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Evaluating Drug Efficacy and Toxicology in Three Dimensions: Using Synthetic Extracellular Matrices in Drug Discovery

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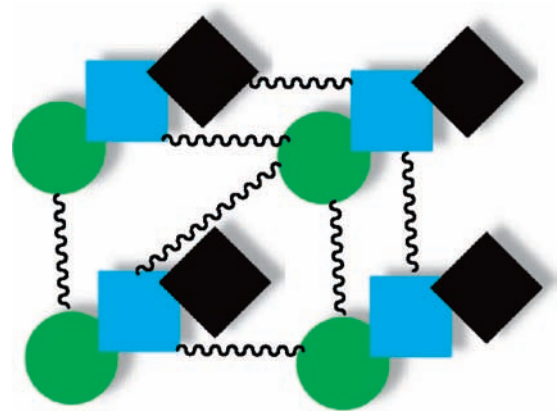

The acceptance of the new paradigm of 3-D cell culture is currently constrained by the lack of a biocompatible material in the marketplace that offers ease of use, experimental flexibility, and a seamless transition from *in vitro* to *in vivo* applications. I describe the development of a covalently cross-linked mimic of the extracellular matrix (sECM), now commercially available, for 3-D culture of cells *in vitro* and for translational use *in vivo*. These bio-inspired, biomimetic materials can be used "as is" in drug discovery, toxicology, cell banking, and, ultimately, medicine. For cell therapy and the development of clinical combination products, the sECM biomaterials must be highly reproducible, manufacturable, approvable, and affordable. To obtain integrated, functional, multicellular systems that recapitulate tissues and organs, the needs of the true end users, physicians and patients, must dictate the key design criteria.

In chemical terms, the sECM consists of chemically-modified hyaluronan (HA), other glycosaminoglycans (GAGs), and ECM polypeptides containing thiol residues that are cross-linked using biocompatible polyvalent electrophiles. For example, co-cross-linking the semisynthetic thiol-modified HA-like GAG with thiol-modified gelatin produces Extracel as a hydrogel. This hydrogel may be formed *in situ* in the presence of cells or tissues to provide an injectable cell-delivery vehicle. Alternately, an Extracel hyrogel can be lyophilized to create a macroporous scaffold, which can then be employed for 3-D cell culture.

In this Account, we describe four applications of sECMs that are relevant to the evaluation of drug efficacy and drug toxicity. First, the uses of sECMs to promote both *in vitro* and *in vivo* growth of healthy cellularized 3-D tissues are summarized. Primary or cell-line-derived cells, including fibroblasts, chondrocytes, hepatocytes, adult and embryonic stem cells, and endothelial and epithelial cells have been used. Second, primary hepatocytes retain their biochemical phenotypes and achieve greater longevity in 3-D culture in Extracel. This constitutes a new 3-D method for rapid evaluation of hepatotoxicity *in vitro*. Third, cancer cell lines are readily grown in 3-D culture in Extracel, offering a method for rapid evaluation of new anticancer agents in a more physiological *ex vivo* tumor model. This system has been used to evaluate signal transduction modifiers obtained from our research on lipid signaling. Fourth, a new "tumor engineering" xenograft model uses orthotopic injection of Extracel-containing tumor cells in nude mice. This approach allows production of patient-specific mice using primary human tumor samples and offers a superior metastatic cancer model.

Future applications of the injectable cell delivery and 3-D cell culture methods include chemoattractant and angiogenesis assays, high-content automated screening of chemical libraries, pharmacogenomic and toxicogenomic studies with cultured organoids, and personalized treatment models. In summary, the sECM technology offers a versatile "translational bridge" from *in vitro* to *in vivo* to facilitate drug discovery in both academic and pharmaceutical laboratories.

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Introduction

The native extracellular matrix (ECM) is a biologist's dream but an organic chemist's nightmare. It consists of a heterogeneous three-dimensional (3D) collection of proteins and glycosaminoglycans (GAGs) linked by covalent and noncovalent molecular interactions. The core proteins of proteoglycans (PGs) are covalently linked to chondroitin sulfate (CS), heparan sulfate (HS), and other sulfated GAGs. Electrostatic associations with ions, hydration of the polysaccharide chains, binding of link modules of PGs to hyaluronan (HA), and triple-helical collagen fibrils comprise some of the noncovalent interactions. HA is the only known nonsulfated GAG and is ubiquitous in all connective tissue as a major constituent of the ECM. We sought to make a chemically tractable 3D system to reconstruct the ECM from its components, with the goal of producing versatile building blocks that would be manufacturable and approvable for human use. To this end, we developed chemically modified HA derivatives that gave biodegradable biomaterials[1,2] with applications in drug delivery[3] and tissue engineering.[1,4] However, HA alone would not support cell attachment and proliferation; other components of the ECM were required. This constraint was ultimately solved by creating a covalently cross-linked synthetic ECM (sECM)[5] that could offer consistent materials in multiple physical forms. The sECMs have experimentally controllable compositions and rigidity, thereby providing tissue-appropriate *in vivo*-like microenvironments for cell culture in 3D and reparative medicine.[6] This Account describes the origins of the sECM technology and the spectrum of potential uses in the drug-discovery process.

Covalent, Cross-linked Equivalents of the Extracellular Matrix

We used hydrazide chemistry[7] to prepare a thiol-modified form of HA, known as HA–DTPH,[8] which could be used to make *in situ* cross-linkable hydrogels[9] with scar-free wound-healing properties. To achieve cell attachment to the HA hydrogels, we used DTPH-modified gelatin to produce biocompatible, cell-seedable disulfide cross-linked gels.[10] To obtain a hydrogel that could be cross-linked in the presence of cells within 5–30 min, we used the bifunctional cytocompatible electrophile poly(ethylene glycol) diacrylate (PEGDA). Alternatively, we covalently incorporated Cys-containing arginine–glycine–aspartate (RGD) peptides,[11] a mixture of three Cys-containing recombinant domains of human fibronectin,[12] or thiol-modified gelatin (Gtn–DTPH) to produce *in situ* cross-linkable hydrogels suitable for 3D cell culture and tissue-engineering applications.[5] Figure 1a shows the first sECM prepared from HA–DTPH (R = H), which supported the growth of healthy tissue *in vivo*.[5]

Adding a third ECM component was required for the retention of growth factors with high avidity. Immobilization of a thiol-modified heparin derivative (HP–DTPH) in the sECM mimicked the heparan sulfate proteoglycans (HSPGs) of native ECMs (Figure 1b).[13] This HSPG-mimetic sECM allowed spatiotemporal control of the delivery of single or dual growth factors, including bFGF, VEGF, angiopoetin-1, and KGF.[13–15] Moreover, these HSPG-like sECMs elicited a formation of interconnected vasculature *in vivo*, in contrast with the incomplete angiogenic response to single growth factors.

To further stabilize HA–DTPH against degradation by hyaluronidases and to increase cross-linking sites, we prepared a carboxymethylated form of HA, known as CMHA-S (Carbylan-S) (Figure 1a). Cross-linking with PEGDA affords a hydrogel that promotes wound healing[2,16] and adhesion prevention.[17]

The sECMs have been used for the engineering and repair of both hard and soft tissues *in vivo*[2] and to support cell attachment, growth, and proliferation in a 3D environment.[18] Preclinical *in vivo* tissue-engineering and repair applications include restoration of viscoelasticity and repair of biopsied vocal folds,[19] accelerated repair of a cortical bone defects,[20] repair of osteochondral defects,[21] and re-epithelialization and revascularization of wounds in diabetic mice.[22] For cartilage repair, the *in situ* cross-linkable, injectable sECM was used to deliver and retain autologous bone-marrow-derived stromal cells.[21]

Variable Composition. The composition of the sECM should be customized for specific cell types for *in vitro* and *in vivo* applications. For example, using an array of 32 combinations of 5 ECM proteins (collagen I, III, and IV, laminin, and Fn) embedded in polyacrylamide, optimal compositions for primary rat hepatocyte function and murine ES cell differentiation were identified.[23] The incorporation of native ECM proteins can also be accomplished with the sECM technology. The addition of native type-I collagen in HA–DTPH–PEGDA afforded noncontracting sECMs suitable for cell growth.[24] Stem cells from adipose and bone marrow seem to prefer HA-rich environments, and thus, a lower gelatin–DTPH:CMHA-S ratio is required. For liver progenitor cells, self-replication occurs on cross-linked CMHA-S hydrogels in the presence of small amounts of type-III collagen, while these cells will differentiate in the presence of type-I collagen or any materials containing significant amounts of type-I collagen.[25] Repair of

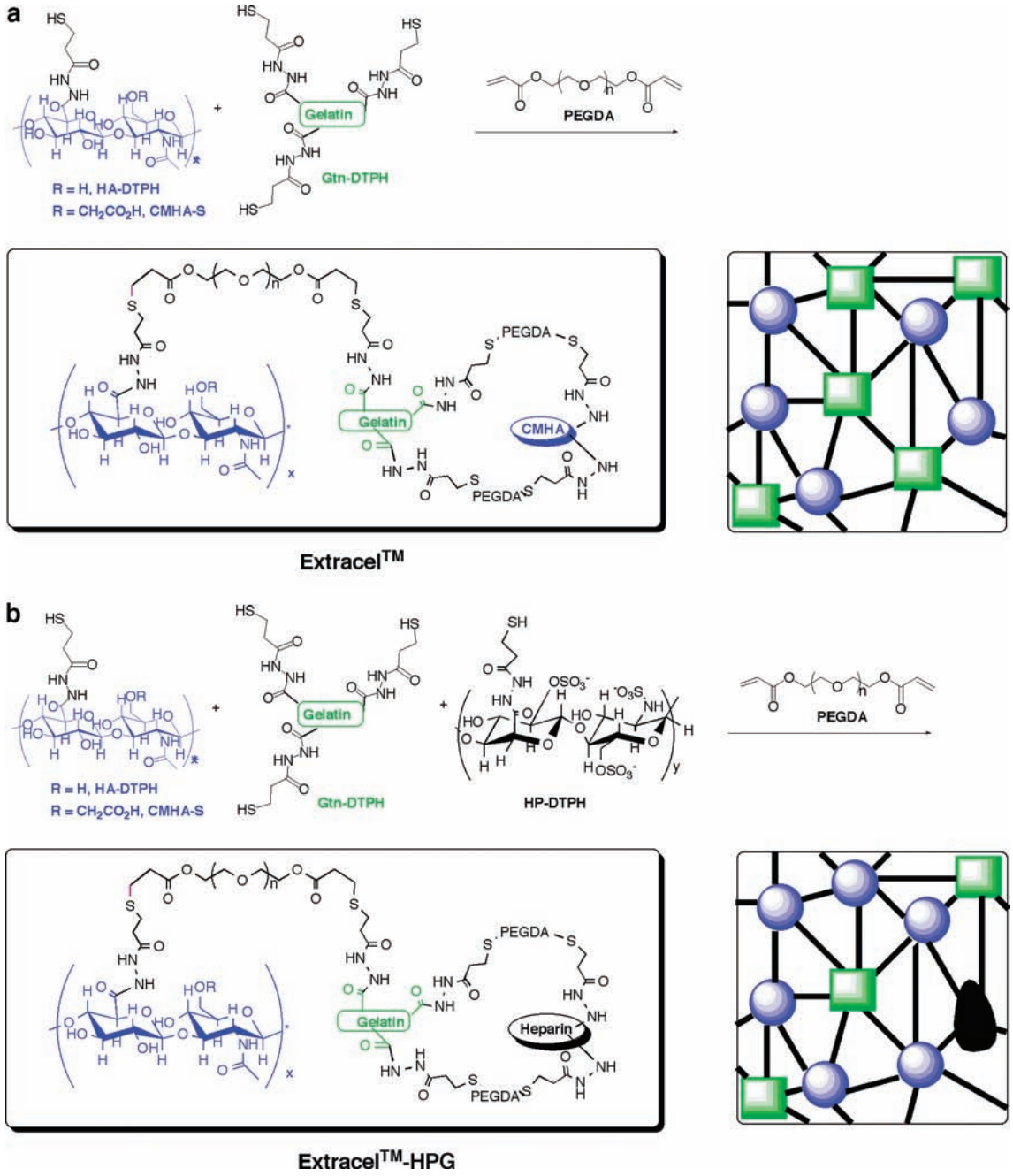

FIGURE 1. (a) Preparation of Extracel, a synthetic, covalent sECM for 3D cell culture and tissue engineering. (b) Preparation of Extracel–HPG. Key: blue circle, thiol-modified HA; green square, thiol-modified gelatin; black diamond, thiol-modified heparin; black lines, PEGDA cross-linkers.

biopsied rabbit vocal folds (a HA-rich environment) was optimal with a gelatin–DTPH:CMHA-S ratio of 5:95.[19]

Variable Rigidity. Cells that form soft tissues or bone are adherent and must attach to the extracellular matrix to spread, grow, and proliferate to form functional tissues. Each cell type achieves its correct morphology and biochemical function in response to a matrix with the same compliance (stiffness) as that of its native tissue source.[26] Matrix elasticity also directs stem cell lineage specification.[27] Thus, mesenchymal stem cells (MSCs) will become neurons on soft matrices (0.1–1 kPa), muscle on matrices of intermediate stiffness (8–17 kPa), and bone on rigid matrices (25–40 kPa). Adult human dermal fibroblasts (HDFs) modified their mechanical response to match substrate stiffness on covalent fibronectin domain-modified sECMs.[28] With PEGDA cross-linker concentrations of 4.5, 1.5, and 0.5%, shear storage moduli of 4270, 550, and 95 Pa were obtained. The HDFs on stiffer substrates had higher moduli and exhibited a more stretched and organized actin

cytoskeleton that those on softer substrates. Gel compliance also plays a critical role in tissue engeering and cell therapy *in vivo*. For example, cartilage repair and soft tissues are best engineered in Extracel hydrogels,[21] while macroporous sponges (i.e., lyophilized hydrogels) appear more suited to bone repair.[20]

Importance of Biodegradation *in Vitro* and *in Vivo.* For tissue repair *in vivo*, a biodegradable material is required. The commonly used polylactides and polyglycolides undergo bulk erosion by slow hydrolysis; variations in the polymer and not the biology dominate the rate of bioerosion. In the HA-based sECMs, the biology of the tissue growth dictates the rate of degradation. As cells proliferate and differentiate, the sECM is degraded by cell-secreted matrix metalloproteinases (MMPs) and GAG hydrolases and the cells secrete a tissue-specific native ECM. Finally, cells can be readily recovered from 3D Extracel constructs with high viability, using trypsin to detach cells and collagenase and hyaluronidase to the digest matrix.

Practical Considerations. The preparation, handling, and availability of materials is crucial when translating from the research laboratory scale to a commercial product. At the Center for Therapeutic Biomaterials, we incorporated many user-friendly features into the Extracel sECM materials.

First, the sECMs can be fabricated into hydrogel films, tubes, porous sponges, nanofibers, and cell-seeded hollow cylinders.[29] The same sECM composition can thus be explored in a variety of *in vitro* and *in vivo* formats, thus separating the effects of composition from mechanical cues.

Second, the sECM technology provides a consistent manufactured product with negligible batch-to-batch variability. Because the sECM is not extracted from an animal tumor, it contains no undefined components or extraneous growth factors. These features are essential to obtain U.S. Food and Drug Administration (FDA) approval of a cell delivery material.

Third, cells can be added to the sECM components in a physiological buffer at ambient or body temperature and then gelled within 5−30 min. This obviates the need to conduct complex manipulations to control rates of gelation. Ease of use is of paramount importance to large-scale adoption by academic researchers, pharmaceutical R&D scientists, and ultimately for clinical use by physicians.

Fourth, the sECM hydrogels exhibit minimal swelling or contraction as ionic strength changes within the physiological range. Unlike collagen gels, no contraction occurs when cells are activated by growth factors.[24]

Fifth, the sECM has been formulated to maximize use in many market niches. The uses of other 3D products are limited by the intrinsic limitations of the materials and cost.

Sixth, the sECM gels and films are transparent at visible and ultraviolet (>256 nm) wavelengths. This facilitates visualization using optical, fluorescence, and confocal microscopy. Many other commercial scaffolds are opaque or translucent, making visualization of cells problematic.

Taken together, the Extracel sECM platform provides flexible experimental parameters, batch-to-batch consistency, multiple physical forms, ease of handling under cell-friendly conditions, resistance to contraction, cost, and transparency. For studies of drug efficacy, toxicology screening, chemogenomic profiling, and proteomic studies of drug−pathway interactions, these materials offer many opportunities not accessible with existing two-dimensional (2D) or 3D cell-culture products.[6]

The Importance of 3D Cell Culture in Drug Discovery

High-Content Screening. Current drug-discovery programs now include biochemically based high-throughput screening (HTS) and cell-based high-content screening (HCS) in the portfolio of approaches for identifying effective agents, validating target selectivity, and eliminating compounds with undesired off-target effects. With few exceptions, cell-based HCS assays employ tissue culture plastic (TCP) as the standard substratum; it is familiar, relatively inexpensive, reproducible, easy to use, and has a long shelf life. However, TCP does not adequately reflect the cellular microenvironment[30] and may be irrelevant to actual *in vivo* conditions.[31] Beginning over 35 years ago, coating surfaces with collagen[32] and later encapsulation in 3D matrices[33] has led to increased appreciation for the differences in cell morphology, mechanics, and behavior in the 3D tissue-equivalent environment.[34] Researchers have begun a paradigm shift from 2D culture on TCP to 3D culture.[18] In pioneering work, Bissell studied the molecular mechanisms of breast cancer and cell invasion using the normal mammary gland as a model system,[35] demonstrating that murine cells cultured in 3D in a laminin-rich gel formed spherical acini and secreted β-casein. Because responses to chemotherapeutic agents depend upon cell architecture and tissue polarity, "...all of a sudden, studying cancer cells in two dimensions appears to be quaint, if not archaic".[36]

The replacement of 2D by 3D methodologies has been hindered by the limitations of the available substrata.[18] The current industry standard, with over 80% market share in 2006, is Matrigel, an murine sarcoma extract that contains proteins, glycoproteins, and growth factors.[37] Matrigel has been successfully utilized in cell growth and differentiation,

angiogenesis, and invasion assays and promotes natural cell morphology and behavior.[38] Matrigel has been used for acinus formation with human breast MCF-10A cells; MDCK cells[39] also form acini in Matrigel. However, limited availability, pathogen content, immunogenicity, and animal tumor origin exclude Matrigel from clinical use. Another dominant 3D material, PureCol (type-I collagen) has limitations attributable to its biochemical and physical characteristics. The apparent market need for a practical, approvable, versatile synthetic ECM for 3D cell culture and tissue engineering led to the development of the Extracel technology.

How can a convenient, affordable, and versatile 3D biocompatible matrix benefit the drug-discovery and drug-evaluation process? Ultimately, end-user needs will drive applications. Potential areas in which a 3D approach could have immediate potential in facilitating the flow through the drug-discovery pipeline are outlined below. These approaches may permit exploration of a much wider spectrum of drug targets.[40] Following this speculative overview, two areas in which my laboratories have achieved initial proof of concept will be described: models for hepatotoxicity and orthotopic xenografts.

Assays for Cell Invasion, Migration, Proliferation, and Angiogenesis. The Extracel technology offers a set of optimizable materials to assess these cell behaviors. New compounds that limit cell proliferation, migration, and invasion are common drug candidates in oncology programs. Identification of leads with potential angiogenic activities for tissue revascularization can be studied *in vitro* or *in vivo* in Extracel-HP.[14] Potential antiangiogenetic compounds for oncology can also be screened in 3D with an analogous approach.

Proteomics and Chemogenomics. Most comparisons of cell–biomaterial interactions have been performed in 2D on nonphysiological substrata,[41] but newer studies show a move toward ECM-mimetic materials. For example, genes in human fetal lung fibroblasts involved in cell signaling, ECM remodeling, inflammation, angiogenesis, and hypoxia were selectively activated in cells on a collagen–GAG mesh.[42] Extracel provides a variety of low-background, covalent ECM-like substrata with defined composition and compliance. Cells could be grown in the presence of drug leads, and the nuances of protein and gene expression would be gleaned by harvesting the cells and profiling cell extracts using microarray technologies. Toxicogenomics is an important subset of chemogenomics, and determination of the entire pattern of gene expression altered in response to a drug candidate should be determined in a physiological 3D environment and not on TCP.

Stem Cell Biology. The rapidly growing field of cell therapy is preoccupied with thorny issues of sourcing, cryopreservation, expansion, differentiation, delivery, retention, and engraftment of stem cells. Extracel has already been validated for 3D growth of stem cells from liver, bone marrow, and adipose compartments, and investigations of stem cells from breast, neural, cardiac, and other compartments are underway. Importantly, Extracel offers the ability to control composition and the potential to translate *in vitro* results into *in vivo* therapies.[18]

Organotypic Culture *in Vitro.* Growing concerns with drug failures in phase-III clinical trials because of cardiotoxicity, neurotoxicity, and hepatotoxicity are driving the pharmaceutical industry to explore *ex vivo* models with human tissues and cells. Many existing early toxicology models using microsomes, TCP-cultured cells, and cell-free extracts are used to weed out toxic compounds. Nonetheless, compounds with serious flaws still enter phase III, often with disastrous human and business consequences. The first area in which tissue engineering may have an important impact, before its impact is significant in reparative medicine, is the development of organotypic cultures for toxicology screening. Pioneering work to integrate cell culture with microfabrication[43] has led to microspherical heart pumps,[44] bioartificial livers[45] and drug response hepatic devices on chips,[46] and patterned arrays of living cells.[47] In many of these examples, a 3D sECM is used or could be incorporated. The coculture of cell types will also be essential in neurological, cardiovascular, hepatic, and other organotypic cultures, and the sECM technology is particularly well-suited for such new designs. The specific use of the Extracel sECM in hepatoxicity models is explored below.

Engineered Human Tissues in Animal Models. One step beyond *ex vivo* organotypic models is the development of whole-organism pharmacokinetic and pharmacodynamic models. Because drug metabolism in rodents and humans differs dramatically, one solution could be the production of mice with engineered human livers. This moves from metabolic profiling in an *ex vivo* human organoid to the study of how the metabolites from the organoid interact within an intact organism. Perhaps such a system might further reduce phase-III failures.

Personalized Medicine Models. The sECM technology allows a vision for personalized medicine in which tissue engineering is connected the determination of drug safety and efficacy, using tissue biopsies. With many pharmaceutical intervention options, pre-evaluation of agents for safety and efficacy *ex vivo* using a patient's own normal and diseased tissues would be ideal. Using the engineered human tissue

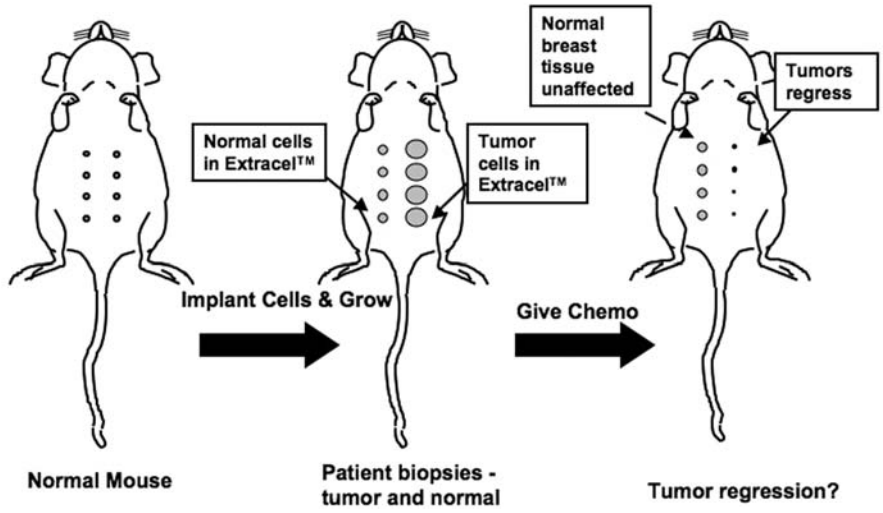

FIGURE 2. Proposed personalized or patient-specific mouse model for the treatment of breast cancer. Cells from healthy breast tissue in Extracel produce four normal breast constructs, while patient breast tumor cells in Extracel would yield four breast tumors. Mice would be used to identify an optimal patient-specific optimal therapy. Original drawing by X. Chen.

model, one can envision a novel efficacy/safety oncology model (Figure 2). Normal breast epithelial cells in Extracel would be injected into the mammary fat pad of a nude mouse to give four normal breast tissue xenografts, which would grow until the sECM was bioresorbed and remodeled with secreted ECM. Then, a breast tumor biopsy from a specific patient, perhaps pretreatment or possibly after cancer has recurred, would provide a heterogeneous pool of tumor and stromal cells that would be suspended in Extracel and injected to give four breast tumors. We might generate 12 mice per patient. Approximately 4 weeks later, large tumor masses would have formed. Then, a variety of treatment options could be explored (a new drug entity, several drug combinations, or a new treatment regimen) to identify a patient-specific optimal therapy. In this model, the patient herself is not the test animal; a patient-specific surrogate allows options to be explored. We describe below the proof-of-concept experiments for the tumor-engineering[48] approach.

Hepatotoxicology Models

Currently, over 20% of new drug candidates fail in phase-III human clinical trials because of hepatotoxicity. Reducing the failure rate at this late stage in drug development, when the most significant investment of time and money has already occurred, has the potential to substantially reduce the overall cost of drug discovery[49] and increase the number of new therapeutics and drug targets evaluated.[40] The cost of bringing a new chemical entity from the laboratory bench to the bedside has been estimated at $1.2 billion, up from a 2003 average of $802 million.[50] "The holy grail of the [pharmaceutical] industry is to be able to predict [drug] toxicity from a cell culture."[51] An *in vitro* method that would accurately predict *in vivo* hepatocytic function could "detoxify" hits, leads, or entire libraries. While measuring cytotoxicity using hepatocytes cultured in 2D may predict acute hepatotoxicity,[52] drugs (~40%) that fail because of *in vivo* bioactivation to a reactive toxin are not identified. This idiosyncratic toxicity cannot be adequately detected until phase-III clinical trials,[52] if at all. Animal testing is currently the only *in vivo* method for xenobiotic bioactivation. However, 80% of compounds that pass animal testing still fail in the clinic, in part because of interspecific difference in hepatic metabolism between rodents and humans.[53] How can 3D tissue-engineering techniques assist in early identification of hepatotoxic compounds?

Hepatocytes cultured in 2D monolayers behave differently than fresh primary hepatocytes,[54] and the sECM technology may offer an opportunity to seamlessly connect *in vitro* and *in vivo* liver toxicology models by culturing human liver cells, from the immature hepatic stem cells to mature hepatocytes, in 3D. With Extracel, compositions can be varied to recapitulate the cellular microenvironment experienced along the maturational lineages.[25] The ability to grow metabolically competent engineered liver tissue in 3D is an important "growth industry", and the sECM technology is unique in enabling both *in vitro* toxicological studies and *in vivo* liver regeneration possibilities.[6]

Hepatocytes absorb drugs from the blood stream and metabolize them through phase-I and phase-II reactions.[53] During phase-I metabolism, 90% of all drugs are oxidized by cytochrome P450 (CYP) isozymes with different substrate selectivities. Seven isoforms account for 95% of this activity

(1A1, 1A2, 2C9, 2C19, 2D6, 2E1, and 3A4), with 3A4 responsible for over 65% of the metabolism of current therapeutic agents.[55] Challenging cultured primary human hepatocytes with drugs is used to determine pharmacological and toxicological profiles, but gene expression for phase-I and phase-II enzymes are different for 2D cultured and fresh cells. CYP isozymes are downregulated within 24 h after plating, and the activity continues to decline in culture.[56] Therefore, hepatocytes are typically cultured for 1 week and used for experiments within 3−4 days after plating.[57] Using human fetal hepatocytes and a supplemented serum-free media, it is possible to culture human hepatocytes for over 1 month on collagen-coated plates.[57] Other *in vitro* approaches to provide a microenvironment for normal function of hepatocytes include encapsulation in alginate microspheres[58] and 3D hyaluronan-based scaffolds.[59]

Extracel sECMs were evaluated for culturing primary hepatocytes in 3D. For proof of concept, freshly harvested rat hepatocytes were cultured under standard 2D conditions on collagen-coated TCP and Extracel sponges.[6] The activity of CYP 1A1 was measured as 7-ethoxyresorufin-*O*-deethylase (EROD). Hepatocytes cultured in 2D lost EROD activity gradually, reaching zero activity at day 9. In contrast, rat hepatocytes cultured in on Extracel sponges showed a cyclical rise and fall of EROD activity that lasted beyond the 17 day test period. Mature human hepatocytes and Hep G2-C3A cells cultured on Extracel hydrogel surfaces or in 3D by encapsulation in Extracel (T. Tandeski and G. Yang, unpublished results) both showed the cyclic production of albumin, urea, and EROD activity for over 28 days. Cyclical EROD activity is a normal property of healthy hepatocytes (L. Reid, personal communication).

Restoration of Liver Function *in Vivo*.[45] Using a 90% liver resection model in a nude mouse, we investigated the ability of hepatocytes encapsulated in Extracel to restore liver function *in vivo* (Figure 3). Primary rat hepatocytes isolated from freshly sacrificed rats were encapsulated in Extracel, and the construct was sutured to the remaining caudate lobe post-resection (Figure 3a). Control mice receiving no treatment, cells only, or Extracel only died within 3 days as glucose levels declined precipitously (Y. Liu, unpublished results). However, all mice receiving a sutured rat hepatocyte-seeded Extracel implant were normoglycemic and survived until sacrifice at 4 days. The gross morphology and histology of the liver showed that viable, functional new tissue had been regenerated. To build on this preliminary success, we seeded 15 million human primary mature hepatocytes[25] onto an Extracel sponge and sutured this to the caudate lobe (Figure 3b). Glucose levels remained normal, and survival of the treated mice was 60% at day 7 (G. Yang, unpublished results). Human albumin was secreted for over 7 days, and the murine liver remnant doubled in size in 7 days (parts c and d of Figure 3).

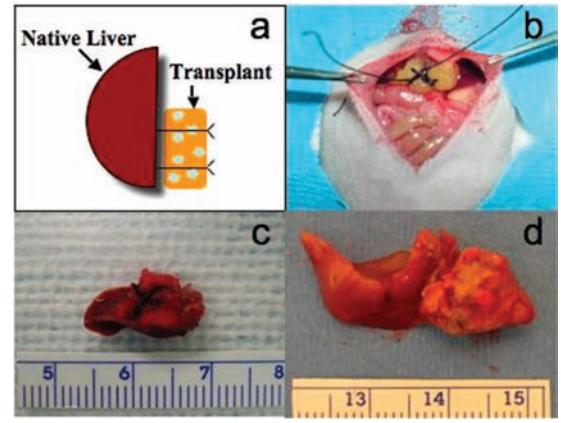

FIGURE 3. Liver repair model. (a) Experimental design, showing human hepatocyte-seeded Extracel implant sutured to the liver remnant. (b) Actual hepatocyte-seeded Extracel implant sutured to the liver remnant of a nude mouse. (c) Liver repair at day 1, with mouse liver at left. (d) Liver repair at day 7, with mouse liver at left. Original drawing by Y. Liu and surgical images from G. Yang.

Engineered Orthotopic Human Tumor Xenografts: Toward Personalized Medicine

Clinically relevant animal models of human cancer are needed to support anticancer drug discovery, and these models must be predictive for translation of preclinical results to efficacy in human patients. Human tumor xenografts in nude mice are common but remain controversial because of high false-positive and false-negative rates.[60] Efforts to develop improved models[61] include injection of cancer cells in buffer or serum-free medium or in Matrigel, a tumor-derived basement membrane extract.[37] Poor "take" is a recurring problem, in that many cell lines or patient-derived cells will not form tumors when injected in buffer or medium. Matrigel increases the incidence of cancer formation *in vivo*, but cost, batch-to-batch variability, viral contamination, and difficulties in handling, particularly for orthotopic injections, have limited wider-scale adoption of this approach. Other alternatives for orthotopic patient-like cancer models include implantation of intact tumor tissue[62] and injection of dissociated cells using a 3D matrix.[63] Cell leakage into the abdominal cavity with concomitant undesired seeding of adjacent tissues or organs can be problematic. Thus, a room-temperature-injectable 3D hydrogel vehicle for cell delivery that would support the formation of robust, vascularized, orthotopic human cancer tissue *in vivo* would have considerable value.

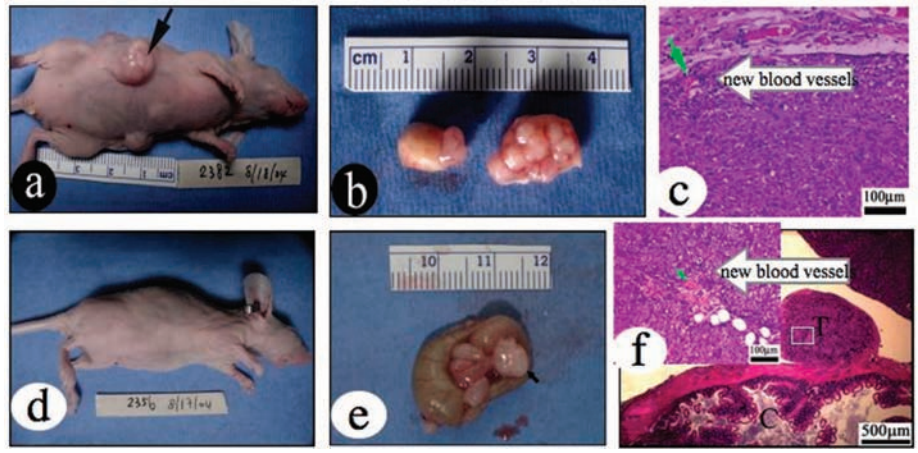

FIGURE 4. Engineered orthotopic breast (top panels) and colon (lower panels) tumor xenografts.[48] Top panels: (a) Gross view of MDA-MB-468 breast cancer cells in Extracel (arrow); (b) two sample tumors, showing vascularity; (c) histology showing new capillaries (green arrow). Bottom panels: (d) Gross view of CaCo-2 colon cancer cells in Extracel injected subserosally; (e) single orthotopic colon tumor; (f) histology showing tumor attached to the colon, with the inset showing newly formed capillaries (green arrow). Images provided by Y. Liu.

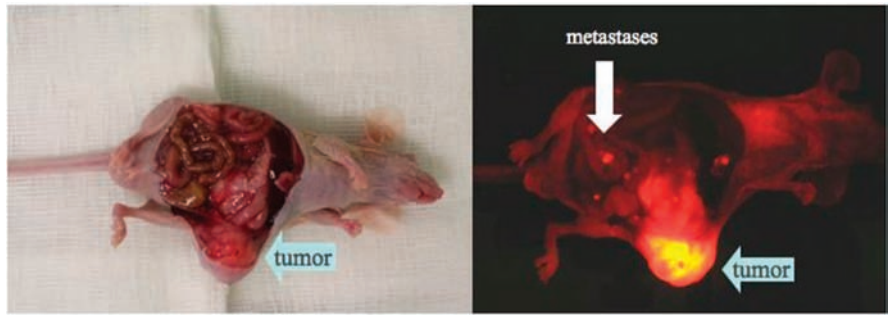

FIGURE 5. Engineered orthotopic metastatic pancreatic tumor xenograft. RFP-labeled MiaPaCa-2 cells in Extracel were injected into the pancreas of a nude mouse and observed after 4 weeks. The primary tumor is evident in the gross view (left), while both the fluorescing tumor and fluorescing metastases are visible by intravital imaging (right). Images provided by C. L. Scaife, J. E. Shea, Q. Dai, M. A. Firpo, and S. Mulvihill.

To this end, Extracel was used to deliver and grow cancer cells *in vivo* in a technique called "tumor engineering".[48] Suspensions of breast, colon, and ovarian cancer cells in Extracel were injected subcutaneously into mammary fat pads, subserosally in colons, and intracapsularly in ovaries, respectively. Two cell lines of different "take" were used for each cancer, and results were compared with orthotopic injection of cells in serum-free medium. Figure 4a shows the gross view following injection of MDA-MB-468 breast cancer cells in Extracel into the mammary fat pad, which afforded uniformly sized, well-spaced tumors. Parts b and c of Figure 4 show two tumors, both richly supplied with blood vessels. Figure 4d shows the gross view following subserosal injection of CaCo-2 colon cancer cells in Extracel; the tumor engineering approach eliminated the distended abdomen and bloody peritoneal fluid characteristic of intraperitoneal injection of Caco-2 cells. Parts e and f of Figure 4e show a single orthotopic colon tumor attached to the colon; the inset shows that capillaries formed within the engineered tumor.

Models for metastatic cancer are even more challenging, because traditional intraperitoneal cell injection methods result in widely disseminated tumor formation following the injection. This is not how metastasis occurs clinically. Establishing an engineered primary tumor, followed by metastasis, would be preferable. Figure 5 illustrates the first example of an engineered, orthotopic metastatic pancreatic tumor xenograft (C. L. Scaife, J. E. Shea, Q. Dai, M. A. Firpo, and S. Mulvihill, unpublished results). Red fluorescent protein (RFP)-labeled MiaPaCa-2 cells were injected in Extracel into the pancreas of a nude mouse. After 4 weeks, the primary tumor was evident under normal light, while both the fluorescing tumor and fluorescing metastases were visible using intravital imaging equipment.

Overall, orthotopic delivery of cancer cells in Extracel showed (a) an increased incidence of cancer formation and reduced variability in tumor size, (b) an enhanced growth of organ-specific cancers with good tumor–tissue integration, (c) an improved vascularization and reduced necrosis in the

tumor, (d) a reduced cancer seeding on adjacent tissues or organs, and (e) a better overall health of the animals. Engineered tumors (injectable, orthotopic, xenografted cancers) offer a clinically meaningful tool for the study of cancer biology, invasion, and metastasis. In ongoing work, we and others are using tumor engineering to investigate new therapeutic agents and treatment protocols.

Conclusions

The sECMs that were originally developed for 3D cell culture and tissue engineering have a myriad of potential applications for drug discovery and target validation under more physiological conditions. The Extracel technology will enable the development of high-content chemogenomic screens and organotypic 3D cultures. Proof-of-concept experiments show that primary hepatocytes retain biochemical function, live longer in 3D in Extracel, and can rescue acute liver failure. Using tumor engineering, oncology drug candidates can be evaluated *in vivo* in orthotopic, vascularized tumors without restrictions on the source of the cancer cells. In short, the sECM technology offers scientists a platform for developing newer and better tools for improving the flow through the drug-discovery pipeline.

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FOOTNOTES

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