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Cancer

Organotypic cultures represent tumor microenvironment for drug testing

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Currently there are no cancer models that can accurately predict the clinical activity of a drug. Compounds are screened and optimized for anticancer activity in a series of *in vitro* and *in vivo* models, each of which has its own strengths and limitations. Organotypic models are three-dimensional *in vitro* representations of tumor microenvironment, which are more biologically relevant and technically challenging than cytotoxicity, clonogenic and spheroid assays, but less in comparison to *in vivo* models.

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Introduction

The time and expense of bringing a single drug to clinical testing necessitate identifying and optimizing lead compounds from a multitude of potential candidates. Rapid screening of libraries to identify active compounds can be accomplished with *in vitro* assays utilizing isolated molecules or tissue culture cells lines. These assays do not incorporate multiple influences in the tumor microenvironment that can affect ultimate drug activity, such as interactions of tumor cells with other cells and with molecules in the extracellular matrix (physical structure supporting the cells). Three-dimensional representations of tumor microenvironment can be provided by organ cultures consisting of tissue slices and organotypic cultures consisting of cells grown in extracellular matrix to mimic tissue (Fig. 1). When used as assays, these models provide the opportunity to select and optimize compounds for evaluation in animals, which provide the most clinically relevant, but expensive step in drug development.

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In vitro models

High-throughput screening assays

The most commonly used *in vitro* models for screening potential anticancer agents are the cytotoxicity and clonogenic assays. In the cytotoxicity assays, monolayer cultures of cancer cells are treated with various drugs and then the living cells remaining are counted by direct staining of the cells with a dye such as sulforhodamine B (SRB). A variation of this assay is to use a tetrazolium dye that changes color upon being metabolized by the remaining living cells. This assay is most often used with established cell lines and is readily adapted for high-throughput screening. The clonogenic assay, also called the human tumor stem cell (HTSC) assay, is used with patient specimens in an effort to choose the best drugs for individualized patient care. This assay involves dispersing human cancer specimens into a single cell suspension and then growing them inside soft agar or methylcellulose, an environment which allows only tumor stem cells, which are capable of self-renal, to form colonies. Stem cells are believed to consist of a small fraction of the tumor mass and to be responsible for tumor recurrence and metastases. Stem cell colonies surviving after drug treatment are counted by staining with the same dies used for the monolayer cytotoxicity assays. The use of soft agar reduces the artifact induced by nonstem cells in the standard cytotoxicity assays. To date, none of these *in vitro* assays has been validated to accurately predict patient response to treatment or the clinical efficacy

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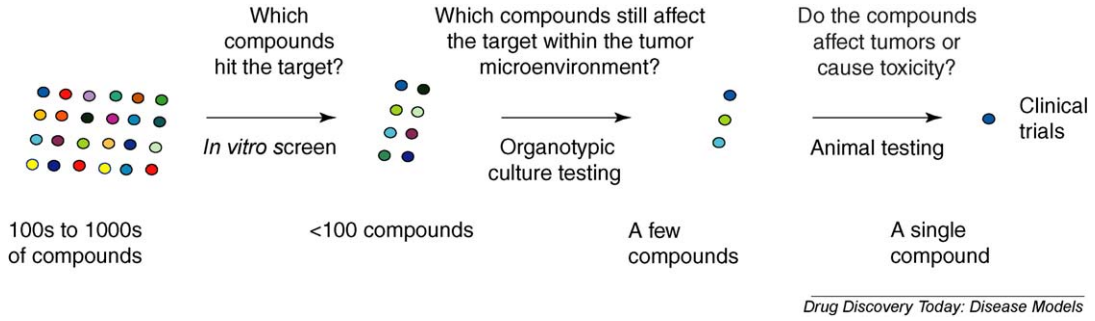

Figure 1. The role of organotypic cultures in cancer pharmaceutical development.

of drugs in general [1], but ongoing clinical trials through the Gynecologic Oncology Group (GOG) and elsewhere are designed to validate the usefulness of these assays in improving patient response and survival.

Relatively new developments in the *in vitro* models include the use of collagen gel droplet [2], and Si-sensor chips, which are able to detect metabolic changes in living cells [3]. Spheroids, which consist of suspended cells grown in clumps represent useful models of cancerous tissue and can be used to study viruses [4], but their biological relevance is limited by the lack of an extracellular matrix.

Organ cultures

There are severe limitations to *in vitro* models used to study drug effects. Cells grown in monolayers on tissue culture dishes lack the three-dimensional (3D) cell–cell and cell–matrix contacts and communication present in intact tissue. To study drugs in a biologically relevant 3D environment, organ cultures were developed in the 1960s by growing 3D tissue explants in tissue culture media. Organ cultures of neural tissue explants were the best characterized and developed models in which neuronal signaling remained functional for long periods of time and functional muscle fibers could be generated [5]. These models are currently being used today in studies of multiple neurological diseases and injuries [6]. Organ cultures also are currently being used for studies of cardiovascular function [7], angiogenesis [8], thymus [9], skin [10], bone [11] and urogenital tissues including kidney and bladder [12].

Organotypic cultures

Preparation of organotypic cultures

Another 3D model of tumor microenvironment is the reconstruction of tissue by growing cells in extracellular matrix. Cultures formed from re-aggregated cells are always called organotypic cultures, whereas tissue explants are called both organ and organotypic culture interchangeably. Organotypic cultures are typically made by suspending stromal cells in growth media containing collagen I, which is liquid at 4°C, and allowing the solution to harden into a gel at room temperature or above. Epithelial cells then are layered on top of the gel and grown in a submerged state until a confluent monolayer is achieved. The gels are then placed at the surface of the liquid media and grown at the 'air–liquid' interface, hence the term 'raft cultures' (Fig. 2). Organotypic cultures are limited by their lack of biological parameters such as immune and vascular systems. Blood vessels have been grown in organotypic culture, but their use has been limited to studying interactions of endothelial cells with fibroblasts.

Living skin equivalent organotypic cultures

Organotypic cultures reconstructed from dissociated cells were first developed in the 1980s. The best-characterized reconstructed organotypic culture is the living skin equivalent developed in the 1980s [13] and is still being used today [14]. Living skin equivalents consisting of fibroblasts inside collagen with epidermal cells on top of the collagen have been successfully grafted onto the donors of the cells to replace damaged skin [13]. Before grafting into the host, the skin equivalents can be grafted into immuno-compromised mice to prime them for accelerated establishment into the human donor [15]. The priming induces the development of well-differentiated tissue architecture including basement membrane structure and barrier function. Skin equivalents are currently being used to study antioxidant protection against photodamage [16], drug metabolism [17], differentiation [18], gene therapy [19] and the immune system [20].

Human papillomavirus (HPV) in organotypic cultures

The skin equivalent organotypic model has been modified to study the life cycle of the human papillomavirus (HPV) in the 1990s [21]. There are multiple types of HPV, which infect skin that covers the outside of our body or similar epithelium that lines the inside of our bodies. The 'high risk' types of HPV are known causes of cancer of the uterine cervical epithelium, thus studies of the HPV viral life cycle are very important to prevention of cervical cancer. HPV infects the basal layer of cells in the epithelium through cracks that can occur in the entire thickness of the epithelium. This layer is capable of proliferating and providing cells for renewal of the upper

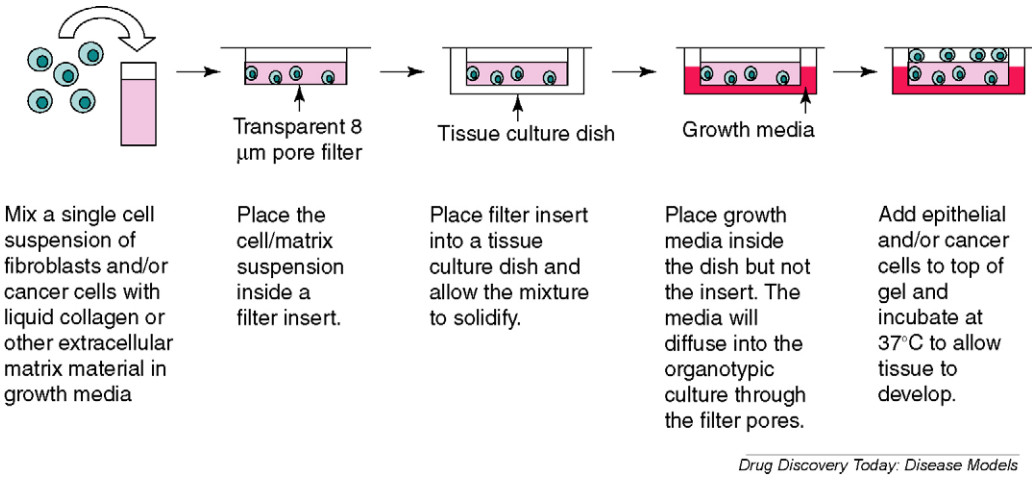

Figure 2. How organotypic cultures are prepared.

epithelial layers. As the cells are pushed into the upper layers they become more differentiated and lose the ability to proliferate. The replication of the HPV DNA genome and synthesis of HPV coat proteins is tightly integrated with this differentiation program of epithelium, so that live virus can emerge from the upper differentiated layers of epithelium. The organotypic model provides the ability to study the viral life cycle because the epithelial layers develop after the organotypic culture has been made, thus providing a dynamic real-time process of epithelial differentiation in which the viral life cycle can be studied. Cell lines capable of producing HPV replication in organotypic culture have been successfully used to study the effects of two commercial preparations of interferon-α (IFN-α) and an antisense oligonucleotide on HPV replication [22,23]. Cervical cancer cell lines grown in organotypic culture have also been successfully used to study pharmaceuticals targeted at specific molecules overexpressed in the cultures similar to the *in vivo* situation [24].

Organotypic cultures of cancer

Although the use of organotypic cultures in drug development has not yet been fully documented, discoveries have already emerged that would have not been apparent if only monolayer cultures had been used. For instance, there are no monolayer culture assays for differentiation of ovarian cancer tissue, but induction of glands by Flexible-Heteroarotinoid (Flex-Het) drugs was observed in ovarian cancer organotypic cultures [25]. The presence of glandular tissue architecture in these cultures allowed the study of the effects of Flex-Hets on both expression levels and distribution of a mucin protein called MUC1. In normal cells, MUC1 expression is limited to the particular side of the cell membrane facing the inside of the gland, called the lumen. In cancer cells, however, MUC1 is abnormally overexpressed on all cell surfaces. Although the Flex-Hets increased MUC1 expression, the MUC1 protein was only present on the lumen surface demonstrating restoration of a normal pattern of differentiation and an observation that could not have been made in monolayer culture. This pattern of MUC1 expression and differentiation was also observed in subsequent studies of xenograft tumors treated with Flex-Hets [26].

Organotypic cultures of carcinogenesis

A major direction in cancer research is to develop drugs that will prevent the development of cancer (carcinogenesis). *In vitro* carcinogenesis models include comparison of normal, immortalized and transformed cells [27]. Evaluation of drug effects on the carcinogenesis process however, requires a dynamic system in which mammalian cells are treated with carcinogens to induce their transformation in real time [28]. An organotypic model of uterine endometrium exhibits the pre-cancer state of hyperplasia when treated with high dose estrogen [29] and is currently being developed as an *in vitro* organotypic model of carcinogenesis.

In vivo models

Hollow fiber assay (HFA) models

Compounds positive in the *in vitro* models are often advanced to screening in the more biologically relevant animal models. The hollow fiber assay (HFA) was developed to provide another level of screening in between cytotoxicity assays and the more expensive and time-consuming xenograft animal models [30]. In the HFA, cell lines are grown inside biocompatible hollow fibers that are then implanted either under the skin or in the peritoneal body cavity of immunocompromised (nude) mice that will not reject the tumor based on species differences. After a short-term treatment period of 4 days, the fibers are removed and evaluated for cell viability using a tetrazolium dye as in the *in vitro* cytotoxicity assay. In addition, the HFA is used to evaluate the effects of

drugs on their designated targets in the cancer cells, which can be retrieved from the fibers after treatment [30].

Xenograft cancer models

The human tumor xenograft model currently is the *in vivo* model that is most widely used to assess anticancer activity. Xenografts are established by injecting human cancer cell lines under the skin of immuno-compromised (nude) mice and monitoring the effects of drugs on the growth of the tumors. The suppression of the immune system is necessary to prevent the mice from rejecting the human tumors because they are from a different species. Multiple human cell lines are commercially available that can be used to provide consistent results when grown as xenografts. In general, it has been observed that *in vivo* xenograft activity does not correlate well with clinical activity [31]. Current efforts are focused on characterizing the cell lines to ensure that the molecular drug targets are expressed and on incorporating pharmacokinetic and pharmacodynamic studies in the *in vivo* testing [1]. Injection of the tumors into the organ site from which they were derived (orthotopic site) is considered to be a more relevant model because it more accurately represents the growth characteristics and pattern of metastases in a particular cancer type [32]. This model is more technically challenging than the subcutaneous model due to the greater difficulty of surgical implantation and measurement of tumors inside the body cavity as opposed to under the skin. A recent study found that 80 tumor explants established in nude mice and treated *in vivo* predicted tumor resistance in 97% and tumor sensitivity in 90% of cases [33].

Syngeneic cancer models

Immune deficiency is a significant limitation of the xenograft model because the immune system plays an important role in eliminating tumor cells. The syngeneic tumor model overcomes this limitation because the animals have a functional immune system. This is possible because the cancer cells injected into the animals are derived from the same species and thus will not be rejected. Very few cell lines have been established that can be used in syngeneic systems. A well-established model consists of the B16 melanoma cell line derived from the C57/BL6 mice. The ID8 ovarian cancer cell line, which is also syngeneic for the C57/BL6 mice and can be grown in the peritoneum or as subcutaneous isograft tumors, is especially important given the recent discovery that intraperitoneal administration of chemotherapy extends survival of ovarian cancer patients by about 16 months [34].

In vivo *models to study cancer development*

In vivo models of carcinogenesis using chemical carcinogens to induce specific types of cancer in animals are routinely used to test promising chemoprevention compounds [35]. Current trends are toward using the more recently developed genetically altered mouse models for *in vivo* studies of carcinogenesis [36]. Other species have also been used to develop *in vivo* models of carcinogenesis. The zebrafish model offers the benefit of being easily manipulated genetically and developed for high-throughput models screening [37].

In silico models

In silico models have been developed and utilized to study drug effects on enzymes and regulation of molecular signaling pathways [38,39]. This approach has not yet reached the level of complexity needed to incorporate the various responses and interactions of the multiple cell types in various stages of the cell cycle and differentiation present in tissue.

Model comparison

Organotypic models represent a step in between monolayer cultures and *in vivo* models (Table 1). Whereas the monolayer cultures are important for high-throughput screening of compounds and can provide information on the molecular mechanisms of drugs, they are at high risk of providing results that might not hold true in the 3D environments of tissues or whole bodies. Organ cultures provide a 3D model of functional tissue. Organ cultures of tissue explants from animals offer benefit over organotypic culture in that they exhibit functional nervous, muscular and cardiovascular systems. Drawbacks of organ cultures are that there are differences between the animals and humans, and human explants are more difficult to obtain. Reconstructed organotypic cultures have not yet been refined to accurately reproduce all of the physiologic systems present in organ cultures and human tissues, but they can be made from human cell lines and are more easily manipulated than organ cultures. *In vivo* models offer the opportunity to evaluate the therapeutic ratio by evaluating toxicity in the same experiment as efficacy. Also the multiple biologic effects that can modulate drugs effects, such as drug metabolism and distribution, will be incorporated in the results obtained from animal models.

Model translation to humans

The major issue with each model is its relevance to the human body. There are obvious differences between humans and other species used in experimental models. Despite the fact that the genomes of various species are highly conserved, the expression patterns of the genomes and posttranslational modifications of the proteins vary considerably resulting in significant biological variability. This results in considerable differences in the distribution, metabolism, excretion and biological effects of drugs between species. This has been noted in differences in toxicity and efficacy between the animal studies and clinical trials. There are also significant differences between humans and rodents in the requirements for transforming a normal cell into a cancer cell [40]. Use of

Table 1. Comparison summary table

Model	Advantages	Disadvantages	Best use of models	Refs
In vitro models				
Cytotoxicity assays	High-throughput is possible Multiple human cell lines are available and provide consistent results	Do not incorporate physiological systems Do not evaluate therapeutic index	Screening	[1]
Clonogenic assays	High-throughput is possible	Do not incorporate physiological systems Do not evaluate therapeutic index	Patient treatment decisions	[1]
Spheroids	Three dimensional interactions Multiple human cell lines are available and provide consistent results	Do not incorporate physiological systems Do not evaluate therapeutic index	Screening and drug mechanistic studies	[4]
Organotypic cultures	Can be use to study viral life cycles More readily manipulated than organ culture Multiple human cell lines are available and provide consistent results Can be used to study some immune functions	Do not fully integrate multiple physiological systems Do not evaluate therapeutic index	Drug mechanistic studies and identifying best compounds for progression to *in vivo* models	[13–29]
Organ cultures	Functional nervous, muscular and vascular systems can be manipulated and monitored	Difficult to obtain human specimens	Drug mechanistic studies and identifying best compounds for progression to *in vivo* models	[5–12]
In vivo models				
Hollow fiber assay	More rapid and easily manipulated than xenograft tumors	More expensive than *in vitro* models	Testing clinical potential of drugs for treatment of cancer	[1,30]
Xenograft tumors	Multiple human cell lines are available and provide consistent results	Do not incorporate immune system Not as easily monitored and manipulated as organ cultures Take longer than HFAs More expensive than HFA and *in vitro* models	Testing clinical potential of drugs for treatment of cancer	[1,31–33]
Syngeneic tumors	All physiological symptoms are intact	Longer tumor development time Not as easily monitored and manipulated as organ cultures More expensive than HFA and *in vitro* models Very few models available	Testing clinical potential of drugs for treatment of cancer	[1,34]
Chemically induced tumors in animals	All physiological symptoms are intact Can be used for carcinogenesis studies	Longest tumor development time More expensive than xenograft or syngeneic models	Testing clinical potential of drugs as chemoprevention agents	[35]
Genetically engineered	All physiological symptoms are intact. Can be used for carcinogenesis studies	More expensive than HFA and *in vitro* models	Testing clinical potential of drugs as chemoprevention agents	[1,36]

human cells and tissue for *in vitro* models is relevant at the cellular and tissue level, but can lead to false results due to the lack of physiological systems.

Conclusions

The drug discovery and development process has many approaches involving screening, structure-function studies and a decision tree that results in a lead compound. The choice of which model to use should be made on the phase of this process and the number of compounds to be tested. Monolayer cultures are amenable to high-throughput screening of large compounds.

Reconstructed organotypic models are intermediate between monolayer cell cultures and *in vivo* models with regard to biological relevance, ease of manipulation and monitoring and cost. There biological relevance is documen-

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ted by the successful use organotypic cultures in transplants. Although organ models have some current benefits over organotypic culture, human tissue slices can be difficult to obtain. Animal models are needed to evaluate *in vivo* activities and toxicities. The best approach is to use multiple models to increase the likelihood of accurate translation to humans and to avoid artifact.

Outstanding issues

- Can organotypic models be developed to incorporate functional physiologic systems?
- Can organotypic models be developed for high-throughput?
- Can a combination model using organotypic models grown as *in vivo* xenografts and host animals treated with drug be developed to increase biological relevance?

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