

Advances in *in vitro* Tumor Models and Genetic Engineering in Cancer Research

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Cancer stands as one of the leading worldwide health threats because it causes approximately 16% of all global deaths, according to the World Health Organization (WHO, 2025). This review discusses the critical role of various tissue culture techniques in studying fundamental and advanced methods used in cancer research in identifying new therapeutic targets. There are several methods to study cancer cell biology and molecular mechanisms, such as traditional two-dimensional (2D), which has long been the gold standard, and more advanced three-dimensional (3D) culture systems that better reflect molecular characteristics, cell-cell interactions, and signaling pathways. Additionally, genetic engineering tools like Clustered regularly interspaced palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9) provide targeted genome editing and RNA interference (RNAi) based approaches such as small interfering RNAs (siRNAs) for the transient gene silencing. These genetic engineering tools offer promising models for investigating the drug response, therapeutic targets, and in cancer research.

Keywords: 2D cultures, 3D bioprinting, 3D cultures, CRISPR/Cas9, Small interfering RNAs (siRNAs).

Cancer is a deadly disease globally due to the genetic and epigenetic alterations. Cancer is one of the largest global health challenges in the 21st century. The World Health Organization reports that there were 9.7 million cancer deaths in 2022, a number that is predicted to rise to over 35 million new cases by 2050, mainly due to an increasing aging population, unhealthy lifestyles, and environmental exposures.¹ Even with considerable improvements in the three pillars of cancer treatment, namely surgery, chemotherapy, and radiotherapy, as well as recent

advances in immunotherapy, many cancers remain poorly treatable.² In preclinical studies, however, existing models show limited relevance to human disease. Conventional two-dimensional (2D) cell cultures cannot mimic the three-dimensional (3D) environment of solid tumors, with their tissue structure and mechanical properties, nor can they account for the complexity and heterogeneity of multicellular tumor ecosystems.³ In this review, we describe three research axes of research and development that are being used in concert to overcome these limitations in cancer modelling and

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drug discovery. We cover tissue culture systems, ranging from classical 2D cell lines to more complex 3D organoids, spheroids, and scaffold-based tissue models, as well as gene editing tools and RNA interference, mainly using the Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9 (CRISPR-Cas9) system for cancer functional genomics. The study of oncogenes and tumor suppressor genes can help understand cancer biology, which regulates cell proliferation, apoptosis, and differentiation. Tissue culture models can investigate the mechanisms *in vitro*. Spheroids and organoids are advanced tumor models that mimic the tumor environment, which helps to understand the cancer biology.⁴

Functional assays such as proliferation, migration, and apoptosis assays are commonly used to understand the nature of the cancer cell and the impact of genetic alterations.⁵ More advanced genetic engineering tools, such as CRISPR-Cas9, are powerful gene-editing tools that aid in the modification of oncogenes and tumor suppressor genes for studying their functional role in cancer.⁶ RNA interference (RNAi) is a gene silencing technique that helps in the identification of gene function and promising therapeutic targets. Both technologies have offered promising methods for cancer research.⁷ Two domains of technological advance, namely, tissue culture systems and genetic engineering, are not mere additional technologies for oncology research. Rather, these two areas are intimately interconnected and form part of an integrated system of research. Tissue culture systems offer experimentally relevant biological systems; genetic engineering allows specific interference with cancer-related pathways.

This review highlights an integrative transformation of advanced *in vitro* culture systems and genome engineering technologies to resolve major constraints in conventional cancer research. By integrating physiologically relevant 3D tumor models such as organoids and spheroids with precise gene-manipulating tools like CRISPR-Cas9 and RNAi, the field is moving towards more advanced cancer therapeutic strategies.

Introduction to Tissue Culture Models

Tissue culture is the fundamental technique in cancer research that enables us to understand the cellular and molecular mechanisms involved in cancer progression. It provides a

controlled environment to assess the effects of drugs, study gene alterations and analyze signaling pathways. There are various tissue culture techniques available to study the role of oncogenes and tumor suppressor genes in cancer biology. Here, we are exploring the key techniques used in cancer biology.

Tissue Culture

Animal cell culture is a standard procedure for culturing and maintaining animal cells, tissue, or organs in an artificial, well-regulated environment.⁸ It is an ideal practice to study cellular processes while avoiding animal model usage. The cells are maintained in standard culture dishes with a modified surface that provides support for cellular attachment and proliferation.⁹ It allows us to control chemical and physical parameters such as temperature and osmolarity, providing an ideal environment for cell growth.¹⁰ Media is an important factor in cell culture technology, as it provides the nutrients and supplements required for cell growth. Cell culture media is classified into natural media and synthetic media, and the latter is most widely used for research. The basal media are used with supplements like fetal bovine serum that facilitate cell growth *in vitro*.¹¹ On the basis of cells cultured, primary cell culture is the one in which the tissue constructs are procured from animals, enzymatically separated using proteolytic enzymes, and strictly maintained under controlled aseptic conditions that favour *in vitro* proliferation.⁹ Secondary cell culture, on the other hand, is established cell lines that are derived primarily after subculture of primary cells once it reaches confluency. Cell culture can be broadly classified into 2D and 3D culture systems based on the substrate used for culturing the cell. Compared to traditional 2D culture, 3D cell cultures mimic the *in vivo* condition in a more sophisticated manner, generating reproducible results. The transition from 2D to 3D cell culture is crucial in studying the tumor microenvironment, cell signaling pathways, and drug response, which are relevant in cancer research.³

Studying the oncogenes and tumor suppressor genes in tissue cultures

Cellular assays in 2D culture environment

Cellular proliferation assays provide a sensitive means to examine cell viability and cell proliferation.¹² Figure 1 depicts the

schematic illustration of the cellular assays. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay utilizes the mitochondrial reductase activity of viable cells to convert a soluble tetrazolium salt MTT, into an insoluble formazan product, which can then be solubilized and quantified spectrophotometrically.¹³ The intensity of the resultant coloured solution is directly correlated with the quantity of live cells.¹⁴ Other assays include CellTiter-Glo, which measures ATP levels using a luminescent signal, and the BrdU (5-bromo-2'-deoxyuridine) assay, which detects newly synthesized DNA through antibody labelling. These assays are essential tools for drug discovery, understanding resistance mechanisms, and personalized medicine approaches.¹⁵⁻¹⁸

Cell migration is the movement of a cell from one site to another between cell-to-cell and cell-extracellular matrix.¹⁹ It is commonly elucidated by assays such as the wound healing assay and the transwell migration assay. In a wound healing assay or scratch assay, a scratch is made in a 100% confluent cell layer, and the closure rate indicates the migratory ability.²⁰ Trans-well invasion assays are used to track the migration and penetration capacity of tumor cells by establishing a chemoattractant gradient.²⁰ The study of migratory properties of oncogenic cells is necessary since the majority of cancer deaths are due to metastasis, and an understanding of cell migration paves the way for targeted therapeutic approaches.²¹

Cell cycle assays are techniques used in examining cell division and DNA replication by analysing the different phases of the cell cycle – G1, S, G2, and M.²² The BrdU incorporation assay helps to measure cell cycle phases, based on the ability of bromodeoxyuridine to integrate into newly synthesized DNA during the S phase of the cell cycle.^{23,24} Another technique, Propidium Iodide (PI) staining, intercalates into DNA and emits fluorescence that is directly proportional to the amount of DNA present.²⁵ Flow cytometry, coupled with PI staining, provides a reliable representation of the cell cycle distribution. Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) is an approach that uses fluorescent proteins to visualize and monitor cell cycle progression.^{26,27} These assays serve as indispensable tools in cancer research, providing

critical insights into the mechanisms driving tumorigenesis and offering opportunities for the development of novel therapeutic strategies.²⁸

Apoptosis is a cellular mechanism that controls cell death and maintains tissue homeostasis.²⁹ Apoptosis is regulated by a group of enzymes known as caspases and diverse apoptotic factors.^{30,31} Caspase assay relies on the intrinsic proteolytic ability of the enzyme caspase, and its quantitative analysis is performed by employing a trans-peptide that flanks caspase-specific cleavage sites, conjugated with a chromophore or fluorophore. Upon the specific enzymatic cleavage, the release of fluorogenic or chromogenic substrate is achieved, and the intensity is quantified, which showcases caspase activity.³² The Annexin V staining method relies on the high binding affinity of annexin to the phosphatidyl serine on the outer membrane of an apoptotic cell. When labelled with a fluorescent probe, the complex becomes a reliable tool to quantify cell death.³³ Acridine orange/Ethidium bromide (AO/EtBr) is a multiplex assay that clearly visualizes and distinguishes different cell phases based on the cell membrane permeability of the dye. Normal and early apoptotic cells emit green fluorescence, while late apoptotic and dead cells emit orange-red fluorescence.³⁴

Advanced Three-Dimensional Culture Models Spheroids

Spheroids are cell or tumor aggregates that are grown in a 3D space. These spheroids mimic *in vivo* tumor niches, and thereby these models serve as crucial *in vitro* models for studying tumor initiation and development.³⁵ Figure 2 depicts the establishment of spheroids and organoids. Spheroids are widely employed as *in vitro* models in various research domains such as tumor biology, cancer biology, and immunology.³⁶ Distinct cellular populations are generated during spheroid formation, with a clear distinction between the outer layer consisting of actively proliferating cells, quiescent cells that dominate the middle intermediate layer, and the core of spheroids populated by necrotic or hypoxic cells.⁸

The hanging drop method is a technique that is used to generate spheroids where small droplets of cell suspension are introduced onto a non-adhesive flat surface that stimulates cells to aggregate towards the bottom of the droplet, forming spheroids.³⁷ A three-dimensional spheroid

of size 1.5 mm from human colorectal carcinoma, HCT116, was developed using the flipped well plate hanging drop technique, and cell-cell and cell-extracellular matrix interaction studies were performed.³⁸ A further improved version of the hanging drop method, where a methyl cellulose polymer is introduced into the hanging drop culture medium, and a pancreatic 3D spheroid model was developed. The modified hanging drop method offers several advantages over the classical hanging drop method. In this pancreatic 3D spheroid model, the molecular aggregates were tightly packed, structurally stable, and resistant to mechanical stress.³⁹ Another approach is the spinner cell culture techniques that foster spheroid formation by employing the usage of certain equipment such as spinner flasks and rotating bioreactors, where the cells are subjected to constant agitation. The constant agitation favours the initiation of cell-to-cell contact and self-assembly of cells in the absence of a cell attachment surface.⁴⁰ A multicellular tumor spheroid model using SK-Hep-1 (Sloan-Kettering Hepatoma-1), human hepatoma cells, was developed by culturing SK-Hep cells in a siliconized cell spin flask. Cellular aggregates were observed during the first 48 hours, and over the following two weeks, the cell aggregates attained a distinct spheroid shape. The model served as an ideal *in vitro* model for nutrients, specifically the glutamine transport rate.⁴¹ A multicellular tumor spheroid of human adenocarcinoma cells, the HT29-based 3D spheroid model was developed using spinner culture technology. The *in vitro* tumor model served as a reliable model for investigating the effects of anticancer drugs and evaluating the efficacy of different drug delivery systems.⁴² In some cases, a scaffold system is utilized for spheroid development that supports cell-to-cell and cell-matrix interactions, guiding tumor formation.²⁸ These scaffolds mimic the extracellular matrix (ECM), rendering physical support, thereby enhancing the growth of the cell. Various factors such as biocompatibility, biodegradability, surface attachment, bioactivity, and their ability to transport oxygen and nutrients should be considered. Hydrogel-based scaffolds are networks of hydrophilic polymers that are derived from natural components such as alginate, gelatin, and chitosan, as well as prepared from synthetic polymers like polylactide (PLA), polyglycolide (PGA),

polycaprolactone (PCL) etc.⁴³ Hydrogels such as Matrigel, with extracellular matrix components such as collagen, are widely employed as a 3D matrix.¹⁰ A 3D bioengineered hydrogel culture was constructed to investigate the interaction between patient-derived glioblastoma cells and a hydrogel with extracellular matrix containing hyaluronic acid. This interaction was crucial in conferring chemotherapeutic resistance in glioblastoma. This model renders valuable insights about apoptosis mechanisms, DNA damage, and proliferation rates after being subjected to chemotherapeutic drugs.⁴⁴ Advanced spheroid development techniques include microfluidics-based cell culture systems wherein the cells are nurtured in chambers with interconnected microchannels. This facilitates oxygen and nutrient transfer and also facilitates precise control of *in vitro* tumor development.⁴⁵ Using microfluidics technology, a tumor microenvironment was constructed by a blend of pancreatic tumor spheroids with stellate cells, which were nourished in interconnected microchannels of the microfluidics device. The model successfully elucidated the paracrine- and juxtacrine-driven cell-cell interactions, the epithelial-to-mesenchymal transition, and the drug resistance mechanism.⁴⁶

Spheroids offer a wide range of applications, especially in cancer research for studying various signaling pathways, metastatic properties of tumors, drug delivery systems, analysing drug efficacy, and elucidating the underlying mechanism of drug resistance.⁴⁷ The well-differentiated layers in spheroids provide more insights into cell signal transduction and cell-to-cell communication, thereby providing a better understanding of tumor progression and tumor metastasis. Spheroids can be used to evaluate the efficacy of anticancer drugs, their mode of action and toxicity, and the data generated are more reliable compared to traditional 2D cultures.

However, certain limitations are associated with spheroid cultures. One of them is the significant increase in dimensionality of the spheroid culture system that may lead to variability in the distribution of oxygen, nutrients, and waste products.⁸ This heterogeneity leads to misinterpretation and limits reproducibility and reliability of results.⁴⁸ There is also a requirement for high-resolution imaging facilities for constant spheroid monitoring.⁴⁹ To

address these limitations associated with spheroid culture, various advancements in biomaterials, microfluidics, and imaging are employed to overcome these constraints of spheroids.

Organoids

Organoids are 3D culture models that are grown in laboratory culture conditions using different cell sources, including adult stem cells (ASCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and patient-derived tumour cells or tissues.⁵⁰ Unlike conventional 2D cell cultures, which grow as flat monolayers, organoids are able to form 3D structures that mimic the real architecture of organs and tissues in the body. So, this potential makes organoids a powerful tool in research field.⁵¹ There are human organoids successfully developed, such as the brain, gut, liver, kidney, thyroid gland, and heart. These developed organoids resemble the structure and functional aspects of the corresponding organ in the body.⁵² ASCs are established by culturing stem or progenitor cells isolated from adult tissue under 3D culture conditions. These organoids are more resemblance to adult stem cells and are useful for studying disease mechanisms, tissue biology, and regenerative medicine. Tumor derived organoids also known as tumoroids are derived from tumor tissue obtained from procedures such as biopsy or surgical resection and it retains the histological, molecular, and genetic characteristics of original tumor. Therefore, tumoroids are valuable models for investigating the tumor biology and discovering potential therapeutic drug response.⁵⁰

Organoids have wide application in developmental biology, tissue engineering, cancer research, and drug discovery. The process of development of organoids involves a stepwise differentiation process. The selection of appropriate stem cells is the initial process. Next, these cells are embedded in a supportive scaffold or matrix, which helps them to grow in three dimensions. Along with these, growth factors and nutrient-rich media will provide the differentiation and maturation of cells into a complete organoid structure.⁵³

Initially, the stem cells are directed to form three germ layers, such as ectoderm, endoderm, and mesoderm. These germ layers are fundamental in embryonic development and give rise to different organs and tissues in the body. The ectoderm is responsible for forming the nervous system and

skin, the endoderm develops into the internal organs such as the liver, pancreas, stomach, lung, thyroid, and intestine, and the mesoderm gives rise to the heart and kidney.⁵⁴ Researchers can generate the organoids by mimicking the natural development process in laboratory conditions. Organoids can be used as valuable tools in research fields, including drug screening. Since organoids can be developed from patient-specific cells, they can be used to predict patient-specific drug responses.⁵⁵ Organoids can help to understand an individual's cells' response to different treatments, so it is often employed in studies associated with personalized medicine. This approach will improve patient outcomes due to disease conditions.⁵⁶ Organoid technology faces a major restriction because it lacks functional blood vessels, which results in tissue death and restricts organoid development to small sizes. All tissues require nutrient and oxygen delivery and waste product removal through complex branching vascular networks.⁵⁶

Enhanced recapitulation of Tumor microenvironment complexity in 3D culture systems

The tumor microenvironment (TME) consists of a group of malignant cells, non-malignant cells, blood cells, immune cells, along with the ECM secreted by the specific cell type. The cellular interaction among these cells mediates the complex cell signaling pathway, permitting cancer metastasis and tumor progression.⁵⁷ Monolayer cultures fail to display cell-cell interaction and cell-extracellular matrix interaction, signalling pathways, and unequal oxygen distribution, leading to tumor hypoxic regions along with the ability to initiate tumor heterogeneity.⁵⁸ The 3D culture models such as spheroids and organoids mimic the hallmarks of TME, the oxygen gradient, vascularization, and cellular heterogeneity.⁵⁹ Spheroids precisely establish the oxygen gradient and ECM architecture, while the organoids recapitulate the cellular heterogeneity.⁵⁹ Vascularization is essential for oxygen and nutrient delivery and the removal of waste in cultures. The integration of vasculature could increase the size of the spheroids and organoids. Vascularization strategies are classified as internal or external. Internal induction relies on cell-driven differentiation and self-organization,

while external induction uses growth factors, medium composition, and hydrogel properties. Both methods will enhance vascularization and tissue integration.^{60,61} The absence of blood vessels prevents organoids from growing beyond three millimetres because their central areas become oxygen deprived and die from lack of oxygen. The development of 3D-bioprinted tumor models using patient-derived tumor cells and microenvironmental and immune cells has solved the essential problem of tissue vasculature.^{60,62}

The 3D bioprinting technology enables the layer-by-layer fabrication of complex tissues and organs with precise structural organization.^{63,64} 3D bioprinters are classified into three main categories: inkjet-based, laser-based, and extrusion-based bioprinting. Inkjet-based bioprinting is a cost-effective and accurate method. It deposits bioink as droplets or microspheres but is limited by nozzle clogging when printing dense bioinks. Laser-based

bioprinting uses a nozzle-free approach, which deposits bioink droplets without contact. This avoids clogging and improves reproducibility. It is not suitable for complex structures. Extrusion-based bioprinting deposits continuous filaments of bioink to form structures. It is the common method and has more advantages, as it is versatile and compatible with many bioinks. Lower resolution and shear force can damage cells, which is one of the disadvantages.⁶⁴⁻⁶⁶

The combination of 3D-assembled structures, including organoids and spheroids, and cell-laden microgels shows great potential for creating vascularized tissues through bioprinting.⁶⁷ The construction of tissue through bottom-up methods involves uniting living cells into structures that resemble natural tissues. Bioinks need to fulfil four essential conditions to be effective: they should maintain their shape during printing while having enough viscosity, they should gel quickly to support

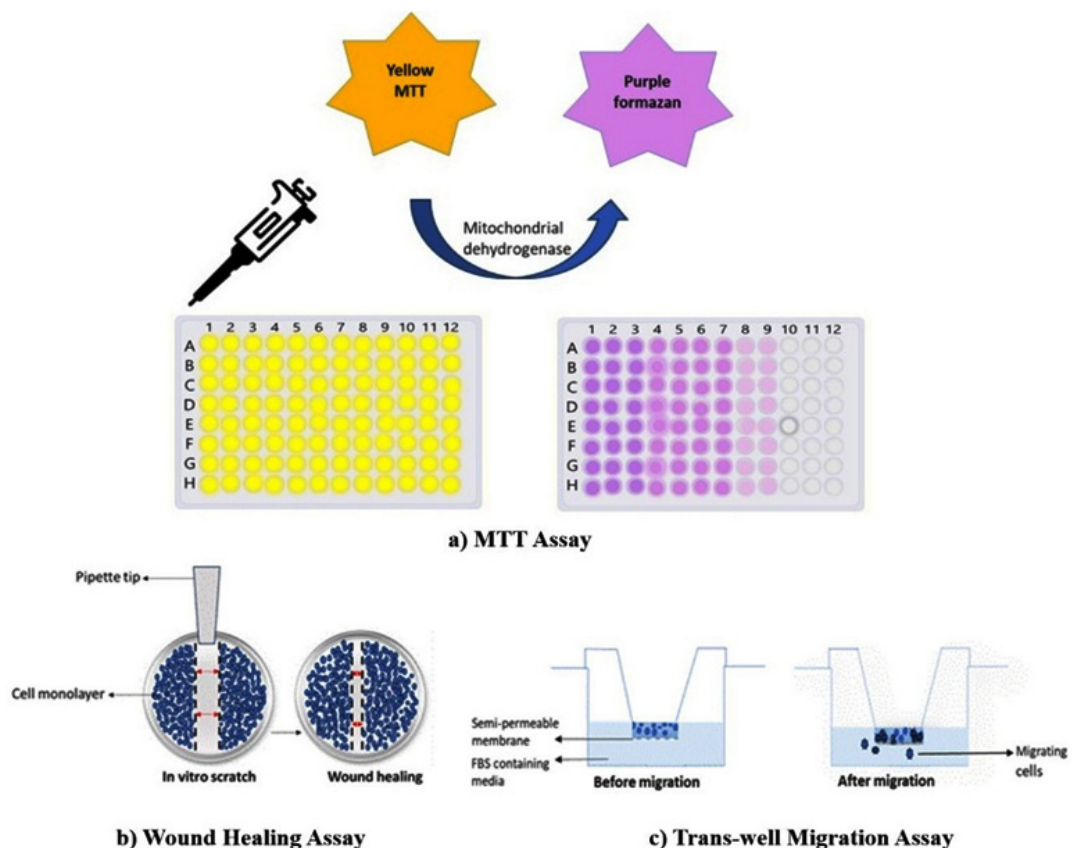


Fig. 1. Depicts the various 2D cellular assays used for cancer research

printed structure stability, they should match the stiffness of natural tissues, and they should be non-toxic for cell survival and operation.⁶² The biocompatibility of natural hydrogels such as collagen, fibrin, and hyaluronic acid is high, but their mechanical properties are insufficient. The mechanical strength of synthetic hydrogels exceeds that of natural hydrogels, but they need chemical treatment to enable cell attachment. Multiple successful bioink formulations use a combination of natural and synthetic materials.

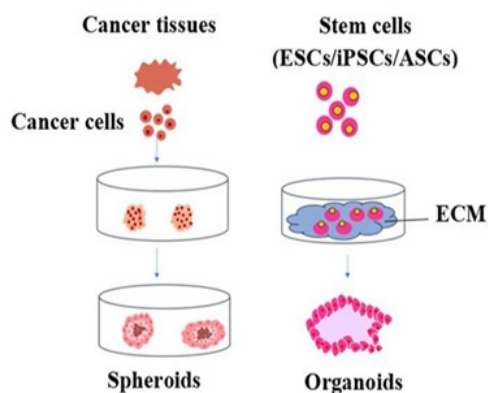


Fig. 2. Depicts the establishment of Spheroids and Organoids

Multi-Barrel Bioprinting and Tumor-on-Chip Integration

A multi-barrel 3D bioprinting method allows researchers to build human glioblastoma cells in the core and endothelial cells in the peripheral layer, which creates an oxygen gradient.⁶⁸ The system places glioblastoma cells in the core area, while endothelial cells receive placement in the outer layer. The specific arrangement of cells in this system produces oxygen, and nutrient levels decrease from the centre to the edges, which matches the hypoxic conditions found in glioblastoma tumors. The construct development leads endothelial cells to form vessel-like structures through vasculogenesis, while the established oxygen gradient causes tumor cells to express genes that match patient tumor hypoxia-adapted phenotypes.⁶⁸

The decellularized stroma method provides distinct benefits because it removes cells from tumor tissue while maintaining the original biochemical elements and structural arrangement of the tumor environment. The combination of tumor-on-a-chip technology with 3D bioprinting allows scientists to create models that duplicate blood vessel perfusion and microcirculation systems.⁶⁹ The microfluidic-assisted bioprinting technique enables researchers to create functional

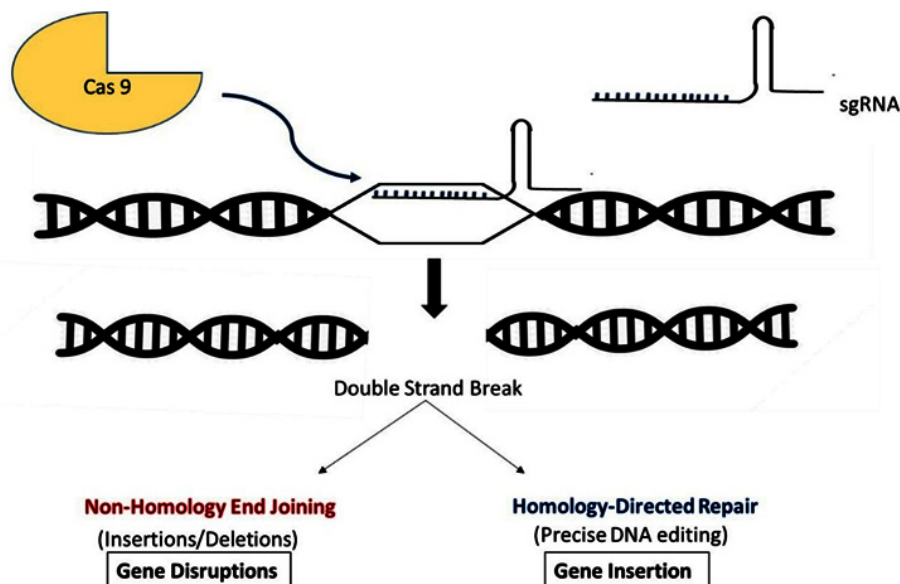


Fig. 3. Represents the CRISPR-Cas9 gene editing mechanism

blood and lymphatic hollow tubes within organ-on-a-chip systems.⁷⁰ The perfusion system performs three essential tasks by delivering nutrients and oxygen to the construct, removing metabolic waste products, and generating shear stress that enhances endothelial cell development and vascular barrier function. The system allows scientists to study cancer cell behaviour during blood vessel entry

and exit processes, which require tumor cells to interact with functioning blood vessels.

Spheroids and organoid 3D culture systems serve as excellent platforms to conduct multiomic approaches, as they are able to recapitulate the tumor under *in vitro* conditions.⁷¹ Multiomics is an interdisciplinary approach that provides high-throughput data on gene

Table 1. Comparison of engineered Cas9 variants and their functional features

Variant	Key feature
SpCas9	• Wild-type Cas9 with high on-target editing efficiency but relatively higher off-target activity. • Cleavage specificity can be affected by single or double mismatches between the gRNA and target DNA.¹¹⁵
eSpCas9	• Engineered high-fidelity Cas9 variant with reduced nonspecific interactions between Cas9 and the DNA backbone. • Retains efficient on-target nuclease activity.¹¹⁶
SpCas9 HF1	• High fidelity variant with modifications intended to decrease non-specific DNA interactions.¹¹⁷
HypaCas9	• Exhibit significant genome-wide specificity without sacrificing on-target activity.¹¹⁸
xCas9	• Can identify a wide variety of PAM sequences, such as NG, GAA, and GAT.¹¹⁹
nCas9	• Cas9 nickase variant with a mutation in one nuclease domain, resulting in single-strand DNA nicking rather than double-strand cleavage. • Provides increased targeting precision and is useful for applications such as base editing and paired-nickase genome engineering.⁹⁵
Sniper-Cas9	• High specificities in human cells without causing on-target cell death. • This enables DNA-free genome editing in a preassembled ribonucleoprotein (RNP) structure.¹²⁰

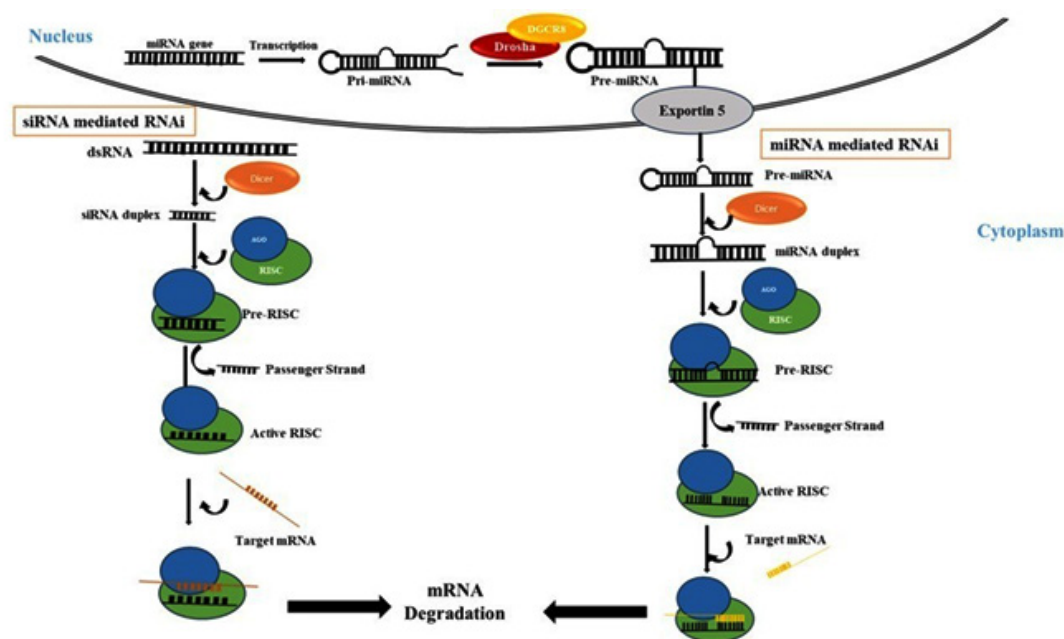


Fig. 4. Shows gene silencing pathway mediated by siRNA and miRNA

expression, proteins, and biological interactions.⁷² Various genomic profiling techniques, such as Next Generation Sequencing (NGS), are used to gain relevant information about the mutational status and differential gene expression patterns of patient-derived 3D spheroids and organoid models. Single-cell sequencing technologies include single-nucleus RNA sequencing (snRNA-seq), a sequencing technique that quantifies transcriptomes of the whole cell genome of a single cell, whereas advanced techniques such as spatial transcriptomics analyze and quantify gene expression within tissues.⁷³

Spatial transcriptomics meets Organoid technology: Mapping cancer ecosystems in 3D

The Spatial transcriptomic technology connects individual cell analysis to tissue structure assessment to show how spatial arrangements affect cellular activities. snRNA-seq delivers detailed molecular information but fails to show where each cell exists within tissue structures.⁷⁴ The RNA is mapped in accordance with specific location and is further sequenced to identify various cell types, generate differential gene expression data, and study intertumoral and intratumor heterogeneity.

Spatial transcriptomics maintains essential location data, which allows scientists to study

gene expression patterns and cellular positions and their interactions with surrounding cells. The combination of high-resolution spatial techniques with bioinformatic tools allows researchers to build 3D spatial transcriptomics atlases from multiple adjacent tissue slices for studying human heart tissue and cardiac organoids and mouse brain samples.⁷⁵ The different spatial transcriptomics methods, including 10x Genomics Visium and Slide-seq and Stereo-seq and MERFISH, offer distinct combinations of spatial detail, gene detection capabilities, and processing speed.⁷⁶ The 10x Genomics Visium platform delivers complete transcriptome analysis through 55-micrometer spots, which can contain multiple cells. The MERFISH technique achieves high-resolution subcellular imaging through sequential fluorescence hybridization while measuring thousands of specific genes. The Slide-seq platform uses 10-micrometer bead-based capture to achieve single-cell spatial resolution while maintaining complete genome-wide coverage.⁷⁶

Integrating Spatial transcriptomics with patient-derived Organoids

Organoid models serve as a platform for conducting spatial transcriptomic experiments.⁷⁷ Organoids constructed of placenta tissue generate scalable and reproducible architectural information

Table 2. Summary of Key Experimental tools and their limitations in Cancer Research

Category	Tools/ Techniques	Application	Advantages	Limitations
2D Culture	Monolayer cell lines	Drug screening, functional assays	Easy, quick growth, reproducible, low cost	Less accuracy, Poor physiological relevance ^{3,8}
Cellular assays	MTT, Brdu, wound healing	Analyze cell metabolic viability, proliferation and migration.	Functional characterization	Prone to experimental errors ¹²
3D Culture	Spheroids, organoids	Tumor modeling, drug response, monitoring signalling pathways.	Mimics TME, heterogeneity	Limited functional blood vessels and oxygen deprivation ^{35,46,47,50,56}
Gene Editing	CRISPR-Cas9	Gene knockout/ knock-in	High precision, scalable	Off-target effects ^{81,84}
RNAi	siRNA/shRNA	Gene silencing using Dicer, RISC mechanisms to degrade mRNA.	Transient modulation	Delivery challenges ^{101,104,111,113}

of transcriptomes, devoid of ethical constraints.⁷⁸ Organoids from gall bladder epithelia were also developed, and by spatial transcriptomics, the gene expression profile was mapped to understand the major stages in cancer progression.⁷⁹ Advanced organoids, such as laminar organoid models, were developed by integrating engineering technology such as lamination-based organoid spatially resolved transcriptomics, and a spatial transcriptomic study was carried out in laminated mouse lung and liver organoids.⁸⁰

The lamination-based organoid spatially resolved transcriptomics method enables automated characterization of primary lung and liver organoid profiles through integrated spatial transcriptomic analysis.⁸⁰ The technique solves a critical problem because previous spatial transcriptomics methods needed to handle small organoid samples through time-consuming sectioning procedures, which damaged the spatial organization of the samples. The lamination method produces flat cell layers from three-dimensional organoids, which allows researchers to use spatial transcriptomics tools directly without needing complicated sectioning methods that could harm sensitive organoid structures.

The 1 cm × 1 cm chip supports four organoids at present, but researchers can expand it to process more than ten organoids at once, which results in decreased sequencing expenses by more than ten times while needing minimal initial material.⁸⁰ The combination of reduced costs and minimal sample requirements through this method makes spatial transcriptomics available to all researchers who study organoids.

Genetic Engineering Tools in Cancer Research **CRISPR-Cas9**

CRISPR-Cas9, or Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein9, is a genome editing tool obtained from the adaptive immune systems of bacteria and archaea.^{81,82} The representation of CRISPR-Cas9 is shown in Figure 3. The CRISPR-Cas9 system lies in its ability to target and modify specific DNA sequences within a genome.⁸³ This is achieved through the use of a guide RNA (gRNA) that directs the Cas9 nuclease to a complementary DNA sequence.⁸⁴ CRISPR-Cas9 technology has revolutionized modern research by offering precision and versatility in genome

editing across diverse organisms and cell types to understand, diagnose, and treat various complex diseases, mainly cancer.⁸⁴ CRISPR-Cas9's ability to induce targeted breaks in DNA has a wide range of applications, including gene knockout, knock-in, gene regulation, epigenome editing, and high-throughput screening.⁸⁵ The mechanism of action is initiated with the creation of a single gRNA, a synthetic RNA molecule comprising a CRISPR RNA (crRNA) sequence complementary to the target DNA sequence and a trans-activating crRNA (tracrRNA) scaffold required for Cas9 binding.⁸⁶ The gRNA, along with Cas9, forms a complex and directs the nuclease to a specific locus by base pairing between crRNA and target DNA.⁸⁷ Upon binding to the target DNA, the Cas9 nuclease induces a double-strand break approximately three base pairs upstream of the protospacer adjacent motif, a short DNA sequence that is essential for Cas9 recognition and binding.⁸⁸ The system's flexibility is further enhanced by the constant development of novel Cas9 variants and delivery methods, extending its applicability in diverse cancer research settings.^{89,90} The different types of CRISPR-Cas9 variants are depicted in Table 1. CRISPR-Cas9 can be used to inactivate oncogenes, such as KRAS (Kirsten rat sarcoma virus), which are often mutated in various cancers, including lung and pancreatic cancer.⁹¹ Loss-of-function screens using CRISPR-Cas9 libraries have identified novel tumor suppressor genes and therapeutic targets.⁸⁹ It is also employed to study the role of non-coding RNAs in cancer development. Cancer dependency genes, which are essential for cancer cell survival but dispensable in normal cells, can be identified through CRISPR-based screens, offering potential targets for cancer-specific therapies. In total, CRISPR-Cas9 serves as a molecular tool, enabling the precise dissection of cancer genomes and the functional characterization of individual genes and regulatory elements. CRISPR-Cas9 has been used in cancer treatments to precisely modify tumor suppressor and oncogene genes, allowing for targeted gene deletion and functional confirmation of cancer drivers. Additionally, it is being investigated in immunotherapy, where CRISPR-engineered T cells (such PD-1 knockdown CAR-T cells) improve treatment efficacy and boost anti-tumor immune responses.⁹² Furthermore, new drug resistance mechanisms and cancer relying

genes have been found using CRISPR-based high-throughput screening techniques, which has made it easier to build personalised cancer treatments.^{93,94}

Advanced CRISPR Technologies: Precision Genome Editing Beyond Cas9 **Cas12a Systems for Multiplexed Editing with Reduced Off-Targets**

The genome editing capabilities of traditional CRISPR-Cas9 systems remain limited because they produce off-target effects.⁹⁵ The CRISPR effector Cas12a operates as a separate class 2 system, which provides better performance than Cas9 through its ability to detect thymine-rich PAM (Protospacer Adjacent Motif) sequences (TTTV, where V represents A, C, or G), its enhanced guide RNA-target DNA mismatch sensitivity and reduced off-target activity, and its ability to perform multiplex gene editing with one crRNA array.⁹⁶ The ability to perform multiplex gene editing with Cas12a proves essential because it allows researchers to use one CRISPR array that produces multiple guide RNAs from a single transcript, which Cas12a cuts into separate functional guides.

The Cas12a-knock-in mouse system allows scientists to perform efficient multiplexed genome editing.⁹⁷ The enAsCas12a variant of *Acidaminococcus* sp. Cas12a shows a strong ability to produce compound genotypes, which include different cancer types that result from tumor suppressor gene inactivation of three separate genes.⁹⁸ The ability of Cas12a-based systems to modify multiple genes at once produces models that better represent natural biological processes.

Base Editing: Single-Nucleotide Precision without DNA breaks

Base editors perform target nucleotide conversions through direct modification without causing DNA double-strand breaks.⁹⁹ Base editors represent a new generation of genome editing tools because they perform single-nucleotide changes without inflicting DNA double-strand breaks. The CRISPR-Cas9 system generates double-strand breaks, which cells repair through faulty mechanisms that produce insertions and deletions together with the desired modifications. The base editors solve these issues through chemical nucleotide modification of individual bases while keeping DNA structures whole.

The three operational sections of

CRISPR base editing include a non-active Cas protein, which attaches to DNA targets without cutting them; a cytidine deaminase enzyme that transforms cytosine into uracil; and a uracil glycosylase inhibitor, which blocks cellular editing systems from reversing the modification. The cell system recognizes uracil as thymine during DNA replication and repair processes, which results in the completion of C-to-T base substitution. The adenine base editors perform adenine-to-inosine conversions through adenosine deaminases, which cells recognize as guanine, to achieve A-to-G base substitutions.⁹⁹ The enzymatic method allows researchers to make exact single-base changes without the DNA damage that occurs when cells repair double-strand breaks.

The Cas12a base editing system allows processing of multiple gRNAs from one transcript, which enables simultaneous base editing at 15 different sites across various human cell types.¹⁰⁰ The combination of Cas homologs with base editors enables researchers to target both cytosine and adenine bases, which could restore about 95% of disease-causing transition mutations.¹⁰¹ The majority of disease-causing single nucleotide variants exist as transition mutations, which involve purine-purine exchanges A<-->G, C<-->G. The clinical development of Beam-101 continues to progress well in Phase I/II clinical trials.¹⁰²

Applications of CRISPR-Cas9 in identifying and validating oncogenic targets

Recent research demonstrates that CRISPR-Cas9 has been widely utilized to find and validate oncogenic targets through genome-wide loss-of-function screens that reveal cancer dependence genes necessary for tumor survival.¹⁰³ Critical vulnerabilities in oncogenic pathways, such as dependencies associated with KRAS-mutant malignancies, have been identified by CRISPR screens. These findings have been confirmed by targeted gene knockout experiments showing decreased tumor growth and proliferation.¹⁰⁴ The therapeutic significance of DNA repair pathways has been confirmed by the identification of effective targets in cancers with BRCA1 and BRCA2 abnormalities using CRISPR-based synthetic lethality techniques.¹⁰⁵ Additionally, by introducing or rectifying mutations and monitoring their direct influence on tumor initiation and progression, CRISPR-engineered tumor models have made it

possible to functionally validate oncogenes and tumor suppressors *in vivo*.⁹²

RNA Interference in cancer research

The process in which specific genes are silenced by small RNA molecules is called RNA interference (RNAi) or gene silencing. RNAi molecules degrade and inhibit the translation of target mRNA. There are different types of RNAi molecules involved in gene silencing, such as small interfering RNAs (siRNAs), short hairpin RNA (shRNAs), microRNAs (miRNAs), and piwi-interacting RNA (piRNAs). siRNAs and miRNAs are vastly studied small RNAs that play a critical role in cell proliferation, cell death, and cancer.^{106,107} siRNAs are synthetic, double-stranded RNAs with a nucleotide length of about 20-25 base pairs, whereas miRNAs are small, endogenous, non-coding single-stranded RNAs naturally present in the genomes of animal cells. The shRNA is artificially synthesized by the vector, which is then processed to form active siRNA. Later, active siRNA and the RNA-induced silencing complex (RISC) together silence the targeted mRNA.¹⁰⁸

siRNA- and miRNA-mediated RNAi

Figure 4 demonstrates the gene silencing pathway of siRNA and miRNA. The double-stranded RNA is processed by the enzyme Dicer into small RNA molecules such as siRNAs, which are then incorporated into RISC, leading to the separation of the passenger strand (sense) and guide strand (antisense). The guide strand then guides the RISC complex to the complementary mRNA for its degradation or blocking of translation. Thereby, it silences the target gene expression.¹⁰⁹

miRNAs are first transcribed as pri-miRNAs in the nucleus. This pri-miRNA is then processed by a microprocessor complex, including Drosha and Digeorge syndrome Critical Region 8 (DGCR8), into pre-miRNAs. The pre-miRNAs translocate from the nucleus to the cytoplasm, where they are converted into mature miRNAs by Dicer and incorporated into RISC, leading to the separation of the passenger strand (sense) and guide strand (antisense). The guide strand then guides the RISC complex to the complementary mRNA for its degradation or blocking of translation. Thereby, it silences the target gene expression.^{110,111}

siRNAs have become attractive agents in cancer therapy because they allow for the sequence-specific silencing of oncogenes implicated in

tumor growth, metastasis, and drug resistance. The stability and targeted distribution of siRNAs to tumor cells have been enhanced by developments in nanoparticle-based delivery systems, increasing therapeutic efficacy while reducing off-target effects.¹¹² Additionally, siRNA-based treatments are being investigated in conjunction with chemotherapy and immunotherapy to produce synergistic anti-cancer effects.¹¹³ RNAi therapy can specifically target and silence the gene expression of multiple genes involved in tumorigenesis. RNAi-based therapy has fewer side effects compared to conventional chemotherapy. It can be used to develop personalized treatment, thus increasing the effectiveness of the therapy.¹¹⁴ Table 2 summarises the relevant techniques used in cancer research as discussed in the manuscript.

Application of genetic tools in organoid systems

Organoids are 3D *in vitro* cultures made from stem or progenitor cells that have an inherent property to replicate a wide range of cell types. Spheroids and organoids are used by researchers to more closely mimic the *in vivo* condition, going beyond conventional flat cultures. By integrating organoids with cutting-edge genetic engineering techniques, researchers can replicate and analyse complex human diseases.^{121,122} This process requires consideration of the method of delivering these genetic tools into the target cells. They could be delivered with a non-viral approach (lipofection or electroporation), or with a viral approach (retrovirus, lentivirus, or adenovirus). Genetic engineering tools such as CRISPR/Cas, RNAi or transposase have been implemented to organoid thereby enhancing disease modelling and enabling developmental research. In organoid genetics, these various methods of genetic engineering are used in organoids to impart required modification on coding regions of genomic sequence.¹²³ Most of the research on organoid genetics has concentrated on improving the knowledge of brain disorders. CRISPR-Cas9-mediated editing can replicate the phenotypes of major neurodegenerative disorders, such as Alzheimer's and Parkinson's, in a three-dimensional organoid environment.¹²¹

CONCLUSION

The landscape of oncology research has undergone an integrative transformation driven

by the cross-disciplinary fusion of advanced *in vitro* culture platforms and next-generation genetic engineering technologies. While traditional 2D culture environments and their associated functional assays remain widely utilized due to their high throughput, reproducibility, and simplicity, they fail to replicate the complex architectural, mechanical, and cellular heterogeneities of human solid tumors. The transition to 3D platforms, specifically multicellular spheroids and patient-derived organoids, successfully addresses these shortcomings by capturing the physical oxygen gradients and true tissue architectures characteristic of the tumor microenvironment. Furthermore, the integration of cutting-edge multi-barrel 3D bioprinting and organ-on-a-chip technologies has paved the way for functional tumor vascularization and micro perfusion modeling. These specialized 3D structures, along with advanced spatial transcriptomics techniques, enable high-resolution mapping of the structural organization and transcriptomic landscapes of evolving cancer ecosystems. In parallel, genome editing has expanded far beyond standard wild-type Cas9 protocols to provide unmatched multi-locus manipulation with minimized off-target effects, and the implementation of specialized Cas12a arrays facilitates highly efficient engineering, while cytidine and adenine base editors enable single-nucleotide precision changes without the cytotoxicity of double-strand DNA breaks. Concurrently, RNA interference strategies via optimized delivery of siRNAs and miRNAs remain indispensable tools for target validation and the sequence-specific silencing of oncogenic pathways. Thus, as discussed in this review, different models offer advantages for cancer research. The 2D culture model is simple, reproducible, and cost-effective, whereas 3D culture models can better mimic the tumour microenvironment. The CRISPR-Cas9 and RNAi provide the researcher with the ability to investigate the gene function. Therefore, combining these approaches with appropriate validation can provide more understanding of cancer biology. Ultimately, these basic as well as advanced techniques act as platforms that accelerate the development of clinically viable, personalized cancer therapeutics and robust drug-screening pipelines.

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