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3D Tissue-Engineered Model of Ewing Sarcoma

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Abstract

Despite longstanding reliance upon monolayer culture for studying cancer cells, and numerous advantages from both a practical and experimental standpoint, a growing body of evidence suggests more complex three-dimensional (3D) models are necessary to properly mimic many of the critical hallmarks associated with the oncogenesis, maintenance and spread of Ewing sarcoma (ES), the second most common pediatric bone tumor. And as clinicians increasingly turn to biologically-targeted therapies that exert their effects not only on the tumor cells themselves, but also on the surrounding extracellular matrix, it is especially important that preclinical models evolve in parallel to reliably measure antineoplastic effects and possible mechanisms of *de novo* and acquired drug resistance. Herein, we highlight a number of innovative methods used to fabricate biomimetic ES tumors, encompassing both the surrounding cellular milieu and extracellular matrix (ECM), and suggest potential applications to advance our understanding of ES biology, preclinical drug testing, and personalized medicine.

Keywords

Ewing sarcoma; MCTS; 3D; tissue-engineering; scaffolds; ECM; tumor model; chemotherapy; biological therapy; preclinical testing

1. Introduction

The Ewing's sarcoma family of tumors (ESFT) is an aggressive form of childhood cancer that has historically included classic Ewing sarcoma (ES), Askin tumors, and peripheral primitive neuroectodermal tumors (PNET) [1-6]. Though previously considered to be

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distinct clinical entities, given subtle variation in their presenting sites and immunophenotype, the World Health Organization now advocates a simplified nomenclature using ES to represent the aforementioned tumors, as they consistently have a round cell morphology, ubiquitously express CD99, and harbor a near-universal pathognomonic chromosomal translocation—affixing the N-terminal EWSR1 gene to a C-terminal ETS gene [7-9]. Although rare in comparison to carcinomas, ES is the second most common pediatric bone tumor, presenting in three cases per million [10]. Racial and gender disparities exist; ES is nine times more common in Caucasians than in African Americans and slightly more prevalent in males than females with a 6:5 ratio [11, 12].

Clinically, ES is an aggressive, rapidly fatal malignancy that can develop in osseous and extraskeletal sites and naturally spreads to lung, bones, and bone marrow if not rapidly treated [11, 13, 14]. In fact, even in the 60-70% of cases where a solitary site is visualized radiographically, micrometastatic disease is presumed to exist within the lung [15]. Fortunately, significant strides in multimodality treatment have enhanced the 5-year survival rate for those with localized tumors from 10% in the pre-chemotherapy era (prior to 1962) to about 75% today [16]. Yet, for inexplicable reasons, this progress in curing patients suffering from localized disease has not extended to those with metastatic or rapidly recurrent disease, and less than 30% of these patients will survive.

In an effort to change that dismal outcome, extensive research has defined key oncogenic events responsible for the growth and maintenance of ES. And not withstanding the lack of a conclusive cell of origin, experimental evidence suggests that ES emanates from a single pluripotent bone marrow-derived mesenchymal stem cell (MSC) that has neuroendocrine features and acquires a specific cancer-causing chromosomal translocation of the EWSR1 gene [17-21]. EWSR1-FLI1, which occurs in 85% of ES, results from the apposition of the N-terminal portion of the EWS gene (located at 22q12) with the C-terminal FLI1 gene of the ETS transcription factor family [22-24]. Less common translocations include EWS-ERG (5-8% of cases) [25] and EWSR1-ETV1 [26], EWSR1-EIAF [27], and EWSR1-FEV [28], which each occur in less than one percent of reported cases [24, 29]. On rare occasions, FUS (one of three TET genes, also known as TLS) can occasionally substitute for EWSR1 to produce a FUS-ERG positive ES [30] and non-ETS pairings with ZSG [31] or NFATc2 [32] have also been reported—the biological and prognostic significance of these exceedingly rare chromosomal aberrations is unknown.

While a number of innovative drugs in preclinical development are aimed squarely at the tumorigenic EWS-FLI1 fusion protein, it has been historically quite challenging to counteract this transcription factor, given its inaccessibility within the cell nucleus. Thus, in addition to conventional chemotherapies that clearly have a prominent role in treatment, most biologically targeted therapies used in the treatment of ES either target downstream signaling cascades induced by EWS-FLI1 activity (e.g., IGF1/IGF-1R, TGF-β, Hedgehog/GLI, Wnt/β-catenin, and Notch/p53) or the surrounding tumor matrix upon which ES cells rely [33-41]. Of particular relevance to the field of tissue-engineering, it is these latter targets and/or processes, which include the extracellular matrix (ECM), nascent blood vessel formation [42, 43], and cell migration or metastasis that will be of greatest relevance since they are poorly modeled by traditional monolayer culture techniques [44].

2D monolayer culture has, of course, been the mainstay for culturing cancer cells for at least five decades, and continues to be the predominant method for testing the antineoplastic drug candidates in the preclinical setting given its many advantages over more complex systems; 2D culture is readily performed using standardized methods, promotes rapid cell growth, uses translucent material amenable to monitoring cells in real-time using light microscopy, requires no special equipment, and is cost effective. Yet, as the cancer biology community can attest, cells placed upon chemically inert flat polystyrene tissue-culture plates under conditions of high oxygen tension and abundant glucose and nutrients poorly mimic how the cells would otherwise behave within their native in vivo host [45]. As a direct result of this iatrogenic effect on cancer cells grown in the laboratory, it is not surprising that the precise protein targets and/or signaling cascades being interrogated in high-throughput anti-cancer drug screens are profoundly different from what occurs in patients and this, in part, explains why the majority of biologically targeted therapies may succeed in the lab but ultimately fail in the clinic. This holds true for traditional cytotoxic chemotherapies as well, since most work by indiscriminately damaging DNA in rapidly dividing cells that grow in monolayers at unparalleled rates unheard of in even their fastest clinical counterparts. Thus, for all the advantages of 2D cell culture for cancer research, if the information gained is unreliable—or even worse, leads to expensive clinical trials that provide false hope to patients—one must reassess whether this preclinical approach is still appropriate when better options exist [46-49].

In recognition of the previously described shortfalls inherent in monolayer culture, and ease with which ES cells/explants can be cultivated in immunocompromised mice, there has been a recent resurgence in the use of mouse models [50, 51]. In part, this gradual pendulum swing back towards tumor xenografts, which had favored in vitro testing over the last several decades, is occurring in an attempt to better mimic elements of the tumor microenvironment (e.g., tumor associated stroma, growth factors, and abundant heterotypic cells). Further, the use of low-passage number explants obtained directly from patients—rather than from long-established cell lines—appears to reduce phenotypic drift from corresponding human tumors and helps to preserve surrounding tumor-associated architecture [52].

Despite the advantages of these so-called patient-derived tumor explants (PDX), they are not without their own challenges: (i) xenografts can still behave differently when placed into a murine host, especially in subsequent generations of engraftment where mouse tissue has replaced the human tumor-associated stroma; (ii) monitoring can be difficult, particularly for orthotopic locations; (iii) specialized surgical skills are required; and (iv) engraftment rates are usually less than 75%. Additionally, because xenografts are placed into immunocompromised mice devoid of a functioning human immune system, it is all but impossible to use them to evaluate the growing list of immunomodulatory drugs, such as checkpoint inhibitors, gaining increased utility in the clinic. Last, PDX models are costly to generate since they require expensive core-needle or open biopsies of human tumors and, subsequently, incur substantial labor costs to perform drug testing.

Overall, challenges associated with current 2D and xenograft tumor models have motivated researchers to develop innovative ex vivo 3D cell culture systems that provide a more

physiologically relevant cellular environment that meets the needs of the basic science and clinical research community (Table 1) [53, 54]. Prominent examples include the culture of cells in relatively simple spheroids [55], pellet cultures [56], or cell-matrix interactions (using protein gels or synthetic polymer scaffolds) [57-59]. More complex 3D cancer models that rely upon co-culture or hybrid culture systems have included encapsulated protein gels seeded with multiple cell types [60], hybrid methods with 3D scaffolds layered upon 2D monolayers [61], and heterotypic cell populations grown as tumor spheroids [62].

Many of the strategies developed originally for tissue-regenerative purposes have recently been adapted for the purpose of modeling human tumors in the preclinical setting, and the increased control of tumor microenvironment has revealed significant advantages over 2D and xenograft culture. In fact, it is this ability for tissue-engineered systems to devolve complex microenvironmental themes (e.g., signaling molecules, biomechanical forces, and metabolic factors) from poorly controlled native/self-organizing cell aggregates that makes their use so appealing. As shown in Figure 1, each component of a tumor's microenvironmental niche can be varied experimentally to determine the relative impact on the cancer cell.

3D culture, for example, can be designed to resemble the in vivo malignancy's shape and environment, which in turn, can influence the behavior and gene expression of the cell, as has been demonstrated in a colorectal cancer model [63]. Further, by manipulating not only the 3D architecture itself, but also the heterotypic cell composition, one can selectively reintroduce key interactions between tumor cells and surrounding stromal cells within an in vivo-like human tumor microenvironment. By creating a more biomimetic 3D environment of cancer, these 3D in vitro models provide important alternatives to both 2D culture and in vivo models by, (i) delivering the applicable matrix constituents in a 3D configuration found clinically, (ii) co-culturing cancer cells, endothelial cells and other stromal associated cells in spatially adequate manner, (iii) examining and adjusting hypoxia to mimic levels found in native in vivo tumor environment and (iv) censoring the release of angiogenic factors by cancer cells in response to hypoxia.

As we delve more deeply into the steady rise of tissue-engineered tumors, and highlight both current applications and future directions they may take in helping to advance our collective understanding of tumor behavior, we take care to distinguish a cancer cell's innate behavior to self-organize (i.e., to form spheroids and other cell aggregates) from their capacity to form complex 3D relationships upon exogenous non-biologically derived tissue-engineered substratum. Though this review highlights the role of tissue-engineered models of ES, we acknowledge the relatively rarity of this bone cancer and, therefore, suspect the concepts presented herein will find more broad spread utility as they relate to other sarcoma subtypes or carcinomas that metastasize to bone or lung.

2. Tumor structures and ECM generation intrinsic to the cancer cells

2.1 Tumor Spheroids

Multicellular tumor spheroids (MCTS) are spherical aggregates of tumor cells that autonomously form when cultured in non-adherent substrates that are devoid of requisite

ECM or growth factors. Since their discovery, MCTS have been demonstrated to more closely resemble the phenotypic behavior of human tumor tissues and, for that reason, have been used extensively to model key elements of malignant tumor behavior [64]. Among numerous examples, they have proven useful to study avascular tumor growth, intracellular tumor hypoxia, and the effects of cell-ECM communication upon drug sensitivity. Within this subsection, we discuss common methods of spheroid formation, highlight their phenotypic resemblance to human tumors, and describe real-world applications that are helping to decipher the complex biology of ES and cancer more broadly.

Since Inch et al. first described the formation of nodular carcinoma MCTS in 1970 using the spinner flask method, the number of methods capable of yielding MCTS has grown significantly and generally fall into two broad categories [65, 66]. The first group relies upon equipment that induces turbulence into the cell culture medium and, thereby, prevents cells from adhering to solid surfaces. In contrast, the second group achieves a similar effect simply by altering the extracellular environment to make it incompatible with cell-ECM adhesion. Prominent examples of the former ‘mechanical’ group include the original spinner flask and rotary wall vessel reactors (Figure 2A), which suspend cells between rotating cylindrical walls to that mimic some elements microgravity [53, 67]. Whereas the latter ‘intrinsically non-adherent group’ places cell suspensions onto non-adherent micro-etched nano-culture plates (NCP) (Figure 2B) or agarose coated Petri dishes (Figure 2C) that prevent cell binding. Newer production techniques include hanging drops (Figure 2D) [68, 69] and microfluidic chips [70], which can produce more uniform spheroid size at the risk of being more specialized and difficult to master.

Regardless of the chosen method to produce them, MCTS evolve from disorganized cell aggregates in the first week into highly symmetric spherical structures by two weeks that have distinct zones: (i) a central core of necrotic cells or ones undergoing apoptosis, (ii) an inner layer of non-proliferating quiescent cells, and (iii) an outer nutrient-rich layer of proliferating cells capable of interacting, albeit in a limited fashion, with surrounding ECM [55, 64]. Though subtle cell-type dependent differences in morphology exist, ranging from simple spheroids uniformly coated in ECM to more intricate structures that have glandular structures resembling ductal tissues, as a class MCTS appear to better mimic human tumors than 2D monolayers with respect to proteomic and genomic expression profiles [71-79]. Lawlor et al., for example, have noted that growth rates, cell morphology, cell-cell junctions, and kinase activation of ES spheroids closely mimic those of primary Ewing tumors [80]. Further, given their contrasts in pH, oxygen tension, and proliferative rates that exist between the inner and outer layers, MCTS can be used to determine layer-dependent antineoplastic effects that couldn't otherwise be observed using traditional monolayers [55, 81-85].

As such, ES spheroids have been used extensively to judge the effectiveness of chemotherapeutic and biologically targeted drugs (Figure 3) [53, 67, 80, 86, 87], to study the impact of cell signaling pathways that regulate ES cell proliferation [53, 80, 88], to investigate the effects of tumor architecture upon immune cell function, and to identify suitable antigens for immunotherapeutic strategies [89, 90]. ES MCTS have also proven useful for modeling micrometastatic disease, contributed to our understanding of anoikis (a

form of cell death that results from lost ECM contact) [53, 88], and served as a platform to evaluate heterotypic interactions between tumor cells and vascular progenitor cells responsible for angiogenesis [77].

2.2 Tumor organoids

Though MCTS grown in nonadherent conditions can reproduce some features of human tissues and/or tumors (e.g. oxygen and drug diffusional gradients, cell-cell contact, etc.) their spherical self-organized structure cannot be said to truly mimic the more complex patterns observed in vivo. Surprisingly, a flurry of research reports published since 2013 have revealed an enormous untapped potential for noncancerous human embryonic pluripotent stem cells (PSC) and/or induced pluripotent stem cells (iPSC) to self-organize into lab-grown organ-like structures (i.e. organoids) when coaxed to do so by external spatiotemporal perturbation using nutrients, growth factors, or rarely heterotopic cells. Prominent examples include ex vivo models of embryonic human brain [91], functional liver buds that resemble human liver [92], and ureteric buds that differentiated towards the renal collecting system (Table 2) [93]. Additionally, a lung organoid has been described that forms beneath the renal capsule of mice in vivo [94]. That human PSCs intrinsically retain the capacity to self-assemble into spatially-complex higher-ordered organ-like structures ex vivo is truly amazing and suggests the genomic or epigenetic information contained within the PSCs is enough to drive organ-level differentiation if augmented by the ‘correct’ microenvironmental cues.

Whilst the referenced organoid models could revolutionize the tissue engineering and regenerative medicine fields by restoring or replacing diseased organs, we foresee novel opportunities to use organoids to study bidirectional regulatory feedback that exists between tumors and their supporting microenvironmental ECM. Among just a few of the examples, one could imagine an ex vivo study of organ-specific malignancies (i.e. brain cancer, colon cancer, etc.) using surrogate organoids that have been corrupted by site directed mutagenesis or less-specific radiation-induced genomic damage to produce tumors. Resulting 3D tissue/tumor hybrids could then be used to screen promising drug candidates, to study metastasis to and from the organoid, and to determine stromal growth factors necessary for tumor maintenance.

2.3 Biologically derived 3D substrates

Despite the advantage spheroids and organoids have over their 2D counterparts, it can be challenging to model their interaction with surrounding ECM, as most are devoid of supporting cells (endothelial cells, fibroblasts, etc.) or soluble growth factors. In an attempt to overcome those limitations, 3D models of ES have integrated naturally occurring substrates derived from human tumors, elements of the basement membrane, and/or gels rich in collagen or laminin [21, 95-98]. By adding back key elements of the surrounding ECM, one can also invoke membrane-bound integrin signaling and, thereby, activate a diverse array of downstream signaling cascades including those responsible for angiogenesis [95, 96, 99], cancer cell motility [97, 100], and drug sensitivity [101, 102].

Of those mentioned above, collagen-supplemented 3D matrices predominate and have been used extensively in several models of metastasis and cancer cell migration that require interaction between cancer cells and physiological cross-linked networks of collagen [103-107]. Collagen 3D matrices can be reproducibly manufactured and modified by a number of factors including their source, crosslinking chemistry, pH, temperature, and monomer concentration without affecting their microstructure. However, minor changes in these factors can significantly alter the resulting matrices and lead to inconsistent results from one laboratory to the next. To minimize these environmental variations and reduce one's reliance upon ill-defined biological derivatives that can lead to irreproducible results, an attractive alternative is to fabricate the tumor niche directly [108].

3. Engineering the tumor niche

The tumor niche, i.e. local microenvironment composed of stromal cells and ECM, plays a well-recognized role in cancer development [109-111]. And just as the niche can influence malignant cells, tumor cells can alter the physical, biochemical, and biomechanical properties of their surroundings to reinforce their malignant phenotype [112-117]. The resulting dysregulated ECM can promote cancer progression by facilitating malignant transformation, local tissue invasion, and subsequent metastasis [118]. Additionally, tumor-induced anomalies in the ECM can alter the behavior of stromal cells and lead to angiogenesis and inflammation that generates a tumorigenic microenvironment [119-135]. An in-depth understanding of the dynamic interplay between a tumor and the niche in which it's found will be critical in the effort to develop innovative antineoplastic therapies that act upon the tumor niche and deprive tumors of oncogenic stimuli.

As a method to delineate the most important interactions between a cancer and its tumor-associated microenvironment, particularly ones that profoundly influence a cancer cell's response to biologically targeted therapy, our laboratory has adapted methods normally employed in the field of regenerative medicine [54]. Surprisingly, the simple transfer of ES cells from a monolayer to 3D scaffold resulted in striking changes in ES cell morphology, behavior, growth kinetics, and sensitivity to antineoplastic drugs. Though we have taken just the first few steps to understand how the tissue-engineered scaffolds achieved those profound effects, we anticipate a tissue-engineered bone tumor niche will better mimic human ES tumors [136-139] and retain many of the advantages inherent in ex vivo preclinical cancer models.

3.1 Tissue-engineered 3D scaffolds

As applied to oncology research, the main purpose of tissue-engineered 3D scaffolds is to recapitulate essential architectural, mechanical, and biochemical elements of the tumor microenvironment in a way that promotes tumor cells to behave as they naturally would if present within a human tumor [140-144]. Also, the tissue-engineered 3D scaffolds should support physiological exchange of nutrients, oxygen, and metabolic waste byproducts, and ideally be compatible with standard experimental techniques (e.g. microscopy, immunohistochemistry, cell proliferation assays). While no scaffold material achieve all of those traits, several have proven useful for oncology research [145].

Defined generically as three-dimensional solid or porous biomaterials conducive for cell growth, the scaffolds can be native or synthetic, permanent or biodegradable, and can vary by other traits, such as porosity or surface functionality that influence their appropriateness for specific preclinical models (Table 3). Moreover, the methods used for fibrous scaffold fabrication can also determine the level of control scientists have over scaffold architecture, with more disordered weaving, knitting, braiding, and electrospinning techniques on one end of the spectrum (defined in Table 4), and well-ordered computer controlled 3D-printed scaffolds on the other. Though less common, scaffolds intended for implantation into animal models must also be safe to the host and biocompatible with the site in which it is inserted. Though a comprehensive discussion of the myriad scaffolds types is beyond the scope of this publication, several reviews cover this topic extensively [146, 147].

Naturally, given this wide assortment of scaffold options, several have proven value in culturing malignant tumors. This includes poly(ε-caprolactone) (PCL), polylactide (PLA), polyglycolide (PGA), and co-polymers (PLGA: poly lactic-co-glycolic acid), which are biodegradable and able to form various structures such as fibers, mesh, sponges (Table 5) [148, 149]. Cancer cell lines have been shown to adhere and grow on these synthetic scaffolds and appear capable of forming 3D structures that are morphologically and histologically similar to in vivo tumors [54, 150-152]. Our own laboratory has employed electrospun PCL as the preferred biomaterial to model the bone niche and found it useful to study ES cell growth kinetics, drug sensitivity, and mechanisms of acquired drug resistance [54]. In our hands, cells attached to the PCL fibers and proliferated throughout the uppermost portion of the scaffolds, reaching a maximum cell number of about 300,000 cells/scaffold and depth of 150μm.

Although the parameters we selected to culture ES within PCL scaffolds would almost certainly need to be adapted if used to model other sarcoma subtypes, this study provides proof of concept that ES cells can attach and grow on unmodified microfiber scaffolds that lack ancillary bioactive moieties (Figure 4). It also brings to light several common findings we have encountered when growing osteosarcoma and ES cells within 3D PCL scaffolds. First, when grown in static culture ES cells eventually reach a maximal cell number and depth of penetration, with preferential growth in the uppermost section that is well oxygenated and more accessible to nutrients [153-158]. Under static conditions, oxygen and nutrient levels drop dramatically as distance from the scaffold surface increases, and viable cell growth is often limited to only about 200 μm [159, 160]. After micropipetting ES cells onto 3D PCL scaffolds, they start to proliferate and eventually migrate throughout this oxygenated zone until a maximum density is reached. More deeply seeded cells within the scaffold will be under metabolic stress and exhibit slower proliferation rates.

As cell number and ECM deposition increases, uneven distribution of nutrients, oxygen, and metabolic waste by-products can emerge even in the uppermost 200 μm of the scaffolds. This, in turn, promotes cell apoptosis/death within unfavorable pockets within the scaffold – a problem exacerbated by the high metabolic demands of cancer cells [161-163]. As previously reported, diffusion gradients within tumor spheroids have been shown to produce this type of varied cell structure and are widely used to study the growth kinetics and hypoxic effects of tumor cells present within avascular tumor micro-regions within irregular

tumor vasculature [72, 164, 165]. When grown under static growth conditions, ES impregnated PCL scaffolds could, therefore, equally serve to mimic the varied stress and oxygenation gradients experienced by ES tumors.

A second notable finding in our scaffold-based model of ES was that cell proliferation within PCL scaffolds more closely approximated the growth kinetics of human tumor xenografts, which were both significantly less than monolayer cultures [54]. As expected, since the majority of cytotoxic chemotherapies act in common upon cells undergoing DNA synthesis or cell division (i.e., actively progressing through the cell cycle), the faster mean doubling time of ES cells cultured as monolayers (24 hours) tends to overstate the true clinical activity later determined if/when a drug reaches the clinic [166]. Thus, if for no other reason than accurately modeling antineoplastic activity in the preclinical setting, scaffold-based cell culture appears to be advantageous over monolayer-based options [167].

In actuality, however, scaffold culture not only affects the proliferative rate but also profoundly impacts the ES morphology, internal signaling patterns, and oftentimes the response to biologically targeted therapies that have become increasingly common for the treatment of all cancer types. Illustrating this fact, our laboratory confirmed that ES cells acquire an in vivo-like round cell morphology when they are placed within PCL scaffolds and continue to express immunohistochemical biomarkers normally expressed by human ES tumors ($CD99^+$, $IGF\text{-}1R^+$, $keratin^-$, and SMA^-) [54]. Though more extensive testing is required in other pathways, the proteomic expression profiles along the IGF-1R/PI3K/mTOR signaling pathway, as measured by Western blotting and flow cytometry, would suggest that 3D PCL scaffolds and murine xenografts can reliably mimic critical signaling cascades of known importance in human ES tumors, a finding also observed in breast cancer cells grown in 3D cultures [168]. Taken together, an engineered ES tumor model reliant upon electrospun PCL fibers scaffolds appears to adequately mimic the morphology, growth kinetics, and protein expression profile of human tumors. Ultimately, one of the most important questions to be resolved is whether the 3D model shares enough fidelity with its human counterpart to advocate its use in a preclinical high-throughput drug-testing platform.

3.2 Exogenously derived ECM that supplements tissue-engineered scaffolds

Though the aforementioned experience culturing ES cells as spheroids or 3D scaffolds was limited to growth upon unmodified surfaces, they serve to lay the groundwork in our understanding of how ES and other cancerous cell types intrinsically interact with a spatially complex biologically inert microenvironment. As these models fail to adequately represent the richer complexity of human biology of proven importance for cancer growth, invasion, and metastasis the next challenge would be to add back key components of the ECM to inert scaffolds and/or employ decellularized biological tissues devoid of cells to isolate the specific effects induced by the residual ECM [136, 169-175].

With respect to the former approach, in which biological ECM supplements the tissue-engineered microenvironment, both Matrigel (a poorly defined gelatinous complex of proteins derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells) and peptides rich in Arginine-Glycine-Aspartic acid (RGD) motifs have been widely reported to affect cell behavior and alter cell self-organization. Matrigel, for example, can induce

interconnected capillary-like networks of endothelial cells that would not otherwise form in monolayer culture and, interestingly, ES-secreted $VEGF_{165}$ enhances this process. As a specific recognition site of integrins for their respective ligands, RGD is a ubiquitous biomimetic peptide present within plasma and ECM that is capable of promoting cell motility and stimulating adhesion between tumor cells and their supporting stromal cells and/or ECM. A number of excellent publications exist that expand on the important roles Matrigel and RGD motifs play in the tumor microenvironment [176-178].

With respect to ES and the other sarcoma subtypes, little is known about the ex vivo cell-derived ECM and this inquiry remains a burgeoning avenue of investigation. Several reports have described the composition of fibronectin, laminin, and collagen in ES cell lines and/or clinical samples [179-181]. Vijayakumar et al., for example, demonstrated low levels of beta-catenin (Wnt-pathway) in monolayer ES cultures [182]. Others have reported ECM/scaffold-related effects of the E-cadherin expression, with high expression by ES cells grown within scaffolds, intermediate expression in spheroids, and low expression in monolayer cultures [53]. Since E-cadherin-dependent co-expression of ErbB4 in ES spheroids appears to up regulate the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and secondarily resistance to anoikis and cytotoxic chemotherapy, it is intriguing whether a similar phenomenon is occurring in our 3D PCL-based scaffold architecture [183]. ECM deposition by human tumors can clearly induce an adverse autocrine effect that promotes malignant cell invasion and metastasis. Thus, the ability to mimic this tumorigenic regulatory loop in ex vivo models of the tumor microenvironment may advance our understanding of dynamic interplay that occurs between tumors and their surrounding ECM, and potentially aid in the development of drugs that target the tumor microenvironment [184, 185].

Accurately isolating the effects of tumor-associated ECM from the effects of surrounding stromal cells would be nearly impossible using conventional 2D or xenograft preclinical models but can readily be accomplished in tissue-engineered systems. Decellularized connective tissues, usually obtained after perfusion with detergents, provide an example of this approach and have recently gained in popularity for cancer research given their unique ability to evaluate the oncogenic effects of naturally derived ECM. The effects of bone-specific ECM has been reported using decellularized PCL scaffolds that had originally supported osteogenic cells [186], and direct chemical conjugation of specific ECM proteins or proteoglycans to the surface of PCL fibers can allow controlled release of high-affinity ligands (e.g., heparin sulfate binding growth factors or cytokines) from the 3D scaffolds [187, 188].

4. Applications for cancer biology and preclinical drug testing

Though a longstanding goal of tissue engineering has been to fabricate artificial tissues and organs capable of replacing diseased human tissues, as of yet, there are still no FDA approved substitutes for major organ systems. Nevertheless, considerable progress has been made and many of the principles used to further that aim have been adapted to model the human tumor niche. As discussed previously, our laboratory has had the greatest success using electrospun scaffolds composed of PCL, a resorbable aliphatic synthetic polymer that

has been extensively used by the tissue engineering and the biomedical community. It offers a number of highly desirable properties, including the following: (a) lacks intrinsic biological or biochemical activity, (b) is inexpensive (c) is readily manipulated to produce fibers of precise diameter, (d) has superior rheological and viscoelastic attributes, (d) is considered safe by the FDA, and (e) exhibits very slow in vivo degradation, which can be finely tuned using copolymer blending to achieve the intended biological effect. Both normal and cancerous cells adhere to PCL, and when electrospun, non-woven mats of 10 μm diameter PCL fibers can be made to resemble the native ECM [189], including physiological bone trabeculae and the bony ES tumor niche [54]. Perhaps most important for our applications, micrometer size electrospun PCL fibers provide the requisite porosity to enable cell infiltration and are compatible with flow perfusion bioreactors [190-195]. As specifically used to model ES, the 3D pattern of electrospun fibers appear to induce pronounced changes in ES cell morphology, growth kinetics, and expression of cancer-related pathways, which in turn, promotes chemosensitivity patterns more closely observed in xenografts and human tumors. Excellent reviews exist that highlight the broad applicability of PCL for tissue engineering, drug delivery, and medical devices [196, 197].

4.1 Morphologic characterization of 3D tumor models

Despite the aforementioned benefits, PCL 3D scaffolds also have their drawbacks. Cells grown within 3D scaffolds can be difficult to dislodge; isolation of RNA, DNA, and protein can be difficult; and low melting temperatures (60°C for PCL) preclude standard tissue embedding with paraffin. And as a class, polymeric 3D scaffolds are usually opaque and poorly amenable to routine light microscopy even when polystyrene or other clear substrates are used. Naturally, this poses a major challenge to monitoring cell morphology and proliferation in real time, which in turn, impairs ones ability to continuously monitor the health and viability of cultured cells. This disadvantage can be mitigated to some extent by simply culturing ES cells on multiple scaffolds concurrently then sectioning some at various time intervals. Once sectioned, they can be evaluated by scanning electron microscopy (SEM) or immunohistochemistry (IHC). Flow cytometry (FC) can be used to complement SEM and IHC analyses but, as with those techniques, interrupts the experiment in the course of completing the analysis (Figure 5). Since the cells are removed from the scaffolds prior to analysis, one cannot evaluate the spatial interrelationship of the cell-cell interaction. Theoretically, the cells extracted from scaffolds for FC analysis could be handled aseptically and replaced within a new scaffold but, in practice, this is uncommon and only performed in the rare case where a distinct subset of cancer cells is required.

4.2 3D models used to assess chemosensitivity

Though cell culture monolayers will continue to offer value to the cancer research community, particularly when used in high-throughput drug screening programs, the data resulting from their use can prove unreliable, or worse, contradictory to what one eventually observes when tested in xenografts or human tumors. Emblematic of this limitation, the NCI-60 (a well characterized set of 60 diverse cancer cell lines) has been extensively used to identify novel antineoplastic agents but poorly predicts whether any single drug will be effective in treating specific cancer type. While this incongruence may, to some extent, stem from artificially high cell division rates that overestimate the benefit of cytotoxic

chemotherapies, marked phenotypic variation between monolayer and 3D culture systems (and human tumors) provides another explanation [53, 54]. In support of this hypothesis, the proteomic signature of human tumor spheroids and 3D scaffold models can be readily distinguished from equivalent cells maintained in monolayer culture. This appears to hold true for ES as well, as cells grown within spheroids or 3D scaffolds (natural and synthetic) more closely approximate the protein expression pattern of human tumors than do cell monolayers cells [53, 54, 80, 86, 90].

Drug companies have taken note and many have added 3D cell culture models to their preclinical testing pipeline while continuing to rely extensively upon xenograft testing to determine a drug candidate's activity, toxicity, and pharmacokinetic profile (i.e., absorption, distribution, metabolism, and elimination). As biologically targeted cancer therapies continue to garner a large share of the drug marketplace, one can anticipate an even greater need for preclinical culture models that more accurately replicate the signaling pathways responsible for cancer progression.

4.3 3D models amenable to high throughput drug screening

Toward that end, a number of techniques—such as hanging drops, micro-etched nano-cultures using NCP, and liquid overlay methods—are among just a handful capable of producing uniform ES spheroids amenable to high throughput drug screens (HTS). Although there is no consensus on what qualifies as a HTS, robotic automation of plate handling and drug administration has made it easier than ever to screen hundreds, if not thousands, of compounds per week in commercial scale laboratories. Such mechanization has also permitted complementary methods, such as RNA interference, that can be used to identify putative mechanisms of resistance. Our laboratory, as an example, has performed spheroid-based HTS in 384-well micro-etched NCP to identify several likely mechanisms by which ES evade mTOR inhibitor activity.

As expected, the use of HTS is critically dependent biometric measurement of drug effects, and at least four main types of assays have emerged for that purpose: (i) assays that measure morphological changes in spheroid volume using automated or semi-automated imaging, (ii) cytotoxicity and cell viability assays that measure cell membrane integrity or intracellular metabolic activity under robust detection readouts (e.g., absorption, luminescence, and fluorescence), (iii) apoptosis assays that reliably assess drug-induced apoptosis and cell death, and (iv) gene expression assays that evaluate specific phenomena like metastatic potential.

5. Future directions

As discussed in the introduction, 3D models ES come in two major varieties: cell aggregates capable of intrinsic self-organization (i.e., tumor spheroids) and multidimensional cell aggregates that grow within artificial substrates. The latter implementation should be especially well suited to answer scientific questions that relate heterotypic cell-cell interactions and provide a unique opportunity to explore autocrine or paracrine effects upon oncogenesis, angiogenesis, metastasis, and immunomodulation. Advances in biomaterials,

such as scaffolds and gels, have provided the essential building blocks necessary to recreate selected aspects of the tumor microenvironment.

5.1 Heterotypic co-cultures

Though the ES cells grow as monotonous sheets that lack features of intrinsic self-organization (ductal structures in breast cancer, for example), microscopic review of ES tumors finds other connective tissue cells (fibroblasts and adipocytes), inflammatory cells (lymphocytes, macrophages, natural killer cells), and vascular cells, including pericytes and endothelial cells (Figure 5). Together with the associated ECM, the tumor microenvironment has been identified as a major factor influencing tumor survival, growth, invasion, metastasis, and response to chemotherapy [50, 198-202]. Because these heterotypic cell interactions, and surrounding ECM, can be selectively introduced into the tissue-engineered 3D scaffolds they serve as ideal platform to study the dynamic effects of cell-cell and cell-ECM contact.

5.1.1 ES-MSC co-culture for modeling Ewing sarcoma—As reported earlier, a wealth of information suggests ES originates from a single primitive MSC that acquired a tumorigenic EWSR1/FLI1 translocation, and as such, this cell type has been extensively used to elucidate the genetic perturbations that promotes malignant transformation [17, 20, 21, 198-201, 203-206]. Equally well-documented, ectopic expression of the resulting EWS/FLI1 fusion protein in permissive MSCs is necessary but insufficient of inducing malignant transformation without co-expression of other dysregulated proteins [207].

Given these facts, and taking into account the profound effects that the cell culture microenvironment has upon the collection, purification, and differentiation of human MSC, confusing or frankly incorrect scientific conclusions could be reached by studying EWSR1-FLI1-transfected hMSCs in monolayer cultures devoid of the relevant 3D architecture, ECM, growth factors, and support cells [208]. Our own experience, albeit starting from pre-established ES cell lines, demonstrated the simple transition from monolayer culture to biologically inert PCL scaffolds induced salient changes in the very same proteins (IGF-1, for example) that are required for malignant transformation. This would suggest that, at the very least, one should consider a more physiological bone tumor niche, whether natural or processed, in prospectively deciphering the critical interchange that exists between EWS-FLI and its coconspirators.

5.1.2 ES-EC co-culture and angiogenesis modeling—Endothelial cells are indispensible to the healthy formation of rich vascular networks, which occurs through a process of angiogenesis, vasculogenesis, and/or tumor cell vascular mimicry [209]. To mimic those processes within a synthetic tissue grown in the laboratory, and thereby improve tissue viability and function, ECs have been used in a wide range of tissue-engineering applications. Unexpectedly, even before a mature vascular network is formed, ECs can affect co-cultured cells and influence the surrounding microenvironment. When mixed with MSCs, for example, ECs enhance osteogenic matrix production within 3D PCL scaffolds [210, 211].

As expected, EC-mediated angiogenesis/vasculogenesis also plays an incredibly important role in tumorigenesis. As occurs for other tumors, ES tumors have been shown to possess the capacity to regulate their own survival and appear to do so by secreting soluble vascular endothelial growth factor (VEGF) that secondarily recruits bone marrow derived cells, pericytes, and endothelial cells into ES tumors [198-201]. A similar phenomenon occurs in preclinical models of ES, as co-culture of human EC and ES cells within an ECM gel can induce vascularized endothelial tubes [50]. Indirectly tied to VEGF expression, the insulin like growth factor-1 receptor (IGF-1R) has also been implicated in the modulation of angiogenesis and vasculogenesis [212] and dual targeting of IGF-1R and its ligand elicits anti-angiogenic effects [50].

To our knowledge, ECs have not been used in co-culture within ES cells within 3D scaffolds but this system would be expected to provide a unique opportunity to decipher the two-way feedback that exits between these cell types. A step in this direction, human MSCs, which could serve as a precursor for ES, and human umbilical vein endothelial cells (HUVECs) were co-cultured in 3D PCL-scaffolds under conditions of flow perfusion. Superior proliferation of both cell lines was achieved and the spatial distribution was more uniform throughout 3D-PCL scaffolds [213]. Since VEGF secretion by human ES tumors has been shown to encourage CD38- primitive MSCs to migrate from the bone marrow to the tumor, where they differentiate into ECs and/or pericytes capable of angiogenesis and vasculogenesis [198-201], it would be intriguing to know whether a similar effect could be replicated within a tissue-engineered ES model. If achievable, one could directly measure the effect of ES-produced IGF-1 and VEGF ligands upon ex vivo vasculogenesis and possibly use this novel model to develop and test antineoplastic agents that intercede with the ES's ability to self-regulate their microenvironment.

5.1.3 Use of the 3D ES model to study immune-mediated therapy—Immune cells, including tumor-infiltrating lymphocytes, macrophages, dendritic cells and NK cells, can either maintain or suppress ES tumor growth depending upon the context in which they interact [214-219]. And though beyond the scope of this review, a handful of biologically targeted therapies designed to stimulate the immune system—either directly or by down-regulating immune checkpoints such as programmed cell death 1 (PD1) or cytotoxic T-lymphocyte antigen 4 (CTLA-4)—have generated incredible acclaim for their remarkable antineoplastic activity in appropriately selected patients. While it remains to be determined if those promising results would hold up when eventually applied to patients suffering from ES, this has become a robust area of research.

NK cells, expanded in vitro, have been shown to provoke significant anti-tumor effects in mice engrafted with ES cells, and in rare instances completely eradicated the disease [216]. Activated NK cells, more so than resting ones, have also shown immunoreactivity in ES tumors [217]. Yet, the regulation of NK cell cytolytic activity is complex and induces both activating and inhibitory receptors as well as induced tolerance to specific self-receptors. A refined understanding of these signals could empower novel therapies for ES.

As a platform for investigating the role of immune cells in suppressing ES tumor growth, one could envision co-culturing ES cells with the specific immune cell of interest (e.g. NK

cells) within a PCL based tissue-engineered scaffold. Though the full complexity of the human and/or mouse immune system can't be modeled in its entirety ex vivo, the exceptional flexibility of the system and greater control of the ES-immune cell interactions may outweigh some of its limitations. There are a number of approaches one might take: (i) utilization of allogenic killer immunoglobulin-like receptor (KIR) incompatible NK cells, (ii) blockade of KIR with mAb, (iii) transfection of NK cells to express tumor reactive T-cell receptors (TCRs) or chimeric antigen receptors (CARs), and (iv) sensitization of ES tumor (or cells) to NK cell killing.

The successful activation of Cytotoxic T-cells (CTL) from healthy human peripheral blood mononuclear cells (PBMC), using a modified EWS-FLI1 peptide, and potent killing of cells bearing the EWS-FLI1 translocation provides renewed hope in developing T-cell mediated immunotherapy for ES treatment [220].

5.2 Multi-organ tissue-engineered systems to study metastasis

Most ES-related deaths occur when malignant cells have metastasized to the lung, grown within that location, and developed de novo or acquired resistance to chemotherapy. Although primary thoracic ES tumors (i.e. Askin tumors) do occur and can spread to adjacent lung tissue by direct extension, the vast majority of ES cells can more reasonably be expected transit to the lung after first leaving the primary site (e.g. bone tissue or less common extraskeletal tissues), entering the circulatory system, and adhering to pulmonary endothelium (Figure 6). Despite the deterministic nature of these steps, little is known about the cellular, biomolecular and environmental factors that explain the predictable nature in which ES cells appear in the lung, at least microscopically, very early in the disease course. For this reason, all patients diagnosed with ES are universally provided chemotherapy with the dual goals of shrinking the macroscopic, clinically detectable tumors and eradicating micrometastatic deposits that are assumed to exist within the lung even when small tumors are effectively treated by surgery or radiation.

Though ES cells intrinsically harbor the capacity to metastasize and can gain even greater metastatic potential by acquiring genetic aberrations, the surrounding stromal cells and ECM are critical partners that influence this continuum from bone to lung. In teasing apart the metastatic triggers intrinsic to the cancer cell from extrinsic ones ascribed to microenvironmental effects highlighted in Figure 1, tissue-engineered tumor models of the bone and pulmonary sites afford scientists previously unimaginable experimental control to adjust biomechanical forces, the signaling milieu, and metabolic stresses. Though not previously used to study ES metastasis, a number of tissue-engineered and/or ex vivo regenerated lung tissue models have been shown to mimic functional lung, and one could envision a closed system that uses microfluidic channels link tissue-engineered bone and lung tissues together in a way that mimics the complete pattern of hematogenous spread. As illustrated in Figure 6, the 3D scaffolds our group and others have reported upon can mimic the bone tumor niche, and both lung-on-a-chip [221] and decellularized lung models [222-227] appear to be conducive to the adhesion and growth of cancer cells. Thus far, only non-small cell lung cancers (NSCLC) have been implanted within these tissue-engineered lung models, however unpublished reports by Dr. Min Kim et al. (personal communication)

indicate ES cells readily form distinct pulmonary nodules when placed within decellularized rat lungs that are maintained under conditions of flow perfusion previously used to grow NSCLC [228]. As a final piece of the puzzle, Ingber et al. demonstrated that miniaturized tissue-engineered organs grown ex vivo can be linked in series to mimic the physiological vascular communications that exist in the human body, so it stands to reason that similar methods could be adapted to join synthesized bone and lung together as an innovative system to study ES metastasis.

Though considerable effort will surely be required to move beyond theoretical possibilities, and recognizing that even the best ex vivo systems lack important factors inherent in living organisms, multi-organ tissue-engineered systems present an enticing chance to interrogate, and hopefully thwart, the critical factors that make the lung microenvironment such a hospitable place for ES and other sarcoma subtypes to grow.

5.3 Personalized Therapy

Given the relative rarity of ES and further molecular sub-classification of patients by their tumor's translocation type and proteomic signature [22-31, 229], it is impossible to adequately test all drugs or rational drug combinations in each patient subset [230]. A partial solution to this problem has been to extensively profile each patient's tumor to identify dysregulated proteomic pathways, genomic mutations, or other ‘-omic’ aberrations responsible for tumorigenesis and to isolate mechanisms of *de novo* and acquired drug resistance [231-233]. This approach, however, must eventually be validated using drug candidates in xenografts or cell lines that truthfully mimic the human tumors from which they derive. Though simple in theory, this latter step has remained a challenge in practice, as pre-established xenografts and/or cell lines devolve over time and eventually lose the phenotypic traits originally present within the respective tumors from which they'd been taken.

In an effort to maintain the intimate link between clinical tumor samples and the derived xenografts and/or cell lines, academic laboratories and pharmaceutical companies alike have made major investments establishing primary cell lines and PDX from patients that have carefully annotated clinical response data available. To ensure PDX maintain a high correlation with the source tumor, early generation explants are used before they lose the surrounding human-derived ECM, considered essential for maintaining fidelity with their human counterparts. Similarly, 3D primary cell culture models are being developed to mirror the native ECM and architectural structures present within human ES tumors in the hopes those elements will preserve, or at least prolong, a differentiated phenotype that is truly representative of the original ES tumor. Admittedly, the scientific community has much less experience growing primary cell lines as spheroids and very few laboratories have the specialized expertise necessary to successfully culture ES cells within tissue-engineered 3D tumor microenvironments.

The limited 3D tissue-engineered tumor models that do exist lack standardization and may need to incorporate subtle changes in the fabricated scaffolds to enable primary cell culture of different cancer types. Thus, a one-size-fits-all tissue-engineered approach for all cancer types is unlikely and wouldn't necessarily be expected given the vast differences sarcomas

and carcinomas have in their proclivity for certain metastatic sites. Sarcomas (including ES), for example, spread more commonly to the lungs and bone, whereas carcinomas usually migrate first to lymph nodes before metastasizing elsewhere. Given this affinity for one tissue type over another, one would contend that a principal advantage tissue-engineered 3D scaffolds have over spheroids and monolayer cultures is their capacity for customization to meet the unique microenvironmental needs of the cancer type of interest. Our laboratory has just begun the time-intensive task of culturing primary ES cells within 3D PCL scaffolds and continues to optimize the techniques required to maintain cell viability within an ex vivo tissue-engineered tumor niche (Supplemental Figure 1). The next step will be to correlate the expression profiles of clinical samples with their paired PDX and cell-embedded 3D scaffolds to ensure they successfully recapitulate the human ES tumors. Subsequently, we anticipate using biomimetic ES tumor models to methodically evaluate biologically targeted therapies in advance of early phase human clinical trials of the most promising drug candidates. Simultaneously, one expects the preclinical ES models will shed new light of drug resistance mechanisms and promote the use of innovative drug combinations that would not have been apparent from more primitive monolayer culture models that lack in vivo-like signaling cascades.

6. Conclusion and perspectives

Complex 3D models of human cancer (including ES) are just emerging in academic labs throughout the country and are anticipated to revolutionize the study of the tumor microenvironment. By providing new tools to manipulate the ex vivo tumor niche of both the primary and metastatic sites in ways not currently possible using murine models, tissue-engineered cancer models could serve as an ideal platform to test new cancer therapeutics. Challenges remain, particularly in scaling up these systems for HTS and adapting them for ubiquitous use by the cancer research community, but these hurdles are not insurmountable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ESFT	Ewing's sarcoma family tumors
ES	Ewing sarcoma
MCTS	multicellular tumor spheroids
3D	three-dimensional
PCL	poly(ε-caprolactone)

ECM	Extracellular Matrix
PDX	patient-derived tumor explants
MSC	mesenchymal stem cells
EC	endothelial cells
NCP	Nano-culture plate
HTS	high throughput screening
NK cell	Natural killer cell
IGF-1R	Insulin like growth factor-1 receptor
mTOR	mammalian target of rapamycin

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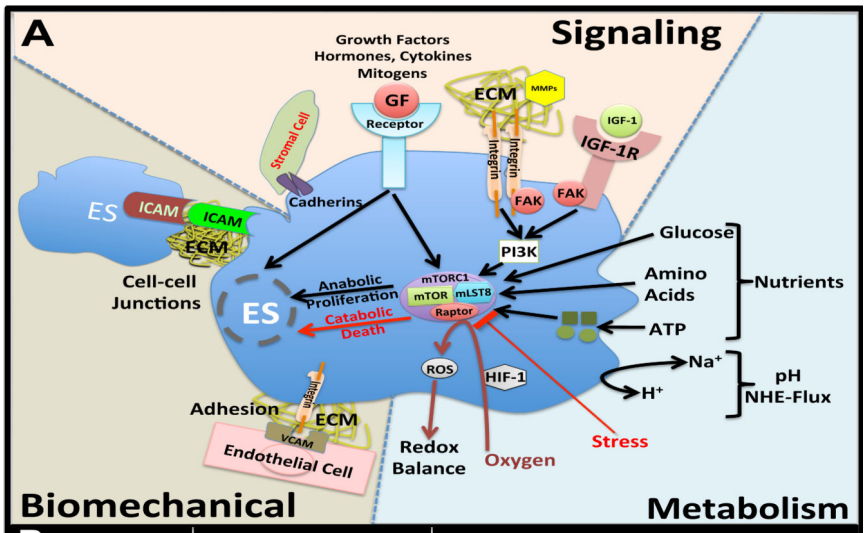

B

Theme	Microenvironment	Key Cell Effectors
Signaling	ECM	Integrins/Proteases
	Growth Factors	Receptors
	Stromal Cells	Cadherins
Biomechanical	Architecture	Cell-cell junctions (e.g. Desmosome)
	Mechanical Forces	Cell-Cell Adhesion (e.g. ICAM, Cadherins) Cell-ECM adhesion (Integrins)
Metabolism	Hypoxia (O2/CO2)	HIF-1/HIF-2
	pH	NHE-Flux
	Nutrients	Glucose, Amino Acids, ATP
	Stress	mTOR Complex

Figure 1.

(A) Principal components of the tumor microenvironment that affect cell behavior. Signaling factors include ECM, GFs, etc. Biomechanical forces include 3D architecture and mechanical loading. Hypoxia, pH, nutrients and stress are affected affecting malignant cell phenotype. **(B)** Key cell effectors in tumor microenvironment. Abbreviations: GF, growth factor; ECM, extracellular matrix; MMPs, metalloproteinases; IGF-1, insulin growth factor 1; IGF-1R, insulin growth factor 1-receptor; FAD, focal adhesion kinase; PI3K, phosphatidylinositide 3-kinases; ATP, Adenosine triphosphate; mTOR, mammalian target of rapamycin; NHE-Flux, sodium-proton exchanger; HIF-1, hypoxia inducible actor-1; ROS, reactive oxygen species; and ICAM, intercellular adhesion molecule 1.

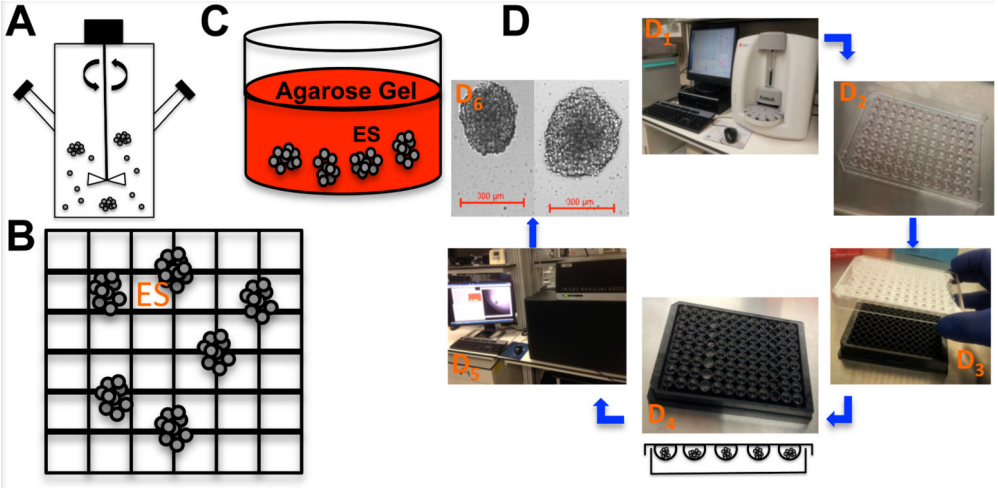

Figure 2. Multicellular tumor spheroids (MCTS) in vitro production techniques of ES

(A) Spinner flask spheroid cultures. **(B)** Micro-etched Nano-cultures. **(C)** Biologically (e.g. Collagen gel) derived 3D matrices cultures. **(D)** ES Hanging-drop cultures. **(D_1)** ES cell line counting using Beckman Coulter Vi-Cell XR Cell Viability Analyzer. **(D_2)** 20µL of cell suspension (100 ES cells) plated on the lid of a Greiner 96-well plate. **(D_3)** The lid was placed back on the 96-well plate containing 100 mL of RPMI (cell culture complete medium) and carefully placed in the incubator for 72 hours. **(D_4)** The lids were removed and 300 µL of RPMI was added to a Nano-Culture® Plate (Scivax NCP-LS) to allow the drop to come in contact with the media, re-incubate for one hour and remove 100 µL. **(D_5)** Spheroids were imaged using the GE InCell Analyzer 6000. **(D_6)** Images of ES spheroids cells at 2×10^4 cells / mL and 5×10^4 cells /mL.

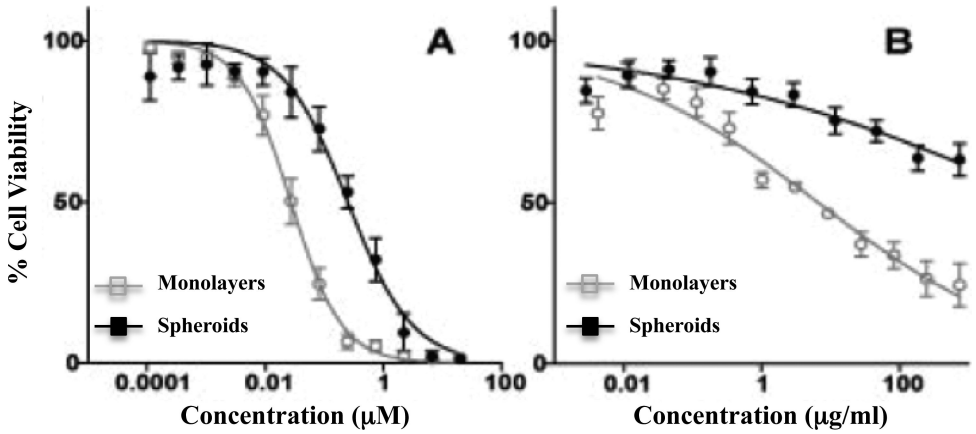

Figure 3. Chemotherapeutic and biologically targeted drug sensitivity testing ES monolayers and spheroids

ES were cultured as both monolayers and spheroids in the presence of chemotherapeutic and biologically targeted drugs. Cell viability data are shown. **(A)** Response to doxorubicin (CID: 31703) **(B)** Response to fully humanized monoclonal antibody R1507 anti-human IGF-1R (Roche).

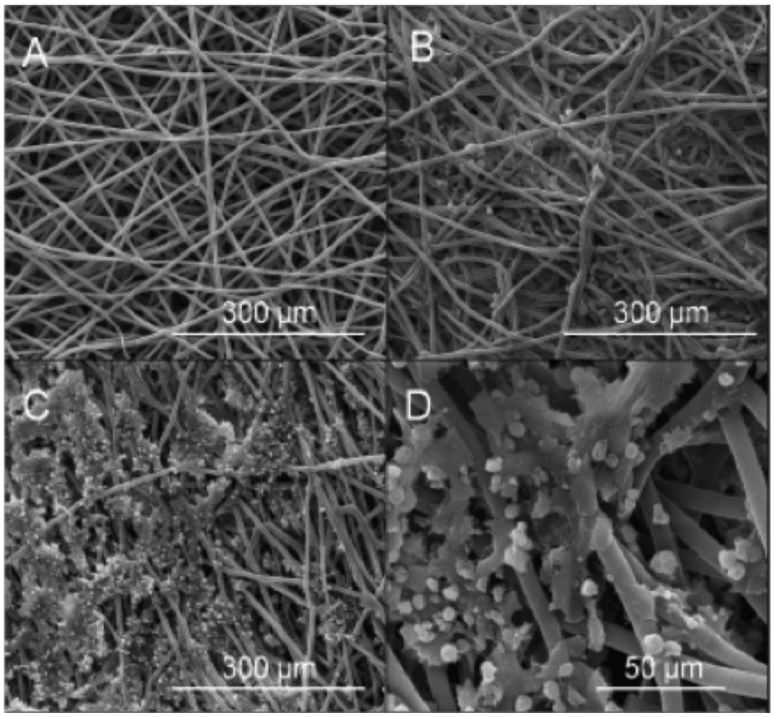

Figure 4. Scanning electron micrographs of PCL microfiber scaffolds

(A) before cell seeding, **(B)** after 2 days in static ES cell culture, **(C&D)** after 24 days in static ES cell culture. After 24 days of static culture, ES exhibit significant 3D cell-cell and cell-matrix contacts.

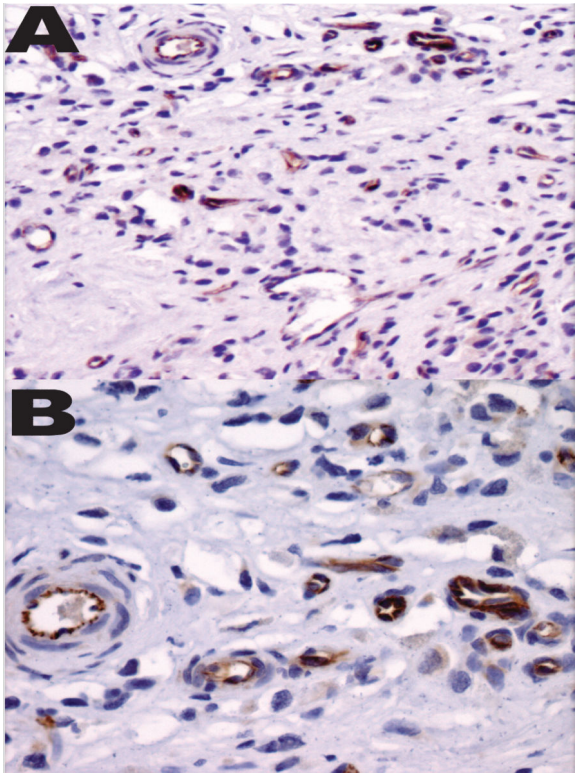

Figure 5.
IHC staining of human ES tumor for VE-Cadherin under low-**(A)** and high- power **(B)** magnification confirming the coexistence of EC.

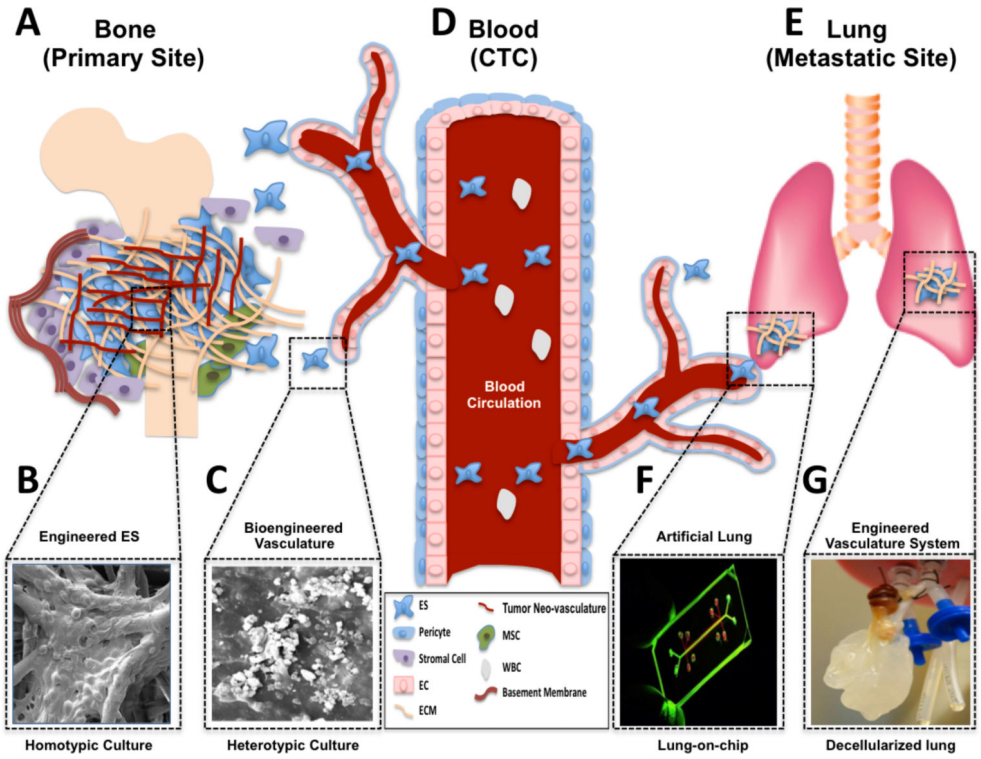

Figure 6.

Bioengineered models that mimic ES microenvironment at the primary, hematogenous and secondary sites. **(A)** Bioengineered preclinical models of ES interacting with osseous-like 3D scaffold **(B)** or synthetic vasculature **(C)**. **(D)** Vascular system by which ES disseminate to lung. **(E)** Lung metastases modeled lung. **(F)** Lung-on-chip or **(G)** Decellularized lung. Abbreviations: CTC, circulating tumor cells; EC, endothelial cell; ECM, extracellular matrix; MSC, mesenchymal stem cell; and WBC, white blood cell.

Table 1

Methods used for ex vivo culture of ES cells

	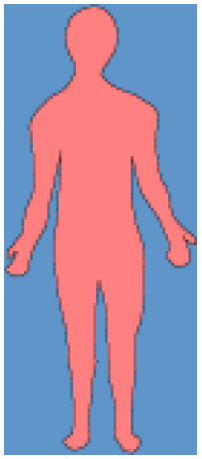		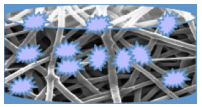	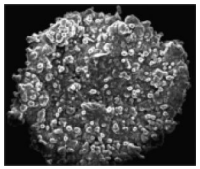	
	Patient	**PDX**	**3D**		**2D**
			Bioengineered 3D Scaffolds	**MCTS**	
Complexity	Organism	Xenograft	Organ/Tissue	Multicellular aggregate	Cellular
Substrate	NA	(Orthotopic/Heterothopic)	Synthetic Fibers (e.g. PCL)	Biological Gels (e.g. Matrigel, Collagen, Laminin rich gels)	Plastic or Glass
Cell Type	NA	Homotypic/Heterotypic	Homotypic/Heterotypic	Homotypic/Heterotypic	Homotypic
Tumor Niche	Physiologic	Chimeric: Animal/Human	Yes	Limited	No
Biology Studies	Limited	Yes (Costly)	Yes (Specialized techniques)	Limited	No
Preclinical Drug Testing (correlation with human trials)	NA	High (Low throughput)	Intermediate (Intermediate throughput)	Intermediate (High throughput)	Low (High throughput)
Biomarker development	Yes	Yes	Unknown	No	No
Personalized Medicine (CLIA-certified)	Yes	Yes	No	No	No

Table 2

Organoids used to study normal physiology and diseases

Organoid Type (Organ)	Disease Modeling	Cell Type	Scaffold Type	Bioreactor	Growth Factor/Nutrients	Reference/Journal
Brain	Microcephaly	hESC, hPSC,	Matrigel	Spinning	hbFGF, RA	Nature[1]
Liver	Cirrhosis	iPSC-LB, iPSC-Hes, HUVECs, hMSC	Matrigel/Collagen IV	NA	hbFGF, hBMP4, HGF, oncostatin-M	Nature[2]
Kidney	Polycystic kidney	hESC, iPSC	Matrigel coated plate	NA	BMP4, FGF2, RA, Activin A, BMP2	Nature Cell Biology[3]
Eye (Retinal)	Degenerative diseases	hESC, hiPSC	Laminin coated dishes	NA	BMP4, WNT3a	PNAS[4]
Intestinal	Gut Defects, IBD, Transplantation,	hESC, iPSC,	Matrigel	NA	FGF4, WNT3a, Activin A, R-Spondin-1, Noggin, EGF	Nature[5]
	colon rectal cancer	Colorectal tumor cells	Collagen coated dishes	NA	EGF, bFGF	Gastroenterology[6]
Lung	Lung cancer	AEC, E14.5 lung single-cell suspension	In vivo Kidney capsule	NA	NA	JCI[7]

Abbreviations: IBD, inflammatory bowel disease; hESC, human embryonic stem cells; hPSC, human pluripotent stem cells; iPSC-LB, induced pluripotent stem cells- liver buds; iPSC-Hes, induced pluripotent stem cells-hepatic endoderm cells, HUVECs, human umbilical vein endothelial cells; hMSCs, human mesenchymal stem cells; hbFGF, human basic fibroblast growth factor, hBMP4, human bone morphogenetic protein 4; HGF, human growth factor; FGF2, fibroblast growth factor 2; RA, retinoic acid; BMP2, Bone morphogenetic protein 2; Wnt3a, wingless-type MMTV integration site family member 3A; FGF4, fibroblast growth factor 4; EGF, endothelial growth factor; AEC, alveolar epithelial cells.

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Table 3

Methods, factors and biomedical application of scaffolds

Method	Examples	Polymers	Properties	Application
Biodegradable porous scaffold: polymeric porous scaffolds with homogenous network	Casting, leaching and foaming methods	PLLA, PLGA, PDLLA, collagen, etc.	Controlled structure & production	Drug delivery, bone & cartilage tissue engineering
Fibrous scaffolds: mimicking the architecture of natural human tissue at the nanometer scale (Nano, micro & nonwoven fiber)	Electrospinning, self-assembly, & phase separation	PCL, PGA, PLA, PLGA	Biomechanical and biocompatible high surface area	Tissue engineering, drug delivery & wound healing
Hydrogel scaffold: shape-retentive polymeric network swollen with a high percentage of water	Microfluidics, micromolding, photolithography, & emulsification	PGS, PEG, PDMS, & Silicon PMMA, HA, PEG, Alginate PMMA, PAA, Fibronectin, chitosan Collagen, gelatin & HA	Biological, mechanical, & physical complexity of structure, shape & size	Microdevices, biochips, cell-based microreactors, etc.
Microsphere scaffold: prepared from a large variety of biodegradable materials, enabling easy control of porosity and pore interconnection	Thermal induction Particle aggregation Solvent evaporation Freezing & drying	PEG, PLLA Chitosan, HAP PLGA & PLAGA Collagen, PLGA, Chitosan	Highly porous for cell transplant Mechanical stability High cellular density Durable & flexible structure	Bone tissue engineering
Ceramic scaffold: useful due to their similarity to bone mineral & their osteo-conductivity and biocompatibility	Sponge replication Calcium phosphate coating	TCP, BCP, PU sponge, calcium phosphate, PLGA, PS, PP, collagens, silk and hair fibers, etc.	Enhanced biocompatibility and bioreactivity	Bone tissue engineering & orthopedic application
Functional scaffold: delivering of substances inducing cell growth	Growth factors, hormones and ligands release	Alginate, gelatin, collagens, fibrin, PLGA, PLA, etc.	Variable structure: hydrogels, membranes, microspheres, foams & membranes	Endothelium interaction, tumor vascular interactions, bone regeneration & wound healing
Acellular scaffold: elimination of the cellular composition without affecting the composition, mechanical integrity and biological activities of the remained ECM	Decellularisation	Biological organs (e.g. lung)	Retain biomechanical properties, anatomical structure & native ECM	Tissue engineering
“Tissue scaffold”: assist in the production of ECM, and possible integration with in vivo tissue growth	Robotic & automated deposition of cells in 3D space	Tubular collagen gel Sodium alginate	Layers deposition of ECM or cells Multicellular composition reconstituting tissues	Printing 3D organs, acellular polymeric scaffolds & biochips development

Abbreviations: PLLA, polylactide; PLGA, poly lactic-*co*-glycolic acid; PDLLA, poly-D,L-lactide; PCL, Polycaprolactone; PGA, polyglycolide; PLA, polylactide; PGS, poly glycerol-sebacate; PEG, Poly ethylene glycol; PDMS, Polydimethylsiloxane; PMMA, polymethyl methacrylate; HA, hyaluronic acid; PAA, Poly acrylic acid; PLAGA, poly-lactide-co-glycolide; TCP, Tricalcium phosphate; BCP, Biphasic Calcium Phosphate; PU, Polyurethane; PS, Polystyrene; PP, Polypropylene

Table 4

Fibrous scaffold fabrication methods

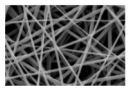
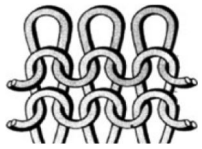
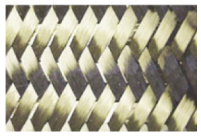
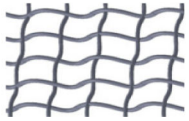
Method	Definition
Electrospinning 	Fibers are drawn under an applied electric field and deposited on a surface forming a fibrous scaffold
Knitting 	Fibers are connected in series of loops
Braiding 	Fibers are braided by intertwining three or more fiber strands
Weaving 	Fibers are weaved by interlacing warp and weft fibers in perpendicular directions

Table 5

Properties of polymers used for bioengineered substrates

Polymer	Biological Activity	Biodegradation Rate (Months)	Biomechanical properties	Area of application
PGA	Inert: anticoagulant, antiviral & plasma cleaning	6 to12	Compressive and flexible Tensile strength 339-394 MPa	Tissue engineering in orthopedics
PLLA	Inert: improvement of tensile & suture, injectable form	>24	Tensile modulus 1.2-16 GPa	Orthopedic & HIV infection
PLDLA	Inert: stimulate regeneration of the whole meniscus	12 to 16	Tensile modulus 1.9-2.4 GPa	Orthopedic
PCL	Inert: bone & cartilage repair	>24	Compressive & modulable Tensile modulus up to 340-360 MPa	Suture coating, dental & orthopedic implants
PEU	Inert: bio-and blood-compatible materials	1-2	Tunable: 5-40 MPa	Cardiovascular devices, artificial organs, tissue replacement, & in vivo restoration of body joints
HA	Inert: angiogenesis, vehicle for osteogenesis, osteoconductive	1-2	Tensile strength 40-100 MPa Bend strength 20-80 MPa Compressive strength 100-900 MPa	Bone graft
PLGA	Inert: delivery of small molecule drugs, proteins and other macromolecules in commercial and research applications	1-12	Compressive & strength Highly tunable	Drug delivery carrier As scaffolds for tissue engineering
PLGA-Collagen	Active: transdermal delivery	1-12	Compressive & strength	Skin tissue engineering, bone substitutes, & artificial blood vessels and valves
Collagen	Active: nanoparticles for gene delivery and basic matrices for cell culture systems	Hours	Elastic modulus of native fibrils around 9 GPa	Gel formulation with liposomes for sustained drug delivery.

Abbreviations: PGA, polyglycolide; PLLA, polylactide; PLDLA, Poly-L/D-lactide; PCL, Polycaprolactone; PEU, Polyesterurethanes; HA, hyaluronic acid; PLGA, poly lactic-*co*-glycolic acid