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A Three-Dimensional in vitro Model of Disease That Improves Preclinical Research by Incorporating Genetic Diversity and Increasing Physiological Accuracy

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ABSTRACT

Biomedical research is essential for the discovery of new medications and treatments, and is built upon the cooperation of preclinical (in vitro/vivo) research and clinical trials. However, 85% of treatments successful in vitro/vivo fail in clinical trials, suggesting that in vitro models are poor indicators of clinical success. The issue lies in conventional "two-dimensional" in vitro models containing genetically identical cells grown on a flat plate, which lack the variety of cell types and cooperation/structure found in real tissue. Moreover, 2D in vitro models do not simulate humans' genotypic variability, which affects both pathophysiology and treatment effectiveness. In contrast, 3D in vitro disease models (e.g. organoids/spheroids) contain the extracellular components, structures, cell-cell interactions, and microenvironment observed in human tissue, resulting in more physiologically accurate disease models. This paper consolidates current research of 3D in vitro models of varying complexities for different diseases to propose an effective and efficient solution for creating 3D in vitro models. Organoids should be the model of interest for organ/tissue-specific diseases and tumors, while patient-derived xenografts formed by implanting organoids into humanized mouse models should be used for studying body-wide disease/treatment effects. Growing organoids in prepared hydrogels allows them to mimic a human extracellular matrix and microenvironment, and adjusting the hydrogel's characteristics allows control over organoid growth/differentiation. Sourcing undifferentiated stem cells from patients of different ethnicities, ages, and socioeconomic statuses allows representation of diverse populations and corresponding epigenetics. Adipose stem cells are abundant and easily accessible with minimally invasive procedures.

KEYWORDS

in vitro • *in vivo* • 3D • disease models • genetic diversity • drug efficacy • organoids

Introduction

Biomedical research focuses on improving our understanding of the cells and tissues of the human body, in the hopes of finding treatments for the diseases that affect it. The current framework of advancing medicine and treatments is known as translational research, and involves preclinical lab research with cells to identify successful interventions/treatments, development of drugs or clinical treatments for use in patients, testing of these drugs in clinical trials, and implementation in mainstream medicine for widespread use. This translational process is essential for the discovery of new medications, drugs, and treatments. Without it, medicine would become a stagnant field, and any hopes of improved prognosis for current diseases would be stifled. Researchers constantly pursue further knowledge in disease and innovation of research methods to improve accuracy of lab findings and applicability of those findings to medicine in practice, to find new cures for diseases and improve the quality of life for patients with diseases.

Despite how simple and streamlined the translational research process may seem, it is not as successful as expected. Despite being preceded by successful, promising results *in vitro* and *in vivo*, about 85% of treatments fail in clinical trials, placing burdens on human test subjects for little in return (Beck & Meyerholz, 2020). While the translational research process has been intuitive, testing a wide variety of treatments in low-cost, consistent cell models of disease before moving forward with promising treatments in clinical trials and human/animal subjects, there is an overlooked issue in *in vitro* models and their correlation to the human population. Within the last two decades, biomedical investigators have realized that *in vitro* studies poorly predict treatment success in humans. Although there are many similarities between how cells, mice, and humans function, they cannot perfectly emulate each other, so they may respond differently to treatments.

While much of the focus of improving biomedical research has been on investigating new drug classes, characterizing new diseases, and developing new treatments, improving the way diseases are modeled at the preclinical level would help reduce wasted time and costs associated with translational research. Furthermore, increasing genetic and physiological similarity of *in vitro* disease models to humans would reduce the failure rate of clinical trials, quickening the discovery of new, successful treatments. Newer types of *in vitro* disease models and new sources of cells can help accomplish this goal by simulating human response to treatments with greater accuracy and better modeling the variation in genetic characteristics in tissue from different populations. With the increasing use of personalized medicine, which heavily prioritizes the genetic characteristics of a population and of a patient in the creation of a treatment plan, it is critical that drugs are tested on a wide variety of populations and that the differences in drug response between populations are acknowledged and factored into care.

Background

Biomedical research starts with preclinical, lab-based *in vitro* research, where disease is induced in cells. Researchers frequently purchase common cell lines from biotechnology companies, and use these to provide a consistent source of genetically identical cells. Traditional *in vitro* research involves

growing these genetically identical cells on a flat plate or well, resembling a Petri dish. The cells are left to grow until they cover the available surface of the plate, after which they are used for testing experimental compounds. The most important elements of *in vitro* research are the direct control over experimental conditions, the interaction with the disease on the cellular level, and the rapid timescale of experiments. Since cells take little time to grow and mature, any treatments, compounds, and ideas can be applied to the cells and tested very quickly *in vitro*. Lab conditions are highly controlled, to minimize confounding variables and external influences on experimental results. This ensures that findings are repeatable and consistent with other experiments in the field, providing validity to the results.

Treatments or molecules that have therapeutic success *in vitro*, by either improving survival or decreasing the severity of the disease, become a focus for further testing in more physiologically complex and accurate animal models *in vivo*. Mice resemble many of the systems, organs, functions, and features present in humans, making them an effective model organism for advanced research of many human diseases. However, since mouse models are more expensive and require more time to grow than pure cell lines, they are only reserved for treatments that had success *in vitro*.

Treatments that are reliably and consistently successful in animal models are candidates for therapeutic drugs that can be given to real patients. Once the treatment has been thoroughly researched and developed, highly successful treatments are administered to humans in clinical trials to gather more detailed information about the action of the drug, any possible side effects, and the most effective dose. Finally, after the drugs demonstrate their success and safety in clinical trials, they are approved by the FDA and can be mass produced by pharmaceutical companies and prescribed by doctors to improve the prognosis of patients (Cavaillon et al., 2020).

As a successful treatment is further developed, the model organism used to test the drug increases in complexity, from cells, to animal models, to humans (Cavaillon et al., 2020). This process, where simple and cost-effective models are used with ambitious, risky treatments, while more expensive and realistic animal models are reserved for promising treatments, is called translational research, and forms a framework for biomedical research that increases efficiency and reduces the time needed for new drugs to enter the industry.

Conventional Two-Dimensional *in vitro* Models

In general, conventional *in vitro* disease models, which consist of a group of homogenous, genetically identical cells spread across a plate, are least successful in simulating the function and characteristics of human tissues and diseases. Méry et al. (2017) describe the specific shortcomings of two-dimensional *in vitro* cancer research, writing:

> The artificial environment limits cell-cell interactions and thus prohibits a number of the physiological processes present in solid tumors… Basic research is also necessary to achieve an understanding of the HNSCC pathological state, but cannot, in itself, provide sufficient information for clinical applications. (p.53)

These shortcomings further elucidate how findings in *in vitro* studies may not truly

represent treatment outcomes in humans. A functioning tissue or system in a living organism has a wide variety of cells that communicate with each other and work together, and the lack of this complexity in two-dimensional *in vitro* models gives rise to its poor predictability in translational research.

Impact of Genetic Variation on Treatment Efficacy

There is consensus that patients of different ages, lifestyles, and genetics experience different levels of success with treatments and different predispositions to disease (Polimanti et al., 2014). However, the patient's genotype, or genetic code, also determines the characteristics and progression of the disease, leading to slightly different pathologies and severities in different patients. Humans are incredibly genetically diverse, encompassing a wide range of genotypes and phenotypes, and may respond to particular treatments in different ways courtesy of this genetic variation, regardless of differences in the state or characteristics of the disease.

In addition to the fact that different ethnicities and races have slightly different genotypes, elements such as the environment a patient grew up in, the food and contaminants they were exposed to during growth and development, and other social and environmental factors can also affect their epigenetics, or modifiable gene expression patterns (Dupras et al., 2014). Gene expression accounts for the relationship between the genotype and phenotype of an organism. Although two individuals may have the same trait coded for in their genes, the intensity or prevalence of the trait may vary as a result of differences in the surrounding environment. The intensity of a trait is a result of the level of protein synthesis of that gene, and the level of protein synthesis can be determined through epigenetics, instead of being coded for in the organism's genes.

Polimanti et al. (2014) highlight the fact that while the field of pharmacogenetics currently investigates the correlation between certain genes and trends in the effectiveness of certain drugs, putting this knowledge into practice is not as straightforward, writing:

> Although hypertension pharmacogenetic data promises to play an important role in patient management, this information is not yet ready to be transferred from the bench to the bedside. Indeed, several confounding factors are present in the correlation between genotype (i.e., genomic background) and phenotype (i.e., response to antihypertensive drugs), such as environment and epigenetics. (p. 157)

Specifically, Polimanti et al. (2014) reviewed medications prescribed for hypertension, and found that genetic and environmental differences between Africans and non-Africans resulted in significantly lower average effectiveness of atenolol and irbesartan, and increased frequency of side effects from irbesartan. The next greatest difference in drug response was found between Europeans and Asians. These findings demonstrate the importance of considering genetic and environmental differences in target populations when researching diseases. Since *in vitro* models of diseases are ubiquitous in preclinical research and are heavily utilized in preliminary experiments of new treatments, they should also replicate the genetic and epigenetic variation observed in the

human population as well, rather than just the conditions of the disease.

A Solution: Three-Dimensional *in vitro* Models

Researchers aiming to improve upon conventional two-dimensional cell cultures and develop more realistic *in vitro* disease models have developed a new type of cell culture that is three-dimensional. Sflomos et al. (2021) describe how three-dimensional cell cultures of cancer cells differ from two-dimensional ones, noting that, "3D culture formats… more faithfully reflect the intra-tumoral heterogeneity and the spatial, biochemical, and mechanical properties of the malignant tumor than 2D plastic dish cultures," emphasizing the structural and molecular complexity that three-dimensional cell cultures are capable of modeling. This complexity is essential for proper function of cells and tissues in real organisms, and must be modeled *in vitro* as well.

Organoids and spheroids are some examples of types of three-dimensional cell cultures. Spheroids are more simple: they assume a spherical shape and are mainly used in tumor modeling. In comparison, organoids form complex, spherical layers that resemble actual organs. Jensen et al. (2021) summarize the popularity of three-dimensional cell cultures, writing, "three-dimensional (3D) cell culture methods have proven to be incredibly useful models to study various types of cancers due to their improved accuracy over 2D culture methods," using cancer as an example to describe the superiority of three-dimensional cell culture models in preclinical research (p. 4). They describe how organoids achieve these properties, "by forming extracellular matrix (ECM) fibers that link single cells together via integrin binding and mimic the microenvironment of certain organs to allow researchers to model human diseases" (Jensen et al., 2021, p. 8). The microenvironment, which is the biochemical environment around and in between the cells that places the tissue in the larger context of the organism, is just as important as the cell-to-cell connections, all of which is missing at the two-dimensional level. The intercellular interactions present in organoids and spheroids are key to accurately resembling the physiology and function of real patient tissue.

Since organoids need some sort of elementary "scaffold" to grow on, in the form of a basic ECM that resembles the tissue, a growing material or surface is necessary to ensure proper growth and development of the organoid. According to Argentati et al. (2018), this biomaterial needs to be biocompatible and biodegradable through bodily functions when needed, and must have resemblance to the structure of the tissue itself. Without taking this step, organoids would not have competent function, and could not perform the role the organ was intended for. Through experimentation with various biomaterials, researchers have found ways to use hydrogels to achieve a successful growing surface for the organoids. Hydrogels are gel-like substances that can hold large amounts of water. A well-known example within the human body is collagen, which connects different tissues in the body and maintains the structural integrity of organs. Other examples include soft contact lenses, gelatin, etc. Hydrogels are important for allowing organoids to grow properly and develop the ECM around them, since they are "unique due to their ability to mimic the ECM while allowing soluble factors such as cytokines and growth factors to travel through the tissue-like gel" (Jensen et al., 2021, p. 9). The cytokines and

growth factors are vital for precise differentiation of the stem cells in organoids, as they assume the various roles that they would in a real organ. As Argentati et al. (2018) explain, "the modification of physical and chemical properties of biomaterials (e.g., dimensions, shape, mechanical properties, and surface structure)… promotes and assists adhesion, proliferation, and differentiation process of stem cells" (Argentati et al., 2018, "Adipose Stem Cells and Tissue Engineering"). These molecular determinants of proper growth and development need to be controlled by scientists, so that the stem cells can differentiate into the correct types of mature cells. From here, the stem cells are able to differentiate, self-organize, position themselves accordingly, and bolster the ECM around them to create a complete, functional organoid. Jensen et al. (2021) found that their organoids "yielded more accurate results, and the effect of the drugs on cells growing in 2D culture was exaggerated… proliferation rates and cell-to-cell interactions in the 3DP model were higher than in the 2D model," further providing validity to the benefits of organoids over two-dimensional cell cultures in modeling tissue function and activity (p. 12). Overall, organoids and spheroids provide researchers with more physiologically accurate model systems, and their growth, characteristics, and function can be controlled with the use of hydrogels. These advancements have allowed researchers to make progress in *in vitro* disease modeling, working toward implementing genetic diversity and performing more realistic experiments.

Implementing Genetic Diversity *in vitro*

Genetic diversity can be modeled in organoids by strategically choosing the source of the stem cells used to grow the organoids, making selections based on ethnicity, age, gender, socioeconomic status, or any other demographics that researchers want to test separately. Researchers can develop organoid resources by making specific sets of stem cells or organoids that represent patients of these demographics or with other medically pertinent characteristics, such as pre-existing conditions or comorbidities (Barbuzano, 2017). As Jensen et al. (2021) elaborate, "Researchers can grow tumor models using organoids through the use of patient-derived tissue cancer cells, effectively allowing scientists to model tumors to test treatments on a patient-to-patient basis," further implicating the benefits that organoids have with regard to simulating diseased patients, rather than just the cells of a disease in general (p. 8). In this manner, organoids would provide a quicker, easier way to determine how patient characteristics affect treatment success, by using a model that is more lifelike and physiologically and structurally accurate than a plate of homogenous cells.

Organoids are also remarkably stable when implanted in *in vivo* models such as mice (Cavaillon et al., 2020). Researchers use this property to develop patient-derived xenograft (PDX) models of disease, which maintain the benefits of human cells while also placing them in the biological context of a living organism, all without the use of real human patients. Stem cells harvested from patients would be implanted in humanized mouse models to study the effects of disease and treatment from the biological perspective of a human. A human organoid can integrate into the mice's living systems and sustain itself, while still functioning as a human body part. By harvesting human stem cells from different populations, experiments can be conducted with the

diversity of human populations in mind. This is especially important when studying interactions between the patient's other body systems, such as the immune system (Cavaillon et al., 2020). The patient's other biological systems are innately unique and tied to the genetic makeup of that patient, adding another layer to the biological context discussed earlier. A particular disease may behave differently through the interactions it has with the host, or the patient, and the patient's interactions with the disease are equally as important to consider when studying treatments and how they treat systemic effects or symptoms in addition to the disease.

The ability to grow organoids relies on the availability of stem cells, which have the ability to mature and differentiate into many different cell types within an organism. They are the reason that a single-celled, newly fertilized zygote can grow and develop into a biologically and structurally complex organism like a human. Since stem cells are precursors to more advanced, specialized cells, harvesting and storing these cells will increase in importance, especially as organoids become more popular and common in *in vitro* research. As the demand for stem cells increases, concerns may arise regarding the ethics of stem cell sources, as well as whether the stem cells must be sourced from the specific organ of interest that is being studied.

Adipose Stem Cells (ASCs) are stem cells found in adipose (fat) tissue, and are commonly used in *in vitro* research and medical applications where tissue composed of mature/advanced cells needs to be regenerated. They are reliable and can differentiate into many types of cells, including fat, bone, cartilage, nerve, skeletal muscle, tendon, cardiac muscle, and vessel lining cells (Argentati et al., 2018). Adipose tissue is relatively abundant in the body, and located in many different parts of the body, providing a rich source of stem cells (Argentati et al., 2018). Due to their abundance and assortment within the body, procedures to harvest the stem cells from adipose tissue are minimally invasive and allow safe, ethical isolation of stem cells. This makes isolation of stem cells practical and feasible, so that organoids can be developed without major difficulties.

Stem cells, including ASCs, have the ability to renew and replenish themselves, in addition to being able to differentiate and mature into specific types of cells. This allows researchers to maintain a consistent source of stem cells after collecting them from a patient once (Argentati et al., 2018). Whether the stem cell differentiates or undergoes self-renewal is controlled by the stem cells' extracellular environment, which can be manipulated by researchers. Including certain signaling molecules can induce differentiation of stem cells into a specific type of cell, resulting in the formation of coordinated tissues. Regenerative medicine and tissue engineering rely on similar biological techniques and concepts to create new tissue. Regenerative medicine uses tissues formed from ASCs to replace diseased and/or dysfunctional tissues in patients (Argentati et al., 2018). The lab-generated tissue is implanted into the patient, and grows alongside the patient's existing tissue. The same concept can be applied to PDX models of disease by implanting the human tissue grown from ASCs into mice. Since harvesting ASCs is less invasive than harvesting stem cells from harder-to-reach areas, harvesting stem cells can become a simpler procedure that can be more widely conducted to gather samples from people of different genetic makeups within a population.

Even if all organoids are not made with ASCs, their relatively simple isolation provides an avenue for simplifying research regarding the impact of genetic diversity on diseases and treatment.

The ability to grow patient-specific organoids by using the patient's stem cells allows clinicians to perform research for the practice of personalized medicine, reducing costs on patients and on the healthcare industry. Personalized medicine is an approach to medicine that recognizes the genetic and epigenetic differences between individuals, as well as the differences in treatment efficacy that come with it. Personalized medicine relies on a reservoir of data and information about specific genetic markers and their effects on drug response to guide clinicians to the most effective treatments and doses for each patient. This data is primarily collected through frequent, large-scale clinical trials, or years of traditional cancer therapy, which are expensive and burdensome to patients (Benz, 2017). Using organoids or PDX models would allow researchers to model a specific disease and organ of interest and gather data to populate these databases, without having to put the patients themselves through treatments. Rather than subjecting the patient to the side effects of a particular drug or treatment, without a guarantee that the treatment will improve a patient's condition, clinicians and medical scientists can use organoids to simulate the patient's tissues or organ systems and apply the drug or treatment to the organoid instead (Barbuzano et al., 2017). Sflomos et al. (2021) express a similar view, claiming that new PDX models of disease "may improve the prediction of the therapeutic efficacy of novel agents" and will "provide understanding and identification of… new therapeutic targets, and conceivably personalized cancer therapy." Stem cells can be harvested from the patient's organs, and the organoid would be grown in the lab. This way, the efficacy of the drug can be tested in a genetically and structurally accurate cellular environment. Many of these organoid drug trial experiments could be conducted simultaneously, testing a variety of drugs or compounds on multiple organoids grown or developed to model certain populations, patients, or characteristics. This would result in precise results, unique to the populations whose cells are used to make organoids, while ensuring an improved quality of life for patients in need of treatment.

Redeeming Qualities of Two-Dimensional Disease Models

While three-dimensional *in vitro* cell culture models can better replicate the physiology, drug response, and genetic diversity of humans, conventional two-dimensional cell culture disease models still have important benefits that prove their utility. Méry et al. (2017) underline some of these benefits, writing, "Advantages… include sample homogeneity and cost as well as it allows to avoid legal and ethical issues associated with animal experimentation. Furthermore, technical improvements have led to successful permanent culture of HNSCC cell lines, involving feasibility and reproducibility" (p. 53). Researchers can quickly experiment with novel, ambitious ideas, since garnering new cells from a cell line for a homogeneous, two-dimensional cell culture is a relatively quick and inexpensive process. In fact, this process is known as high-throughput screening, and is commonly used at the *in vitro* level. High-throughput screening is a research method in which a certain experimental procedure, like testing the

effect of a drug on cells, is standardized and repeated for many different treatments, with experiments being conducted in parallel. Robots can also be used to automate some of the repetitive, tedious steps of these experiments (Falk, 2020). High-throughput screening helps eliminate treatments molecule by molecule, ultimately narrowing down effective drug classes so that scientists can specifically study these in more detailed, thorough experiments using "three-dimensional" cell cultures or *in vivo* disease models. Unlike a three-dimensional cell culture or *in vivo* model, two-dimensional cell cultures do not need time and extraordinary maintenance to grow and develop before the treatment can be applied.

Furthermore, two-dimensional *in vitro* models are superior with respect to the number of experimental variables or factors that can be controlled. With a plethora of established, historically reliable cell lines with isolated genotypes and characteristics, researchers can purchase specific cell lines with premade characteristics for use in their study (Méry et al., 2017). Any variations that come with how stem cells grow into three-dimensional organoids, or how mice models incorporate an implanted organoid, will not be present in two-dimensional cell culture models, reducing sources of error and making results more precise and consistent. Because of this control over exact cell, tissue, genetic characteristics, and environmental/cellular conditions, a lower sample size can lead to similarly robust results, saving time, money, and resources in preliminary experimentation (Beck & Meyerholz, 2020).

Despite these benefits, organoids are comparatively more dynamic and realistic, allowing generation of cancer models with genetic variation. However, a major concern with organoids is whether their phenotype, genetic profile, and characteristics can remain consistent enough over the course of a study (Lee et al., 2021). In diseases like cancer, for which researchers may be interested in identifying broad disease or treatment effects, such as altered cell survival, alterations to the cell cycle, and other simple phenomena on the cellular level, two-dimensional cell culture models are sufficient (Méry et al., 2017). This is because two-dimensional cell cultures are better able to reveal specific cellular responses to conditions, disease, and treatments. In addition, their chemical secretions, biological activity, and growth patterns can be analyzed in shorter experiments than in organoids.

Thus, preclinical research can be broken down into two main areas: the study of cellular mechanisms for disease research and the study of new treatments or medicinal compounds for patient improvement and survival. While both of these types of research areas use the translational research framework, taking advantage of cells *in vitro*, animal models *in vivo*, and humans in clinical trials, it is important to differentiate these types of research at the *in vitro* level. When the objective is to characterize a certain disease, two-dimensional cell cultures and highly controlled cellular/lab conditions should be used. When the objective is to devise treatments that have a therapeutic effect on the human health while limiting side effects, three-dimensional cell cultures and physiological accuracy in a larger biological context should be used. Drugs and treatments need to treat humans who have disease, not just the diseased cells themselves. Thus, the objective of the research determines the research approach and the type of *in vitro*

model used, to best suit the goals of the study.

Conclusion

The concept of *in vitro* research is still very essential and important. However, the specific method/model used to carry out *in vitro* experiments should be changed from the traditional two-dimensional cell culture to a more representative one: a three-dimensional cell culture. With importance being placed on having genetic diversity in translational research and optimizing treatment success, organoids and patient-derived xenografts offer the best options for achieving an *in vitro* model as close to real patient disease states as currently possible. Three-dimensional cell cultures continue to dominate *in vitro* research due to the ability to transplant them into model organisms. Adipose tissue is a particularly useful source of stem cells for this purpose.

Some researchers wish to further expand the role of organoids in biomedical and translational research, advocating for diminishing the role of expensive animal models and valuable human subjects in experimental studies of drugs and treatments. According to Cavaillon et al. (2020), the "refinement, reduction, and replacement of animal models should be strongly encouraged. *In vitro* or *ex vivo* studies can be performed directly on human cells and tissues, and… complemented by more sophisticated and robust human organoid and organ-on-chip models." Developing more accurate and resilient organoids, as well as developing additional methods with hydrogels to more finely customize the characteristics of the organoids may decrease the need for highly specialized mouse models, helping bridge the gap between preliminary *in vitro* research and clinical trials. The customization of these three-dimensional models would make translational research even more time-effective and cost-efficient.

In the future, the development of new treatments might take a small fraction of the resources they do today, and successful drug candidates may emerge on a regular basis. This progress, combined with advancements in the field of personalized medicine, hopes to ensure that each patient is given the most effective drug and dose possible. Soon, cancer, dementia, Alzheimer's, and other diseases that have challenged medicine for decades may have their very own drugs that were identified, researched, and developed with the help of organoids.

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