Insights on the differences between two- and three-dimensional culture systems in tumor models (Review)

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Abstract. Traditional cancer research generally utilizes commercial immortalized cancer cell lines cultivated in two-dimensional (2D) culture systems. However, as cell-cell/cell-matrix interactions and the microenvironment cannot be explored *in vivo*, 2D cell culture models inadequately replicate the phenotype and physiology of original tissues. Therefore, three-dimensional (3D) cell culture technologies, such as organoids, which present potential for mimicking the features of primary solid tumors *in vivo*, may be useful in cancer research. By embedding them into special medium, cancer cell lines can be propagated to form tumor organoids. Notably, cells in tumor organoids are different from their original 2D counterparts. During organoid or spheroid formation, crucial aspects including cancer biology, transcriptome, proteome, signal pathways and drug sensitivity, undergo alterations. The present review summarizes the disparities between 2D cancer cells culture and 3D tumor organoids or spheroids with the aim to guide researchers in selecting optimal models for scientific investigations.

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1. Introduction

Malignant tumors have imposed a notable burden on human society (1). Despite extensive efforts in cancer management (2-4), effective treatments and early diagnosis remain crucial to reducing cancer-related mortality. Advances in novel chemical compounds and neoadjuvant targeted therapies offer promise. Progress in tumor therapy and diagnosis depends on fundamental tumor biology studies, often using *in vitro* and *in vivo* models. Tumor cell culture is a fundamental technique with broad applications, including investigations into biological properties and drug screening.

The first human cancer cell line, HeLa, was established from a cervical carcinoma tissue in 1951 (5,6). Subsequently, numerous cancer cell lines have been successfully established, including ovarian (7), breast (8), colorectal (9), pancreatic (10), prostate (11), kidney (12) and bladder (13) carcinoma cell lines. HeLa cells, which are readily available and commonly cultured in two-dimensional (2D) monolayers, serve as vital preclinical models in cancer biology-related research and drug development. However, emerging three-dimensional (3D) culture techniques enable the formation of HeLa spheroids that mimic *in vivo* tumor characteristics and exhibit distinct behaviors to a greater extent, such as increased drug resistance compared with 2D cell culture (14-18). These differences are attributed to factors such as cytoskeletal reorganization (16), overexpression of specific genes (SLC2A1, ALDOC and PFKFB4) driving drug resistance (17), and the ability to model tumor microenvironment (TME) complexity, such as through co-culture with fibroblasts (18).

Whilst 2D culture systems, valued for their simplicity and low cost, have notably contributed to cancer therapy, the limitations are increasingly recognized. Results from 2D models often fail to predict patient responses due to the lack of cell-cell/cell-matrix interactions and altered metabolism and gene expression when cancer cells are cultured on a plane plastic or glass bottle/dish (19,20). Moreover, 2D models

cannot capture the inherent heterogeneity of *in vivo* tumors, which comprise diverse cell types, such as cancer stem cells (CSCs), differentiated cancer cells, stroma cells, immune cells and fibroblasts (21-23).

Consequently, more representative research models are needed. 3D culture approaches, including a large class of culture methods, also called 'organoid' culture technologies, have been exploited (24-27). Based on a 3D structure formed by Hydrogel or artificial scaffold, cell lines are capable of self-renewal and differentiation, for example embryonic stem cells, induced pluripotent stem cells and cancer cell lines, can be propagated to generate organoids in 3D culture medium with special bioactive proteins (28). 3D organoids can maintain the architectural characteristics, gene expression profiles, signal pathways and drug sensitivities of the primary tissues (24,29-32). Therefore, 3D organoids appear to be more effective research implements for cancer related investigations.

Currently, different types of 3D culture systems are being researched, and there is mounting interest in the field of drug discovery, drug screening and tumor biology research (29,33-36). Patient derived cells, induced pluripotent stem cells, patient derived tissue and commercial cancer cell lines are the main sources of organoid generation (28). Due to the easy propagation and convenient manipulation of cancer cell lines, several research teams have used immortalized cancer cell lines to construct tumor 3D spheroids or organoids for scientific investigations (37-39). Moreover, drastic changes in 3D organoid cells have been reported when comparing the same cell lines cultured in 2D models (40-44).

To improve the understanding of the underlying mechanisms, the present review describes the recent progress in tumor 3D organoid development and systematically evaluates the alterations in gene expression profiles, metabolism and response to chemotherapy during the transition from 2D to 3D culture.

2. Progress and status of 3D tumor culture

There has been notable progress in 3D tumor organoid technology for cancer research (45). Tumor 3D organoids are *in vitro* multicellular structures derived from resected tumor tissues or types of cells capable of self-renewing, including immortalized cancer cell lines, induced pluripotent stem cells and CSCs. One of the earliest 3D multicellular spheres was established in 1907 by Wilson (46). Subsequently, 3D-culture technologies have continuously evolved (Fig. 1).

Current 3D organoid generation methods fall into two main categories: Scaffold-free and scaffold-based technologies. Among scaffold-free approaches, the hanging droplet method is widely utilized. This technique relies on gravity to enable suspended cancer cells in culture medium droplets to proliferate, polarize and self-assemble into spheroids (47,48). Its simplicity and replicability make it popular for spheroid formation (49,50). However, scaffold-based systems are increasingly favored, as they are more effective at replicating the critical cell-to-extracellular matrix (ECM) essential for physiological functions. Currently, diverse biomaterials are applied for scaffolds, such as type-I collagen (51,52), polymeric nanofiber (53), hyaluronic acid (54,55), laminin-rich ECM (lrECM) (56,57), gelatin (42,58,59), poly-Caprolactone (60), poly-Hydroxyethyl Methacrylate (poly-HEMA) (61-63) and Matrigel (64,65). A landmark advancement occurred in 2009 with the development of a Matrigel-based 3D intestinal organoid system by Sato *et al* (66) using LGR5-positive adult intestinal stem cells. When $LGR5^+$ intestinal stem cells were propagated in this Matrigel culture system, certain essential growth factors, such as EGF, FGF, Wnt3a, Noggin, R-spondin 1 and N-acetylcysteine, triggered a number of signal pathways to facilitate cells to proliferate, differentiate, alter the gene expression files and phenotype to form intestinal crypt-villus units. This foundational protocol has since been adapted to generate diverse organoids associated with the liver (67,68), lung (69,70), colorectal (71-73), gastric (74-77), pancreatic (78-80), prostate (81-85), kidney (86,87) and breast (88,89).

Previous research has focused on establishing 3D organoid/spheroid systems; future studies should prioritize developing more physiologically relevant models. Emerging integrated platforms offer promise. The tumor-on-chip, which combines microfluidic chips, integrates dynamic flow, ECM, cancer cells to simulate vascularized microenvironments (90), and enable real-time study of metastasis mechanisms (91), immune-tumor interactions (92), high-throughput drug screening via concentration gradient systems (93). Furthermore, 3D bioprinting enables precise spatial patterning of multicellular components critical for tumor modeling: tumor cells establish core malignancy (94,95); stromal cells-including cancer-associated fibroblasts (96) and adipocytes (97)-reconstruct the TME; and vascular networks facilitate nutrient/immune cell delivery (98). Notably, this technology enhances drug resistance in multicellular models vs. monocultures (94,95). Furthermore, patient-derived bioinks enable personalized drug response prediction (99).

Whilst *in vivo* testing, particularly patient-derived xenografts (PDXs) in mice, remains a preclinical standard for retaining tumor histology and heterogeneity, it faces limitations. Mouse stromal and immune cells rapidly replace human counterparts in PDX models, creating a microenvironment distinct from human tumors (100). 3D tumor organoids offer a compelling alternative. By simulating tumor heterogeneity, microenvironment components, vascularization and immune interactions, complex human-derived organoid models show notable potential to reduce reliance on animal testing. This aligns with the ethical 3R principles (Replacement, Reduction and Refinement) mandated by regulatory frameworks.

In summary, platform selection must align with research objectives, balancing physiological fidelity against practical constraints (Table I). High-throughput drug screening prioritizes scalability: Scaffold-free systems (such as hanging drop and micropatterned arrays) enable higher throughput than bio-printed models (47-50,93) but simplify microenvironment complexity. TME interaction studies require ECM integration: Matrigel-based organoids (66) or bio-printed co-cultures (94-97) restore cell-matrix signaling yet introduce batch variability (65) or bioink artifacts (101). Immunotherapy or metastasis research demands dynamics: Microfluidic chips with endothelial barriers optimally model immune cell trafficking and shear stress (90-92), despite increased costs compared with static systems. Personalized medicine relies on patient fidelity: Bio-printed patient-derived organoids better preserve human stroma than PDXs (100) but require >4 weeks

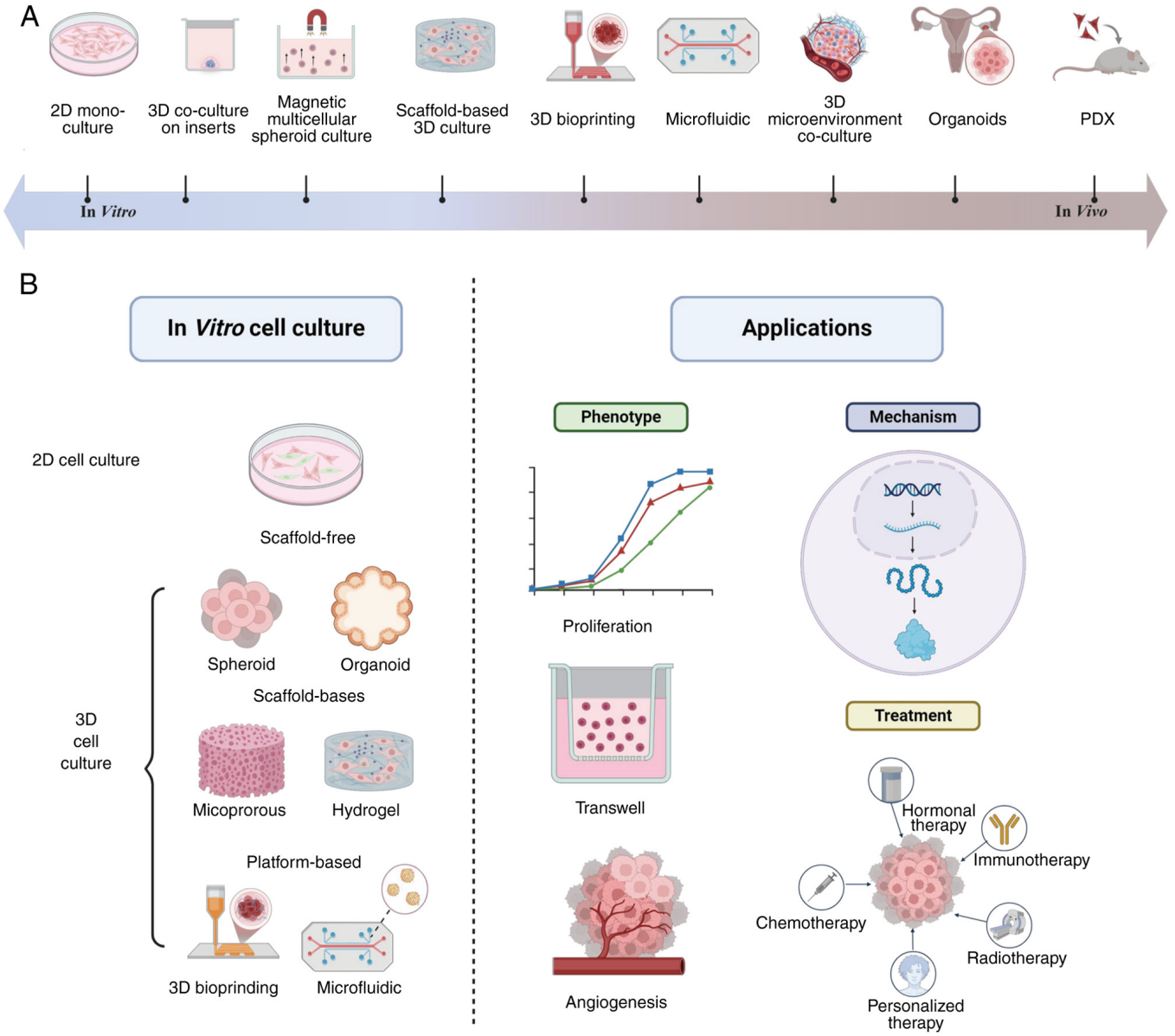

Figure 1. 3D tumor culture technology and applications. (A) Evolution of tumor modeling platforms. (B) *In vitro* tumor culture systems: Classification and applications. 3D tumor cultures are classified as scaffold-free, scaffold-based or in combination with other platforms (left). Translational applications (phenotyping, mechanisms, and therapies) (right). Figure was created by Bio Render and Power Point.

culture periods, limiting acute clinical utility. Collectively, optimal platform choice necessitates strategic trade-offs among physiological relevance, throughput, cost and timeline.

3. Distinct biological properties of 3D tumor models

Cancer cells cultured in 3D systems exhibit distinct biological behaviors and morphology compared with 2D systems. A hallmark change is a reduced proliferation rate and cell viability across several cancer types (Table II). For example, 3D spheroids of HCT-116 (a metastatic colorectal carcinoma cell line) have demonstrated downregulated DNA replication/cell cycle genes and reduced Ki-67 expression compared with 2D monolayers (102). Moreover, colorectal (56), breast (103), osteosarcoma (103), Ewing sarcoma (60), hepatocellular carcinoma (104) and neuroblastoma (105) cells consistently demonstrate lower proliferation in several 3D systems. Furthermore, breast cancer cell viability in poly-HEMA 3D scaffolds was reported to be <50% of 2D levels (106). Migration has also been reported to decrease in breast cancer and osteosarcoma cells cultured in 3D conditions (103).

Notably, multiple myeloma (MM) cells have been reported to proliferate faster in 3D tissue-engineered bone marrow models than in 2D (107); however, MM proliferation decreases in Matrigel-based 3D systems (108,109), highlighting model-dependent effects.

3D tumor culture also induces profound cellular structural changes. Breast cancer cells undergo marked chromatin reorganization in Matrigel 3D systems (43). Lung and head and neck carcinoma cell lines exhibit altered morphology, cytoskeletal architecture and increased heterochromatin in 3D compared with in 2D models (110). This is associated with reduced histone H3 acetylation and elevated HP1-α expression. These structural modifications may contribute to

Table I. Comparative analysis of 3D tumor culture platforms.

Technique	Representative methods	Advantages	Limitations	Optimal use cases	(Refs.)
Scaffold-Free	Hanging Drop	Low cost Suitable for high-throughput drug screening Artificial hypoxia gradients	Absence of physiological ECM signaling Artificial hypoxia gradients Cannot model stromal invasion	Initial drug screening	(47-50)
Natural Matrix Scaffolds	Type-I collagen Hyaluronic acid lrECM Gelatin Matrigel	Native ECM bioactivity Supports stemness maintenance Organotypic morphology	Batch-to-batch variability Xenogeneic interference (murine components) Poor mechanical control	Cancer stem cell studies Mechanistic research	(51,52), (54,55), (56,57), (42,58,59), (64-66)
Synthetic Scaffolds	Polymeric nanofiber Poly-ε-caprolactone Poly-HEMA	Tunable stiffness/degradability Chemically defined Sterilization compatibility	Lack of bioactive motifs Reduced cell adhesion Potential cytotoxic byproducts	Biomechanical studies Long-term toxicity assays	(53), (60), (61-63)
Organoids	Sato Protocol	Preserves tumor heterogeneity Patient-derived modeling Long-term expandability	Absence of vasculature/immune cells Prolonged culture (>4 weeks) Technical complexity	Precision oncology Drug resistance mechanisms	(66)
Microfluidic	Tumor-on-Chip	Physiological fluid shear stress Multi-tissue interactions Real-time imaging	High equipment costs Specialized training required Low throughput	Metastasis research Immunotherapy evaluation	(90-93)
3D Bioprinting	Extrusion Bioprinting	Precise spatial control Vascular network design Multi-cellular TME recapitulation Patient-specific architectures	High equipment cost Limited resolution (50-200 μm) Bioink viscosity constraints Long optimization cycles	Vascularized tumor models High-content drug screening Personalized medicine platforms	(94)

ECM, extracellular matrix; lrECM, laminin-rich ECM; Poly-HEMA, poly-hydroxyethyl methacrylate.

Table II. Differences in proliferation and viability between 2D and 3D culture.

Authors, year	Cancer type	Culture system	Cell line	3D vs. 2D		(Refs.)
				Proliferation	Viability	
Luca *et al*, 2013	CRC	lrECM	SW-480, HT-29, DLD-1, LOVO, CACO-2, COLO-205, COLO-206F	-	-	(56)
Karlsson *et al*, 2012	CRC	NanoCulture plate	HCT-116	-	NR	(102)
Ramaiahgari *et al*, 2014	HCC	Matrigel	HepG2	-	NR	(104)
Breslin *et al*, 2016	BC	Poly-HEMA	BT474, HCC1954 and EFM192A	NR	-	(106)
Fallica *et al*, 2012	BC	Matrigel	MCF-7	-	NR	(103)
Fallica *et al*, 2012	OS	Matrigel	U2OS	-	NR	(103)
de La Puente *et al*, 2015	MM	3DTEBM cultures	MM1s, H929 and RPMI8226	+	NR	(107)
Lamanuzzi *et al*, 2021	MM	Matrigel	U266, MM1S and OPM2	-	-	(108)
Fong *et al*, 2013	ES	poly-caprolactone	TC-71	-	NR	(60)
Hua *et al*, 2012	NB	poly-HEMA	IMR32, CHP134, LAI-55N and COL	-	NR	(105)

CRC, colorectal carcinoma; HCC, hepatocellular carcinoma; BC, breast carcinoma; OS, osteosarcoma; MM, multiple myeloma; ES, Ewing sarcoma; NB, neuroblastoma; lrECM, laminin-rich extracellular matrix; 3DTEBM, 3D tissue-engineered bone marrow cultures; '-', decrease; '+', increase; NR, not reported.

enhanced cells survival under stress, such as radiation therapy, by reducing DNA double-strand breaks and lethal chromosomal aberrations.

In summary, transitioning to 3D culture typically reduces cancer cell proliferation and viability. However, MM demonstrates a significant deviation from this trend: within bone-like 3D microenvironments, MM cells display accelerated proliferation-markedly diverging from their inhibited proliferation in Matrigel cultures. This divergence exposes a fundamental constraint: Proliferative outcomes are scaffold-dependent, challenging the dogma that 3D culture intrinsically inhibits growth. Additionally, 3D spatial organization induces chromatin compaction, a structural change that diminishes DNA damage susceptibility and enhances treatment resistance. Although this improved simulation of *in vivo* stress adaptation is valuable, it introduces a key compromise: 3D systems may disproportionately amplify resistance pathways whose clinical relevance remains uncertain.

4. Phenotypic alterations in 3D cultured cells

Cell culture conditions markedly influence cellular morphology and phenotype. Unlike 2D systems, 3D spheroids or organoids can more effectively recapitulate the cell-to-cell and cell-to-ECM interactions of parent tissue, enabling cancer cells to maintain morphological and (epi)genetic heterogeneity. Crucially, transitioning cancer cell lines from 2D to 3D culture usually induces a shift towards mesenchymal and stem cell phenotypes (Table III). In colorectal cancer, HT-29 (a P53 gene mutated, K-RAS wild type, microsatellite stable colorectal carcinoma cell line) organoids exhibit elevated αSMA (a mesenchymal marker) and loss of E-cadherin (an epithelial marker) in comparison with their 2D counterparts (53). Additionally, HT-29 and HCT-116 tumor organoids have demonstrated increased expression of Snail, an epithelial-mesenchymal transition (EMT) marker, at both the transcription and protein level in comparison with 2D cultures. HCT-116 organoids display strong N-cadherin (a mesenchymal marker) staining, absent in 2D. Key proteins (ZO1, β-catenin, E-cadherin and vinculin) translocate from membrane interfaces in 2D to cytoplasm/nucleus in 3D, implicating pathways such as WNT/β-catenin in EMT (111). Epithelial ovarian carcinoma spheroids also show loss of E-cadherin and gain of vimentin, confirming EMT (112).

Furthermore, HT-29 tumor organoids express CSC markers including NANOG, OCT4, LGR5 and SOX2. HCT-116 organoids demonstrate increased aldehyde dehydrogenase enzyme activity, which was also a hallmark of CSC, compared with in 2D culture. Additionally, MMP9 (an aggressiveness marker) expression is elevated in HCT-116 3D cultures compared with in 2D, suggesting enhanced invasiveness. Furthermore, epithelial ovarian carcinoma spheroids show increased MMP9 immunofluorescence, indicating greater aggressiveness in 3D (112).

In summary, 3D-culture systems uniquely preserve the heterogeneity of parental tissue, facilitating the expression of clinically relevant phenotypes (EMT, stemness and invasiveness), often absent in 2D. This phenotypic fidelity necessitates careful selection of culture models for research associated with cell phenotype and morphology.

5. Altered gene and protein expression in 3D cell culture

Cell culture conditions markedly impact gene and protein expression profiles associated with cell function and

Table III. Differences in phenotype between 2D and 3D culture.

				3D vs. 2D				
Authors, year	Cancer type	Culture system	Cell line	Epithelial	Mesenchymal	Stem cell	Aggressive	(Refs.)
Quarni *et al*, 2019	CRC	Polymeric nanofiber scaffold	HT-29	-	√	√	NR	(53)
Quarni *et al*, 2019	CRC	Polymeric nanofiber scaffold	HCT-116	-	NR	√	NR	(53)
Skardal *et al*, 2015	CRC	Hyaluronic acid-coated microcarriers	HCT-116	-	√	NR	√	(111)
Karlsson *et al*, 2012	CRC	Nano-culture plate	HCT-116	√	-	NR	NR	(102)
Ramaiahgari *et al*, 2014	HCC	Matrigel	HepG2	√	-	NR	NR	(104)
Tanaka *et al*, 2022	PAAD	Matrigel	S2-013	-	√	NR	NR	(116)
Loessner *et al*, 2010	OV	Polyethylene glycol-based hydrogel	OV-MZ-6	NR	NR	NR	√	(112)

CRC, colorectal carcinoma; HCC, hepatocellular carcinoma; PAAD, pancreatic adenocarcinoma; OV, ovarian cancer; '-', no; '√', yes; NR, not reported.

physiology (Table IV). Notably, 3D systems are more effective at replicating tissue-specific functions. HepG2 or HepaRG cell lines regain hepatocellular functions and metabolic capabilities in 3D systems, showing upregulated gluconeogenesis enzymes (G6Pase and PEPCK2), lipid metabolism genes (apoB, apoE and apoA-I) and phase I/II xenobiotic-metabolizing enzymes (such as CYP3A4, CYP1A2, UGT1A1 and UGT1A6) (42,104,114). Furthermore, the expression of tumor-related genes (for example ALB, AFP, CD133, IL-8 and β-TGF) are enhanced in HepG2 spheroids.

3D culture induces widespread expression shifts. Colorectal carcinoma spheroids generated by HCT-116 cells have been reported to upregulate hypoxia and adhesion genes but downregulate DNA replicate and cell cycle genes compared with in 2D culture (102). Breast cancer spheroids have demonstrated elevated cytochrome p450 enzymes and P-glycoprotein related to drug metabolism (106). Moreover, 3D breast cancer models have been reported to exhibit increased apoptotic markers such as caspase 3/7/9, decreased pErk (viability), yet elevated survival proteins including Akt, pAkt, Erk and EGFR family members (106,113). Hypoxia in spheroid cores also drives HIF-1 induction, activating multi-drug resistance-1 gene (coding P-glycoprotein) and its targets, including PDK1, PGK1 and VEGF (113).

In other cancer type 3D culture research, differential expression of genes has been detected between 2D and 3D cells. For example, oral squamous cell carcinoma cell spheroids have been reported to upregulate interleukin 8 expression (115). Furthermore, in an Ewing sarcoma 3D culture model, IGF-1R, phosphorylated IGF-1R, c-kit and HER2 were notably higher than in the 2D monolayer culture (60). Neuroblastoma 3D spheroids also exhibit an elevated expression of HER4 (105). In addition, a marked transcriptomic and epigenetic divergence has been reported by single cell analysis methods when pancreatic adenocarcinoma cell lines were cultured in 3D conditions (23).

Taken together, 3D culture environments could alter gene/protein expression, enabling cells to recapitulate the functions and physiology of original tissue *in vitro*. However, these advantages are offset by inherent limitations. A primary concern is the hypoxic core within 3D spheroids, which disproportionately induces HIF-1-mediated drug resistance pathways (for example, MDR-1, PDK1 and VEGF), potentially distorting therapeutic response predictions. Furthermore, attempts to model *in vivo* complexity can generate model-specific distortions: Breast cancer systems may exhibit conflicting survival signals (simultaneous pro-apoptotic caspase activation and pro-survival Akt/EGFR upregulation), whilst Ewing sarcoma models display amplified IGF-1R/HER2 signaling that could exaggerate perceived target vulnerabilities. Single-cell analyses add another layer of complexity, revealing that 3D-induced transcriptomic and epigenetic heterogeneity can mask critical subpopulation dynamics, complicating interpretation. Consequently, whilst 3D platforms overcome fundamental metabolic limitations of 2D cultures, their variable oxygen gradients and context-driven pathway activation necessitate rigorous context-dependent validation for translational relevance.

6. Differential signaling pathway in 3D culture

Cell culture surroundings have substantial impact on cell behaviors and functions, largely mediated by alterations in gene expression and signal pathway (44). As depicted in Fig. 2, the alterations in signal pathways are mainly concentrated on the downstream of the EGFR family pathways and WNT/β-catenin pathway.

WNT/β-catenin signaling, one of the most common pathways involved in EMT, is elevated when cancer cell lines are transferred from 2D to 3D culture (44,111,116). In 3D liver-colorectal hybrid carcinoma organoids constructed by Skardal *et al* (111), WNT/β-catenin pathway activation

Table IV. Differences in expression levels of genes or proteins between 2D and 3Dculture.

Authors, year	Cancer type	Culture system	Cell line	3D vs. 2D		(Refs.)
				Increase	Decrease	
Quarni *et al*, 2019	Colorectal cancer	Polymeric nanofiber scaffold	HT-29	αSMA, snail, NANOG, OCT4, LGR5 and SOX2	E-cadherin	(53)
Quarni *et al*, 2019	Colorectal cancer	Polymeric nanofiber scaffold	HCT-116	ALDH	NR	(53)
Skardal *et al*, 2015	Colorectal cancer	Hyaluronic Acid-Coated Microcarriers	HCT-116	N-cadherin and MMP9	NR	(111)
Luca *et al*, 2013	Colorectal cancer	Laminin-rich extracellular matrix	HCT-29, CACO-2	phosphorylated and total p42/44	Phosphorylated AKT, EGFR	(56)
Karlsson *et al*, 2012	Colorectal cancer	Nanoculture plate	HCT-116	E-cadherin, p21, CD44 and laminin; genes associated with hypoxia and cell adhesion	Ki-67; genes implicated in DNA replication and cell cycle.	(102)
Storch *et al*, 2010	Lung cancer	Matrigel	A549	HP-1α	NR	(110)
Storch *et al*, 2010	Head and neck cancer	Matrigel	UTSCC15	HP-1α	NR	(110)
Sun *et al*, 2020	Liver cancer	Gelatin; 3D bioprint	HepG2	ALB, AFP, CD133, IL-8, EpCAM, CD24, and β-TGF. AAT, TTR, TAT, CYP2D6, and CYP3A4	NR	(42)
Takahashi *et al*, 2015	Liver cancer	Hanging drop	HepG2, HepaRG	ApoB, Albumin and cytochrome P450, CYP7A1, CYP8B1 and ABCB11; genes related to drug (CYP1A2, CYP2B6 and CYP3A4), glucose (glucose-6-phosphatase, phosphoenolpyruvate) and lipid (SREBP1, SCD1 and DGAT2, apoE and apoA-1) metabolism.	NR	(114)
Ramaiahgari *et al*, 2014	Liver cancer	Matrigel	HepG2	Phase I (CYP3A4, CYP1A2, CYP2E1, CYP2C9, CYP2C19, CYP4F3 and CYP2D6), II (UGT1A1, UGT1A6, UGT1A3, UGT2B4, SULT2A1) and III (OAT2 and OAT7) metabolic enzymes; nuclear receptors AhR, CAR and PXR; OATP1B3 and MRP2.	Ki67	(104)
Breslin *et al*, 2016	Breast cancer	Poly-HEMA	BT474	Akt, pAkt, Erk, HER2, HER3, HER4; EGFR; multidrug resistance p-glycoprotein (PGP) CYP3A4; Caspase 3, Caspase 7 and Caspase 9.	pErk, pHER2	(106)

Table IV. Continued.

Authors, year	Cancer type	Culture system	Cell line	3D vs. 2D		(Refs.)
				Increase	Decrease	
Breslin *et al*, 2016	Breast cancer	Poly-HEMA	HCC1954	Akt, pAkt, Erk, EGFR, HER2, HER3, HER4; multidrug resistance p-glycoprotein (PGP) CYP3A4.	pErk	(106)
Breslin *et al*, 2016	Breast cancer	Poly-HEMA	EFM192A	Erk, HER3, HER4; pHER2; multidrug resistance p-glycoprotein (PGP) CYP3A4	Akt, pErk	(106)
Doublier *et al*, 2012	Breast cancer	Spheroid	MCF-7	HIF-1α; PDK1, PGK1, and VEGF; P-glycoprotein	NR	(113)
Monberg *et al*, 2022	Pancreatic cancer	Matrigel	Panc 1	SMAD3, SMAD4, TGFB1, and DDIT3, SOD1, ID1, and NDUFV2 (reactive oxygen species and metabolism)	NR	(44)
Loessner *et al*, 2010	Ovarian cancer	Polyethylene glycol-based hydrogel	OV-MZ-6	α3/α5/β1 integrin and MMP9	NR	(112)
Fischbach *et al*, 2009	Tongue cancer	Hydrogel	OSCC-3	interleukin 8	NR	(115)
de la Puente *et al*, 2015	Multiple myeloma	3DTEBM	MM1s, H929, RPMI8226, MM1s-GFP-Luc	IL-1α, ANG, MIP-1δ, TNF-α, TNF-β, OPN PARC and eotaxin 3	NR	(107)
Fong *et al*, 2013	Ewing sarcoma cancer	poly-caprolactone	TC-71	IGF-1R, phosphorylated IGF-1R, c-kit and HER2	NR	(60)
Hua *et al*, 2012	Neuroblastoma	poly-HEMA	IMR32, CHP134, LAI-55N, COL	HER4	cyclin D	(105)

3DTEBM, 3D tissue-engineered bone marrow cultures; NR, not reported.

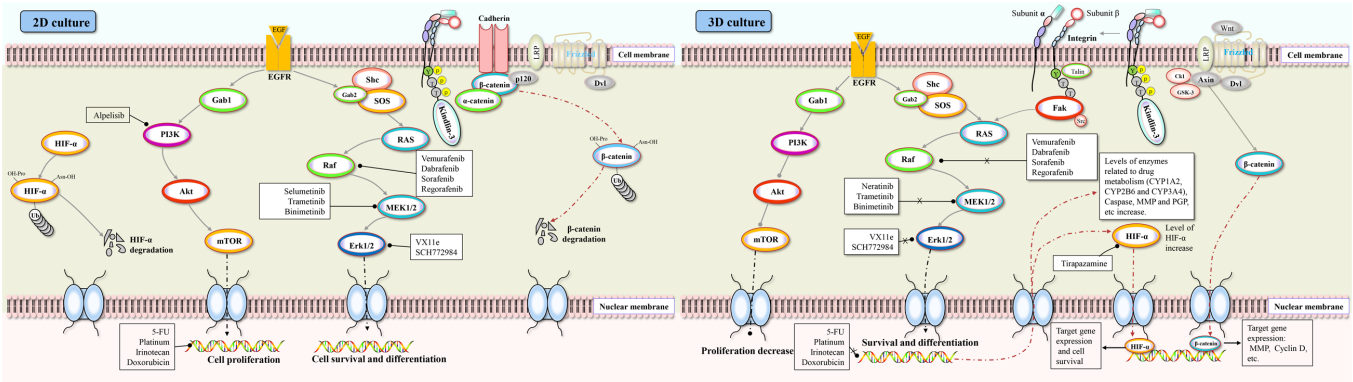

Figure 2. Differences in signal pathways and drug responses between 2D tumor culture and the 3D counterparts. Figure was created by Bio Render and Power Point.

participated in negatively regulating the viability and proliferation rate of colorectal cancer cells, whilst no such phenomena were detected in the corresponding 2D cultured cells (111). In addition, WNT/β-catenin pathway activation also promoted colorectal cell resistance to 5-FU in 3D liver-tumor organoids. In comparison with 2D cultures, colorectal cancer cells within an ECM-derived hydrogel 3D culture system (117) and breast cancer poly-HEMA spheroids (106,118) presented higher levels of total EGFR and phosphorylated EGFR. By contrast, EGFR signal pathways were suppressed in colorectal cancer cells under 3D lrECM circumstances by decreased levels of EGFR and phosphorylated EGFR (56).

Other signal pathways involved in regulating cell proliferation, survival and metabolism, are also reported to be differentially activated between cells in 3D and 2D cultures. Reduced PI3K/mTOR signaling in 3D osteosarcoma cells lowers proliferation and motility (103). Conversely, elevated levels of total Akt and phosphorylated Akt were detected, indicating upregulation of PI3K/Akt signal pathways in poly-HEMA 3D cultures established by Breslin and O'Driscoll (106). Protein expression levels of IGF-1R and phosphorylated IGF-1R were also reported to be upregulated in 3D cultured Ewing sarcoma cells compared with in cells in 2D plastic, suggesting augmented IGF-1R/mTOR signaling in 3D scaffolds (60). Additionally, angiogenesis signal pathways, such as VEGF, hepatocyte growth factor and IL-8, could be induced by hypoxia and at a higher level in MM 3D cultures compared with in 2D cultures. Moreover, pathways in which MAPK is involved are found at different levels of activity in cells between 3D and 2D culture conditions. The rates of phosphorylated and total p42/p44 MAPK are elevated in colorectal carcinoma cell lines (HT-29, SW480 and CACO-2) within 3D conditions (56,117). In 3D lrECM, breast cancer cell line AU565 cells have been reported to exhibit activation of MAPK pathway as phosphorylation of MEK1/2 not Akt is detected. By contrast, in 2D cultures, only Akt is phosphorylated. Notably, for the SKBR3 cell line, both PI3K/Akt and RAS/MAPK pathways were reported to be activated in 2D conditions, whereas in 3D lrECM, SKBR3 cells only demonstrated activation of the RAS-MAPK pathway in line with AU565 cells in 3D lrECM. Based on binding with its ligand, HER2 supports cell proliferation and survival by activation of the lower tyrosine kinase through RAS-to-MAPK and PI3K-to-Akt pathways, respectively (119-121). In accordance with the aforementioned results, raised activation of MAPK and attenuated PI3K/Akt signal pathways caused by HER2 homodimerization have been reported in breast cancer cell spheroids compared with in 2D cell culture (118). Furthermore, Weigelt *et al* (57) reported that culture systems had an impact on PI3K/Akt and RAS/MAPK pathways, even without the participation of HER2. However, the level of phosphorylated HER2 was attenuated in 3D lrECM compared with in 2D cultures.

The transition from 2D to 3D culture significantly alters oncogenic signaling pathways, though scaffold-specific variations complicate mechanistic extrapolation. WNT/β-catenin activation consistently promotes EMT and chemoresistance across 3D models. By contrast, EGFR responses show marked scaffold dependence: Signaling amplifies in hydrogel/poly-HEMA matrices but attenuates in lrECM, directly linking extracellular matrix composition to receptor regulation. Similarly, PI3K/Akt pathway activity varies contextually. It is reduced in osteosarcoma 3D models yet elevated in poly-HEMA breast cancer spheroids and Ewing sarcoma 3D scaffolds. Notably, 3D architectures can uncouple canonical pathway hierarchies, as demonstrated by lrECM-induced MAPK activation concurrent with PI3K/Akt suppression in HER2 positivity breast cancer, independent of HER2 engagement. These observations collectively suggest that pathway rewiring arises from complex ECM-receptor-integrin crosstalk rather than dimensional change alone, highlighting the need for standardized scaffold characterization to enhance translational relevance.

7. Changes in drug response under 3D culture environment

Drug discovery and screening serve an important role in the fields of personal and precise medicine. Whilst 2D cell culture remains prevalent, its models often exhibit oversensitivity to drug treatment compared with the parental tissue *in vivo* due to the lack of cell-cell and cell-ECM interaction. This discrepancy drives the adoption of 3D research models, where altered expression of drug metabolism genes, including cytochrome p450 enzymes, p-glycoprotein and EGFR family members, leads to distinct drug responses in 2D models (Fig. 2 and Table V) (42,95,113-115).

Table V. Differences of drug sensitivities between 2D and 3D culture.

Authors, year	Cancer type	Culture system	Cell line	3D vs. 2D		(Refs.)
				Sensitive	Resistant	
Goudar *et al*, 2021	Colorectal cancer	Poly(dimethylsiloxane) co-culture with NIH3T3 fibroblasts	HCT-8	NR	5-FU and Regorafenib	(40)
Forsythe *et al*, 2020	Colorectal cancer	Hydrogel	HCT-116, HT-29, Caco-2 and SW480	Regorafenib, Sorafenib, Dabrafenib and Trametinib	5-FU, Cisplatin, Irinotecan and Oxaliplatin	(117)
Ramamoorthy *et al*, 2019	Colorectal cancer	mTiD	HCT-116, SW480, DLD-1	NR	5-FU, Irinotecan, Oxaliplatin	(38)
Karlsson *et al*, 2012	Colorectal cancer	NanoCulture plate	HCT-116	NR	Melphalan, Irinotecan, Oxaliplatin, 5-FU	(102)
Li *et al*, 2020	Lung cancer	Matrigel	H1299, H460 and H1650	Harmine and Berberine	Cantharidin	(127)
CHOI *et al*, 2019	Lung cancer	Micropillar chip	A549	NR	Resistant to chemotherapeutic agent	(124)
Mazzocchi *et al*, 2022	Lung cancer	Hydrogel; co-culture with fibroblast	Pleural Effusion, H460	NR	Cisplatin + Pemetrexed, Carboplatin + Pemetrexed and Crizotinib	(125)
Wu *et al*, 2018	Lung cancer	Droplet spheroid	A549, H1299	NR	Cisplatin	(126)
Sun *et al*, 2020	Liver cancer	Gelatin; 3D bioprint	HepG2	NR	Cisplatin, Sorafenib, and Regorafenib	(42)
Takai *et al*, 2016	Liver cancer	AlgiMatrix	Huh1	NR	5-FU and Doxorubicin	(128)
Phan *et al*, 2020	Breast cancer	Matrigel	VN9, VN9CSC	HO-MeOHE and Tirapazamine	Doxorubicin	(133)
Breslin *et al*, 2016	Breast cancer	Poly-HEMA	BT474, HCC1954 and EFM192A	NR	Neratinib and Docetaxel	(106)
Lovitt *et al*, 2015	Breast cancer	Matrigel	MCF-7, MDA-MB-231	NR	Epirubicin, Vinorelbine and Paclitaxel	(122)
Doublier *et al*, 2012	Breast cancer	Spheroid	MCF-7	NR	Doxorubicin	(113)
Weigelt *et al*, 2010	Breast cancer	Laminin-rich extracellular matrix	AU565	Trastuzumab	Pertuzumab	(57)
Weigelt *et al*, 2010	Breast cancer	Laminin-rich extracellular matrix	SKBR3	NR	Trastuzumab	(57)
Weigelt *et al*, 2010	Breast cancer	Laminin-rich extracellular matrix	HCC1569	Pertuzumab and Lapatinib	NR	(57)
Dhiman *et al*, 2005	Breast cancer	Polymer chitosan	MCF-7	NR	Tamoxifen	(123)
Loessner *et al*, 2010	Ovarian cancer	Polyethylene glycol-based hydrogel	OV-MZ-6	NR	Paclitaxel	(112)
Wei *et al*, 2022	Bladder cancer	Matrigel	RT4 and HT1197	NR	Cisplatin, Venetoclax and S63845	(130)
Vincent-Chong *et al*, 2020	Tongue cancer	Matrigel	RP-MOC1	NR	Radiation	(129)

Table V. Continued.

Authors, year	Cancer type	Culture system	Cell line	3D vs. 2D		(Refs.)
				Sensitive	Resistant	
de la Puente *et al*, 2015	Multiple myeloma	3DTEBM	MM1s, H929, RPMI8226, MM1s-GFP-Luc	NR	Bortezomib and carfilzomib	(107)
Wen *et al*, 2013	Pancreatic cancer		MIAPaCa-2 and PANC-1	NR	Gemcitabine and 5-FU	(132)
Fong *et al*, 2013	Ewing sarcoma cancer	Poly-caprolactone	TC-71	NR	Doxorubicin	(60)
Chitcholtan *et al*, 2012	Endometrial cancer	Poly-HEMA	Ishikawa, RL95-2, KLE	NR	Doxorubicin	(131)
Hua *et al*, 2012	Neuroblastoma	Poly-HEMA	IMR32, CHP134, LAI-55N, COL	NR	Cisplatin, Doxorubicin, Etoposide, and 4-hydroxy-Ifosfamide	(105)

mTiD, metastatic tumor-in-a-Dish; 3DTEBM, 3D tissue-engineered bone marrow cultures; NR, not reported.

In comparison with 2D models, 3D colorectal cancer cell culture generally shows a marked increase in resistance to classes of chemotherapeutic agents, targeting DNA synthesis and repairment, including platinum-based drugs, 5-FU, melphalan and irinotecan (38,102,111,117). Similarly, several types of anticancer drugs, such as platinum-based drugs, monoclonal antibodies, multityrosine kinase inhibitors and other chemical drugs, are often more effective in breast carcinoma cells cultured in 2D plane systems than the 3D counterparts (57,106,113,122,123). Furthermore, other types of cancer, including lung (124-127), liver (42,128), ovarian (112,118), oral (129), bladder (130), endometrial (131), pancreatic carcinoma (132), MM (107), Ewing sarcoma (60) and neuroblastoma (105), are usually more responsive to chemotherapeutics in 3D culture circumstances than in 2D conditions.

Notably, multi-kinase inhibitors targeting downstream VEGFR, FGR and EGFR receptor pathways, such as regorafenib, sorafenib, dabrafenib and trametinib, induce a higher degree of cell death in 3D culture systems than 2D cultures (117). As the expression of HIF-1 is augmented in 3D culture, tirapazamine, which is an inhibitor targeting the HIF-1 downstream pathway, has been reported to have higher efficacy in 3D culture than in 2D (133). Moreover, trastuzumab, a commercial monoclonal antibody against HER-2 downstream pathway, has exhibited higher efficacy against the HER2-amplified cell line AU565 under 3D lrECM than in 2D cultures (57). However, the efficacies of kinase inhibitors, regorafenib, AG148 and AG1478, were reduced in 3D CRC cell system compared with in a 2D model, due to reduced EGFR expression in IrECM (40,56,57). Trastuzumab has also been shown to be ineffective against SKBR3 cells in 3D lrECM due to downregulated HER2 phosphorylation (57).

The drug reaction is associated with the 3D culture matrix. Goudar *et al* (40) co-cultured HCT-8 colorectal cancer cell line with NIH3T3 fibroblasts to set up 3D chimeric tumor spheroids (CTSs), which were associated with a decline in EGFR expression as fibroblasts can secrete several types of ECM including type-I collagen and laminin (134,135). A further study demonstrated that the CTSs had enhanced resistance to tyrosine kinase inhibitors, in line with the aforementioned results (56). 3D SKBR3 cells in poly-HEMA culture system regain trastuzumab sensitively as 3D poly-HEMA conditions support anchorage-independent cell growth under the absence of an artificial ECM (117). Additionally, acinar polarity, which exists in cells 3D lrECM, is not observed in 3D poly-HEMA culture systems, highlighting the crucial influence of the ECM.

In summary, 3D-culture systems better replicate *in vivo* drug response profiles than 2D models, particularly for conventional chemotherapeutics where heightened resistance reflects clinical observations across multiple carcinomas. However, significant context-dependent variations exist. Targeted therapeutics exhibit class-specific divergence: Multi-kinase inhibitors (regorafenib/sorafenib) and hypoxia-activated agents (tirapazamine) demonstrate superior efficacy in 3D environments, whereas certain tyrosine kinase inhibitors and monoclonal antibodies show reduced activity due to ECM-mediated receptor modulation. Crucially, ECM biochemistry governs therapeutic outcomes: IrECM suppresses HER2/EGFR signaling

and drug sensitivity, whilst scaffold-free poly-HEMA systems preserve target vulnerability. This matrix-driven heterogeneity necessitates rigorous microenvironmental characterization in preclinical studies to ensure accurate interpretation of drug responses. Standardized documentation of scaffold properties would substantially improve translational predictability.

8. Conclusions and future perspectives

The present review compares key differences in cell phenotypes, behaviors, gene expression profiles, signal pathways and drug sensitivities between 2D and 3D cancer cell cultures. 3D tumor culture systems directly address the fundamental limitations of 2D models-artificial metabolism, loss of tumor heterogeneity and poor clinical predictability-by reconstructing critical TME elements: Cell-matrix interactions, spatial architecture and hypoxia gradients. This transformative advancement evolves from early spheroids to contemporary scaffold-defined organoids, enabling physiologically relevant carcinoma modeling. Crucially, 3D platforms restore malignancy hallmarks absent in 2D systems: Preserved tumor heterogeneity, EMT progression, stemness programs (OCT4/SOX2) and hypoxia-driven resistance (HIF-1/MDR-1). Whilst therapeutic predictability improves significantly for conventional agents such as 5-FU, matrix-dependent efficacy variations in targeted therapies (for example, lrECM-diminished HER2 inhibition) represent persistent modeling challenges that require resolution.

Nevertheless, persistent constraints reintroduce the very biases 3D systems aim to resolve: Microenvironmental artifacts from amplified hypoxia hyperactivate resistance pathways, whilst biomaterial inconsistencies (Matrigel variability, bioink artifacts) compromise reproducibility. The scaffold-response paradox-exemplified by context-dependent proliferation kinetics in MM (bone-mimetic enhancement vs. Matrigel suppression) and opposing EGFR pharmacodynamics-highlights fundamental complexities in tumor modeling. Translational barriers similarly mirror historical gaps: Extended patient-derived organoid maturation (>4 weeks) hinders clinical adoption, whilst multidimensional heterogeneity risks obscuring critical subclones as noted in single-cell analyses.

Addressing these challenges necessitates: i) Standardization via synthetic ECM and quantitative material registries (mechanical/ligand/degradation metrics) to resolve biomaterial limitations; ii) Dynamic integration of vascularized microfluidics-bioprinting hybrids mitigating hypoxia artifacts, combined with immunocompetent interfaces for immunotherapy screening; iii) Clinical translation acceleration employing automation to compress workflows to <2 weeks, validated by spatial multi-omics. This interdisciplinary convergence will ultimately deliver on 3D modeling's original promise articulated in the introduction: Bridging the translational gap through quantifiable, patient-specific therapeutic platforms that transform precision oncology.

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Authors' contributions

GJZ, QDL and YSW designed the review. GJZ and QDL collected the citations and drafted the manuscript. YFW mainly helped to revise the manuscript. YSW revised the manuscript. All authors read and approved the final version of the manuscript. Data authentication in not applicable.

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Competing interests

The authors declare that they have no competing interests.

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