loss of epithelial cell polarity has been proven to lead to increased cell proliferation and tumourigenesis. Many cells types, in 3D multicellular culture models, were found to assume a more or less normal cellular architecture and exhibit gene expression profiles that were reflective of an authentic differentiated phenotype [7, 10]. As disorganization of cellular architecture is a common feature of most solid human breast tumours, cells that fail to differentiate, as would be the case in human breast cancers, can easily be distinguished from normal cells [11]. Cancerous human breast epithelial cells are apolar, exhibit disorganized cell–cell junctions, contain distorted cell nuclei, and fail to organize into ductal structures. Cell shape and tissue architecture is partly dependent on various adhesion molecules, and so any alterations in these molecules have been implicated in cancer progression [12]. However, growth of normal epithelial cells as monolayers on artificial supports leads to partial loss of the original epithelial cell characteristics, and also the quality of the monolayer is strongly influenced by the physico-chemical properties of the support.

Advantages over 2D monolayers

The advantage 3D cultures have over 2D monolayers are their well defined geometry, which makes it possible to directly relate structure to function and in turn enable theoretical analyses. The presence and support from stromal elements and ECM together with its close resemblance with micrometastases and initial avascular regions of tumours augments their authenticity. Three-dimensional cultures are composed of cells with different phenotypes such as proliferating, non-proliferating and necrotic cells very similar to the situation within intact human tumours. Phenotypes present more closely resemble those present *in vivo*. Cellular heterogeneity within these tumour models caused by mass transport limitations, resembling the multiple phenotypes found in solid epithelial tumours, is far more realistic than the cellular homogeneity found in monolayer culture. As 3D cultures support co-cultivation of multiple cell types, interaction between epithelial and stromal cells which regulate normal and neoplastic development can be studied. Given the crucial importance of the tumour–stromal interaction in cancer biology, a more realistic environment is created better able to elicit an *in vivo* like response to cancer therapies. Tumour cell adherence and migration in spheroid co-cultures reflect the distinct metastatic potential of (different) breast tumour cells.

Three-dimensional *in vitro* systems have been shown to recapitulate the drug sensitivity patterns of tumour cells *in vivo*. MDA-231 spheroids exhibited a much lower IC_{50} to cisplatinum when plated in monolayer cultures than as suspended spheroids. In a study by [13] upregulation of TGF-βs in tumour cells when treated with DNA damaging therapeutic strategies such as cisplatinum have been shown to be protective. Treatment of MDA-231 spheroids, but not MDA-231 monolayers, by cisplatinum demonstrated upregulation of TGF-β1 mRNA and protein which is highly predictive of the patterns of drug response of tumour cells *in vivo*.

With its many advantages over conventional monolayer cultures, 3D cultures more closely resemble the *in vivo* situation with regard to cell shape and its environment. This is important as it is recognised that cell shape and environment can determine the behaviour and gene expression of the cell. For example, K-*ras* mutations, frequently found in colorectal tumours, have been noted to occur more consistently during transition from early to intermediate adenoma stage which coincides at what time tumours acquire 3D growth patterns [14].

As animal models and *in vivo* studies are costly and complex with problems of unpredictable characteristics and ethical approval, physiological 3D model systems using human cells to create an authentic model is an obvious choice. With the recent advances in tissue engineering, 3D cultures are now more morphologically and functionally differentiated (lobulo-alveolar structures that express and secrete milk) and can be produced in larger quantities as a result of greater control over culture composition, new bioreactors and greater choice in the method of inducing 3D growth.

Tumour microenvironment

With the increasingly recognised fact that tissue microenvironment is very important in the regulation of normal cell function, we need to develop models to test how structural and biochemical cues provided by the tissue microenvironment play critical roles in the suppression of tumourigenic phenotypes. These extracellular cues themselves influence intracellular gene expression that in turn result in fundamental altera-

tions in the composition of the microenvironment [15]. Inappropriate alterations of cell–microenvironment interactions can result in abnormal cellular behaviour, as seen in tumour progression [16]. An interesting example of cell environment affecting differentiation occurs with ectopic implantation of embryonic cells which transforms them into malignant tissues while the same cells located in the uterus undergo normal embryogenesis [17]. The disease progression in breast and lung disease present good examples of how targeting the tumour microenvironment may prevent tumourigenesis. As fibrosis in both organs is associated with an increased risk of cancer formation, targeting fibrotic stromal reactions may decrease the likelihood of cancer formation.

Tumour–stromal interactions

The fact that while normal stroma can be protective in delaying or preventing tumour formation, abnormal stroma can promote tumourigenesis, underlines the crucial importance/role of stroma in tumourigenesis. Stroma is well known to support and respond to tumourigenesis directly influencing the tumour process (Figure 1). This was demonstrated by culturing primary human breast epithelial tumours cells, in combination with stromal elements, in 3D collagen Type 1 matrices. The addition of stromal elements caused the tumour to spread and become invasive, with greater concentrations of stromal elements having a proportional effect on tumour growth [18]. Recent evidence even shows that stroma and its products can be oncogenic in that it can transform adjacent cells, in the absence of malignant epithelial cells, to induce different phenotypic and genomic changes in epithelial cells [19]. Also tumour–stromal interactions such as fibroblast associated alteration in tumour cell morphology and extracellular matrix distribution have been implied [20]. Oncogenic signals from tumour derived fibroblast can change non-malignant epithelial cell populations to malignant ones. Furthermore only transient stimulation is required to transform cells into tumourigenic lesions. Stroma is thought to promote oncogenic activity through a combination of ways including manipulation of matrix metalloproteinases, recruitment of inflammatory cell and viral alteration of stromal signals [18]. In addition stroma exposed to carcinogens through irradiation has been shown to accelerate mammary epithelial cell tumour formation and progression [21].

The interaction of endothelial and tumour cells in 3D co-culture spheroids provide important clues to the behaviour of capillaries in tumour. With the well known fact that the centre of tumour spheroids is either necrotic or quiescent, tumour–endothelial co-culture spheroids provide a way of studying whether oxygen and nutrients are delivered to the centre by the endothelial cells in a similar way to *in vivo* tumours. Also accumulating evidence points to the local environment particularly interaction with adjacent cells and ECM, as being a major factor in endothelial cell differentiation. Heterogeneity of mature endothelial cell phenotypes is proof different organs produce different endothelial phenotypes. The study of how changes in stroma can affect tumour formation represents a new direction in tumour therapeutics.

Cell signalling and adhesion

Study of intercellular adhesion and signalling between cells within spheroids has provided evidence that anchorage-independent growth may not be essential for tumour progression. It is now thought signalling between cells through adhesion molecules may provide the crucial elements needed for sustaining cell viability. Recent functional studies of integrins in a human model system provides evidence to support the idea that the structural stability afforded by integrin-mediated cell–ECM interactions is an important determinant of normal cell behaviour, and that alterations in tissue structure can give rise to tumourigenic progression [8, 9, 12, 22, 23]. For instance expression of the cell–cell adhesion molecule E-cadherin promotes differentiation and inhibits metastasis of several cancer cell lines [24]. Three-dimensional cell growth may induce cell differentiation through enhanced expression of E-cadherin and other cell adhesion molecules. Loss of E-cadherins at adherens junctions have been implicated in many tumours. Loss or decreased function of E-cadherin in association with increased expression of other cadherins has been shown in desmoplasia, invasive tumours and metastasis [25].

The use of 3D spheroids has enabled better understanding of tumour microenvironments leading to the fact that cell fate is dependent not only on adhesion mechanisms but also on the interaction of these adhesion molecules with soluble growth regulatory factors such as hormones, cytokines and growth factors. For example, there is evidence of bidirectional cross modulation between epidermal growth factors (EGRF) and β1-integrin in human mammary tumour cultures. Over

expression of β1-integrin and EGRF in malignant human breast epithelial cells, but not in normal epithelial cells, when cultured in 3D matrix, demonstrates that the two-way signalling between cells, ECM and EGRF only occurs in 3D [26]. There is evidence signal transduction in 3D differs from 2D as well as being dependent on the physical state of the 3D substrate. Taking signalling of the focal adhesion molecule (focal adhesion kinase) as an example, tyrosine phosphorylation is down regulated in 3D co-cultures with suggestions that it may even be independent of the FAK regulated pathway in comparison to 2D monolayers [27].

Although it is well recognised that 3D environments promote polarity and differentiation in normal epithelial cells, in fibroblasts some 3D matrix interactions also display enhanced cell biological activities and narrowed integrin usage compared to 2D substrates. Cell derived matrices were better than artificial 3D matrices and 2D substrates in promoting cell adhesion, migration and *in vivo* like morphogenesis [27]. In normal breast stromal fibroblasts secrete a collagenous ECM however in breast cancer; cells are mixed in between all the stromal cells producing a disorganized mass. It is important to reiterate here that the ECM produced by spheroids is of tumour cell origin, while *in vivo* it originates mostly from host cells. This implies a differential expression of gene producing ECM constituents.

These intrinsic changes in cell function profoundly affect the response of a tissue model to external agents. For example, mouse mammary tumour cells exhibit greater drug resistance to melphalan and 5-fluorouracil as multicellular aggregates than as monolayers [28]. Due to greater understanding of important ECM constituents, receptors in the microenvironment of tumour cells and signalling pathways that operate pre-dominantly in 3D, new therapies such as Herceptin® (blocks signalling by EGFR ERBB2 or HER2) and Gleevec® (KIT-selective tyrosine kinase inhibitor) have been manufactured. These new therapies that specifically target signalling pathways could be used to alter or modify microenvironment initiated signals that control whether tumour cells differentiate or apoptose.

Cell culture methods

This review summarizes existing 3D culture methods of breast cancer and highlights their benefits for *in vitro* testing of novel tumour therapies. Multicellular co-cultures allow cellular analysis in a controlled and reproducible format as well as providing a realistic portrayal of the *in vivo* response to experimental treatment. Even with the complexities and limitations of 3D multicellular co-cultures, these models provide an important adjunct to the use of animal models, particularly when human cells are used. Models that closely mimic breast cancer are essential to the development of new treatments. Improved models will hopefully expedite the discovery of a cure for breast cancer.

In recent years breast cancer models, based on 3D cell culture methods, have greatly increased in number due to new culturing techniques emerging from the field of tissue engineering, which has lead to improved models. Previously spontaneous aggregation, liquid overlay cultures and spinner flasks were the most popular methods used to produce 3D *in vitro* breast cancer models. More recently pre-fabricated scaffold based cultures, the NASA developed Rotary Cell Culture System and pre-engineered collagen scaffold have been developed. The scaffold based culture system is gaining in popularity as the 3D matrix is used to promote multilayer growth of cells derived from breast cancers. The design of the Rotary Cell Culture System is a vast improvement on the spinner flask in that larger more morphologically and functionally differentiated breast cancer spheroids can be produced.

Spontaneous cell aggregation

Spheroid cultures were found to be functionally distinct from their monolayer counterparts. In principle, 3D spheroids were obtained either by exploiting spontaneous cell aggregation, generating more or less spherical cellular conglomerates, or by culturing cells on artificial substrates that induce cellular differentiation and maintain cellular function. The ability of malignant cells to adhere to each other (homotypic aggregation) and other cells (heterotypic aggregation) as well as to endothelial cells and exposed basement membrane are considered critical during the early stages of cancer metastasis. Some breast cancer and breast cell lines will spontaneously aggregate during cell culture, to form tissue like spheroids. For example, it is well documented that human breast carcinoma cell MDA-MB-435 undergoes homotypic cell aggregation [29]. The breast carcinoma cell line DU4475, when grown in suspension forms clusters and cords which are attached chiefly by desmosomes [30]. An important feature of metastatic cancer cells is multicellular aggregate formation, which directly correlates with their increased survival potential *in vitro*

[31] and metastatic propensity *in vivo* [32]. Fortunately the majority of the established tumour lines are derived from metastases. These multicellular tumour spheroids are similar to avascular tumour nodules and or microregions of solid tumour *in situ* in terms of growth kinetics, with discrete cell populations consisting of proliferating, quiescent and necrotic cells. Due to mass transport limitations, toxic metabolic waste build up and lack of nutrient penetration at progressively greater depths within the spheroid, proliferating cells were mostly present on or near the surface. Therefore this model successfully recreates the situation observed in micrometastases prior to vascularization and intervascular microregions of large tumours [33].

Liquid overlay culture

For cells to grow as 3D aggregates or spheroids they require conditions in which the adhesive forces between the cells are greater than for the substrate it is plated on. Other methods such as spinner flasks and gyrators also prevent adherence by inhibiting meaningful contact with the culture vessel wall. The simplest way to achieve this condition is by using liquid overlay techniques which prevents matrix deposition. With this technique many tumour cell lines will undergo spontaneous homotypic aggregation. Spheroid formation can even be induced in tumour cells that do not spontaneously aggregate or are difficult to aggregate. Essentially cells are grown over an agar base in media with spheroid formation following a biphasic process; firstly cells migrate towards each other on the agar and aggregate into spheroids. Secondly cell growth results in an increase of spheroid size. Cells do not adhere to the substratum but grow on it. Other substrates, such as agarose and reconstituted basement membrane (Matrigel®), can be inserted instead of agar [34].

Spheroids generated in liquid overlay cultures maintain the cellular composition and differentiation of intact tissue. In addition to acquiring several features of a differentiated epithelium (lobulo-alveolar structures), they also display enhanced differentiated function such as the ability to express and secrete milk found in functionally differentiated lactational cells. However an important point of note is that cell adhesion behaviour is altered due to cell contact with the substratum. Kunz-Schughart et al. [20] employed liquid overlay cultures to manufacture a heterologous 3D co-culture spheroid model of breast tumour cells and fibroblasts. This model mimicked solid scirrhous breast carcinomas which are well documented for areas of extensive desmoplastic reaction. The phenomenon of desmoplasia is still not fully understood, but it has been characterized by enhanced fibroblast accumulation and proliferation together with altered ECM production [35–37]. Thus this model specifically created to mimic the tumour stromal interactions that are present in scirrhous breast cancer will allow the study of tumour and normal fibroblast differentiation and its role in tumour development.

The type of ECM and media used are important factors in the way they support and promote cell growth and differentiation, specifically the arrangement of ECM and the constituent make up of the media. For example, generous amounts of thick matrices promote differentiation in human mammary tumour cell cultures. However when cultured on a thin layer of 3D ECM tumour cells fail to show differentiated phenotypes forming disorganized and apolar cellular masses [4]. This data points to the fact that structural and biochemical cues provided by the ECM as well as the cell–ECM interaction have a major factor in determining cellular phenotype. It is also critical that serum and other components in media do not interfere with the process of cell differentiation. Until now liquid overlay techniques were the most widely used technique to manufacture 3D aggregates.

Gyratory rotation and spinner flask spheroid cultures

Spinner flasks are used for growing cells as a suspension culture in liquid media. While the static environment of liquid overlay cultures are useful in studying individual spheroids, greater numbers of spheroids can be cultivated in dynamic suspension when spinner flasks are used. They are stirred tank bioreactors, in which impeller mixing maintains the cells in suspension. The fluid movement is thought to aid in mass transport of nutrients to and wastes from the spheroids. Spinner flask culture (developed by [38] in the 1970s) was the most widely used method for culturing large numbers of multicellular tumour spheroids, but other methods such as roller tubes and gyratory shakers were used with varying degrees of success. The gyratory rotation technique involves placing a cell suspension in an Erlenmeyer flask containing a specific amount of media. This flask is rotated in a gyratory rotation incubator until spheroids of required size are produced. Note the cells do not come into contact with any substrates. Although spinner flasks were the preferred method for growing spheroids,

static liquid overlay in 96 well plates were also popular for monitoring individual spheroid growths and co-culturing.

Scaffold based cultures

Three-dimensional *in vitro* breast cultures can be manufactured by growing on pre-fabricated scaffolds. Cells attach to and migrate along intertwined scaffold fibres. As the cells divide, they fill the interstices within the scaffold to form 3D cultures [39, 40]. The constituents of the scaffold can profoundly affect the properties of the culture. The most commonly used scaffold is collagen, however other ECM proteins can also be used with the possibility of adding growth and other regulatory factors. Of course the scaffold can be inoculated with primary or established cells as well as small pieces of tissue. Use of 3D collagen gels is even thought to produce more differentiated phenotypes. There is now evidence that collagen scaffolds provide signalling as well as a physical contribution to produce spheroid organization and differentiation [41].

Pre-fabricated engineered scaffolds

Recently 3D, biodegradable, engineered scaffolds have been introduced as an improved method of simulating the ECM and providing physical/structural support (Figure 2). These structures consist of natural molecules and/or synthetic polymers that can be used as a 3D physical support matrix for *in vitro* cell culture as well as *in vivo* tissue regeneration. These scaffolds bring great potential in recreating the natural physical and structural environment of living tissue [42]. This is of great advantage as ECM molecules have been shown to promote signalling pathways that influence key cell functions such as migration, proliferation and

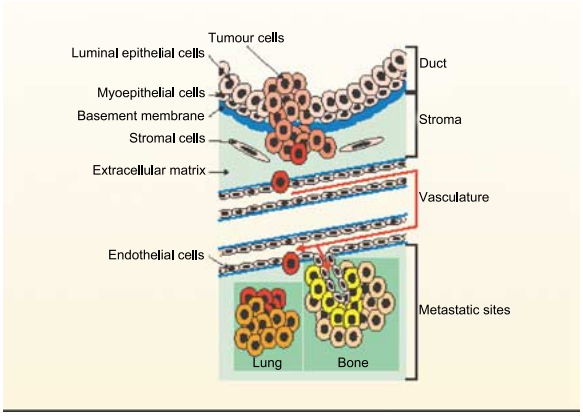

Figure 1. Breast cancer invasion and metastasis.

Figure 2. Nanostructured composite collagen–chitosan matrices.

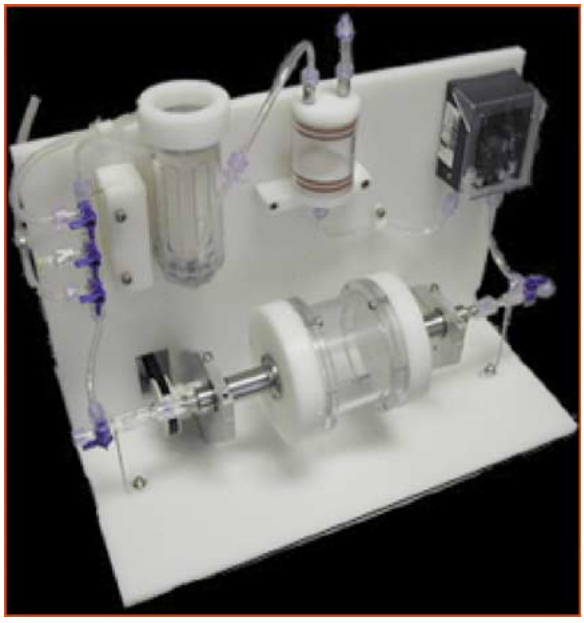

Figure 3. Rotary Cell Culture System.

differentiation. Currently they present one of the most promising experimental approaches for regenerating the native structural and functional properties of living tissue.

Rotary Cell Culture System

The NASA developed Rotary Cell Culture System introduces a revolutionary concept (Figure 3). By simulating microgravity, cells are maintained in a dynamic fluid suspension in liquid media mixed by minimal hydrodynamic forces. The culture flask

(vessel) rotates whole on its horizontal axis, providing end over end mixing of the cells. Fluid turbulence and shear forces are minimized by the vessel being completely filled with media and providing aeration through a semi-permeable membrane which eliminates bubbles producing hydrodynamic forces. This system successfully integrates cellular co-localization, 3D stromal/epithelial/matrix interactions, and low shear forces. This device provides a very quiescent environment for 3D spheroid cell culture with adequate mixing for mass transport. Results show that reduced turbulence has profound effects on the physical properties of a culture. Not only were the spheroids larger in size, but significantly more differentiated morphologically and phenotypically than in spinner flasks. This is a great advantage as higher fluid turbulence in the spinner flask has been shown to damage fragile animal cells affecting membrane integrity and metabolism [43]. Another advantage of the Rotary Cell Culture System is the ability to co-culture multiple cell types in forming 3D *in vitro* structures. To assist in 3D structuring, inert material scaffolds, microcarrier beads coated in various substrates are used for cells to attach. By co-localizing cells in 3D for an extended period of time, there is an opportunity to build tissue with characteristics that are very much different to that grown in 2D.

Advances in tissue engineering have improved the variety, fidelity and quantity of breast cancer models. These models are vital in cancer research as they are currently widely used to estimate dosage of new therapies, evaluate penetration into solid tumours, assess the efficacy of drug cocktails and to develop tumour vaccines. Cellular composition of the model affects the relevance greatly in that human cells and tissue vary greatly from animal tissues, as well as tumour cell behaviour widely varying between them. Application of primary human mammary stromal cells such as fibroblasts and endothelial cells takes the model one step closer to the *in vivo* situation.

Discussion and the future

The concept of the 3D *in vitro* co-culture system was invented due to the many advantages it gave to cancer research. These models can reproducibly reflect the distinct invasive behaviour of breast tumour cells, mimic the cell–stromal interactions of breast carcinomas and allow for systematic investigation into the multiple unknown regulatory feedback mechanisms between breast tumour and stromal cells in a well defined 3D environment. Multicellular tumour spheroids have a well organized spherical symmetry of morphological and physiological features including complex cell–cell and cell–matrix interactions resembling avascular tumour sites and or micrometastatic regions *in vivo* and thus are a very useful model in tumour biology.

Currently heterotypic co-cultures of luminal and myoepithelial cells; tumour and fibroblasts; tumour and endothelial cells are available. However a lack of more complex models involving more than two cell types still persists. This is important as human mammary epithelial cells when injected alone into mammary fat pads do not support ductal structure formation [44]. With the recent availability of immortalised primary human mammary stromal cells; fibroblasts and endothelial cells, it is now possible with the aid of new 3D culture techniques to develop a 'humanized' or more human heterologous 3D *in vitro* breast cancer model. Both endothelial cells and fibroblasts were immortalised using a combination of retroviral transduction of the catalytic subunit of human telomerase plus mutant variants of the SV40 T-antigen gene. Despite its name the large T protein does not transform the stromal cells but it does unlock their indefinite proliferation, provided that telomeric erosion is prevented by the telomerase activity present in the cells. Neither gene singly was capable of full immortalization of these cells. These stromal cells are immortal and thus have an unlimited growth potential. They seem to have no other genetic abnormalities and thus remain to all intents and purposes normal providing a readily available source of relevant stromal cells. The availability of cells that are conditionally immortalised (temperature-sensitive) as well as non-temperature sensitive variants from the same individual donor stocks enables different combinations of quiescent and proliferatively active cells to be generated. In this way the response of tumour cells to continued stromal proliferation (equivalent to desmoplasia and neoangiogenesis) can be examined, as well as the response of quiescent stromal cells to the presence of proliferating tumour [45]. Preliminary results show 3D co-culture spheroids comprising breast tumour lines, human fibroblasts and endothelial cells can be created using zero gravity tissue culture methods (Table 1). This model represents a complex heterologous multicellular model. Such organotypic cultures consisting of multiple cell types, matrices and other environmental factors can simulate a total organ

Table 1. Tissue culture methods for spheroid manufacture

Tissue culture method	Advantages	Disadvantages
Spontaneous aggregation	Cheap	Only occurs in a small number of cell types
	Little work involved	Forms clusters rather than strict spheroids
Liquid overlay cultures	Cheap	Produces only a small number of spheroids
	Easy to set up	
	Static method	
	Concentrate on individual spheroids	
	Rapid and easy screening	
Scaffold based cultures	Good extracellular support	Expensive
	Provides 3D support	
	Easy to set up	
Gyratory and spinner flasks	Cheap	High shear stress forces
	Simple to use	Architecture within spheroids not as well preserved
	Long-term culture	
	Produce large quantities of spheroids	
	Allows accurate control of physico-chemical parameters such as pH, nutrient concentration, glucose and oxygenation	
Pre-engineered collagen composite scaffolds	Biodegradable	Expensive
	Pre-engineered to simulate extracellular matrix	Difficulty seeding and culturing sensitive cell lines
	Use *in vitro* and *in vivo*	
Rotary Cell Culture System	Very low shear stress forces	Expensive
	Minimal contact with vessel wall	
	Simulated zero gravity	
	Quick production of spheroids	
	Culture multiple cell types	
	Produce more differentiated complex epithelial like architecture	

environment in culture in comparison to monotypic 3D cell cultures which only approximates the biochemical and spatial dimensions. Development of such a model would mean autocrine and paracrine interactions can be studied in their molecular and cellular terms. Ideally appropriate ECM, immunocompetent cells, and blood supply are required; nonetheless it brings us a step closer to manufacturing a more authentic organotypic tumour model.

As the intricate macromolecular network of extracellular matrix is pre-dominantly produced and secreted by stromal cells, it is now possible to investigate its crucial role in cell/epithelial differentiation and tissue-specific gene expression acting as a positive or negative regulator. These heterologous co-cultures incorporating stromal cells present a novel way of reconstructing the breast organ architecture *in vitro*. This is very timely as it is now emerging that carcinomas may be regulated by their connective tissue environment. The example of bi-directional integrin/EGFR interaction suppressing transformation of breast cancer cells to a malignant phenotype underlines the dominance of cellular and tissue architecture over other factors in regulating tumour progression. Recently it is has been noted that human stromal fibroblasts, when transplanted in combination with human mammary epithelial cells into the mammary fat pad of nude mice, promote differentiation into infiltrating ductal tree systems which are responsive to lactogenic cues. This is an exciting discovery as human mammary epithelial cells xenografted into fat pads do not normally elaborate ductal structures. Also the implantation of tumour/endothelial cell co-cultures into nude mice allows us to study the penetration and formation of neovessels in tumour spheroids. A study by [46] demonstrates the invasion and efficient neovascularization of tumour spheroids implanted in nude mice. In addition a human mammary cell line from

the luminal epithelial cell compartment with progenitor like properties, immortalised by the transduction of the E6/E7 genes from human papilloma virus type 16, has been isolated. These cells formed structures resembling terminal ductal units when implanted either in Matrigel or orthotopically in mice [47]. It is thought that these cells may function as precursor cells of terminal duct lobular units in the human breast. Thus rather than xenografting human tumours whole we could reconstruct the human mammary gland, using human mammary epithelial progenitor and stromal cells, and then implant various different tumour lines.

With all the advantages of 3D heterologous cultures, and the insights they provide we will be able to increase our understanding of the tumour micromilieu. They will be an invaluable model for developing and testing new cancer therapies *in vitro*, particularly as there are two targets now: the tumour cell and its environment. The next step would be the development 3D co-cultures incorporating pluri- or omnipotent embryonic stem cells providing the potential for differentiation into cells necessary for tumour survival or proliferation. Also further research into how immunocompetent cells affect tumour cell growth and propagation is required.

Acknowledgements

Ludwig Institute for Cancer Research for its support and Funding, and Dr Suzanne Eccles for her help and advice.

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Address for offprints and correspondence: Dr Jong Bin Kim, Department of Surgery, First Floor – Breast Cancer Laboratory, Royal Free and University College London Medical School, Ludwig Institute for Cancer Research/University College London, Charles Bell House, 67-73 Riding House Street, London W1W 7EJ, UK; *Tel.:* +44-207-679-9044; *Fax:* +44-207-679-9508; *E-mail:* jong.kim@ucl.ac.uk