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Nanostructured Biomaterials for In Vitro Models of Bone Metastasis Cancer

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Abstract

In recent years, tissue engineering approaches are commonly used to develop *in vitro* models of cancer for both the primary sites of cancer as well as metastatic sites of cancer. Here, we review advanced nanocomposite materials and scaffolds used for the design of *in vitro* models of cancer. These models recapitulate the tumorigenic phenomena, including epithelial to mesenchymal transition, mesenchymal to epithelial transition, migratory characteristics of metastasis, and chemoresistance for creating environments. We specifically illustrate the use of tissue-engineered bone as *in vitro* models for metastatic prostate and breast cancer, since these two types of cancer have the propensity to metastasize to bone. We also discuss the significant pros and cons related to each biomaterial and their improvement methods, thus highlighting the role of scaffolds in tumor evaluation and future directions for modulating cellular phenotype in 3D disease models.

Introduction

According to the World Health Organization, cancer is the second leading cause of death globally, resulting in an estimated 9.6 million deaths in 2018. Often, no effective treatment is available when cancer spreads to distant organs through a process called metastasis [1-3]. Preclinical studies on cancer drug developments often rely on conventional two-dimensional (2D) cultures, which do not faithfully recapitulate the three-dimensional (3D) tumor microenvironment, thus failing to capture realistic drug response, leading to ineffective translation of preclinical studies to clinical trials [4]. In 2D monolayer cultures, cell-cell, cell-matrix interactions are limited compared to in vivo, leading to poor phenotypic retention [5]. *In vivo* models mimic native 3D microenvironment and provide real-time insights into the mechanism of tumor initiation, progression, and drug response. However, these models are costly, time-consuming, and often fail to reflect human response due

to their immune-deficiency and species difference. Also, these models fail to achieve metastasis before the animal dies. Recently, 3D *in vitro* model systems have been at the forefront of cancer research due to attempts to mimic in vivo tissue while showing a good correlation with clinical outcomes [6]. These efforts are particularly crucial for the creation of microenvironments of metastasis.

Tissue engineering approaches are commonly used to develop 3D disease models of various cancers efficiently. The tissue engineering approach includes isolation and culture of specific cell types onto 3D scaffolds to generate tissues for implantation into the patient to restore or augment tissue function [7]. The scaffolds are usually made up of degradable or non-degradable biomaterials. They can provide biophysical and biochemical cues to the cells to facilitate tissue formation, while porous microstructure helps nutrient supply and waste removal throughout tissue growth [8]. Natural materials such as chitosan, alginate, silk have been successfully used as biomaterials for tissue engineering applications. Synthetic materials such as polycaprolactone, polyurethane, polyethylene glycol, poly-L-lactide have also been used to develop scaffolds with improved mechanical properties compared to natural materials. Tissue engineering approaches help to improve cell attachment, proliferation, differentiation, and extracellular matrix (ECM) formation *in vitro* [9,10]. Some studies have incorporated nanoparticles within the polymer matrix to mimic the nanocomposite structure of natural tissue while improving the scaffolds' mechanical properties. Nanostructured biomaterials scaffolds have been shown to replicate tumorigenic phenomena in the form of 3D *in vitro* models, which could be used for the discovery and development of anti-cancer therapeutics.

In this review, recent examples of how advanced nanomaterials are utilized to recapitulate tumorigenic phenomena, including epithelial to mesenchymal transition, mesenchymal to

epithelial transition, metastasis, and chemoresistance to create environments at both the primary and metastatic sites of cancer are shown. We specifically illustrate the use of tissue-engineered bone as *in vitro* models for metastatic prostate and breast cancer, since these two types of cancer have the propensity to metastasize to bone. We also discuss the significant pros and cons related to each biomaterial, thus highlighting the role of scaffolds in tissue-engineered tumor models and future directions for modulating cellular phenotype in 3D disease models.

Hydroxyapatite-based scaffolds:

The mineral hydroxyapatite (HAP) ($Ca_{10}(PO4)_6(OH)_2$), with stoichiometries mimicking bone-like, Ca/P ratios of ~1.67 has been well investigated for bone tissue engineering. The current literature also suggests some application of HAP for a 3D *in vitro* cancer model, in particular for bone metastatic cancer such as breast cancer [11], prostate cancer [12], and osteosarcoma [13].

Hydroxyapatite is a ceramic material, and it is challenging to fabricate 3D in-vitro models from them directly. Thus, HAP is frequently combined with other polymeric materials to construct composite scaffolds. At the nano level, HAP possesses a high surface area, which is known to be critical for cell-biomaterial interactions. The critical nano-sized HAP particles have also been evidenced for their antitumor activity [14]. Nano HAP particles were shown to promote the adsorption of serum proteins onto the scaffold surface that leads to increased breast cancer cell adhesion and growth compared to crystalline HA nanoparticles [15]. In a recent study, it is shown that nanocrystalline HAP stimulates the malignancy of ductal breast carcinoma cells (**Figure 1)**. The exact cause of such enhancement in breast cancer malignancy is attributed to non-stoichiometric hydroxyapatite associated calcification. The results showed increased expression of pro-tumorigenic cytokine IL-8 in MCF10.com breast cancer cells grown on HAP loaded

poly(lactide-co-glycolide) (PLG) scaffolds compared to control (PLG scaffolds without HAP) samples signifying that homing of breast cancer cells on HAP-based scaffolds provide suitable microcalcification environment that triggers their malignancy rate [11].

Tricalcium phosphate-based scaffolds:

Tricalcium phosphate (TCP) chemically defined as $Ca_3(PO_4)_2$ comprises of two phases, α-phase, and β-phase. Generally, β-TCP is more stable and has a higher biodegradation rate than α-TCP. However, in contrast to HA, β-TCP is less stable yet possesses a higher degradation rate. Previously, β-TCP has been considered as an excellent material for bone tissue-engineered constructs due to its higher biocompatibility and osteoconductive properties. These properties are mainly attributed to its Ca/P ratio of 1.5 that closely resembles the concentration of bone minerals. However, recently TCP has been investigated extensively in creating 3D *in vitro* and *in vivo* bone metastatic cancer models [16-18].

Further, β-TCP is known to exhibit a high resorbable interlocking network within the bone defect site to promote its healing. A recent study demonstrated enhanced ectopic bone formation by the PCL/ TCP scaffolds generated by the electrospinning technique due to the excellent integration of scaffold into host bone tissue of NOD/SCID mice. Next, to mimic invasion of breast cancer cells on a naïve bone tissue, breast cancer cells were implanted adjacent to scaffold that led to the induction of osteoclastic bone reaction in bone tissue-engineered construct [17].

Several fabrication methods have been employed to improve scaffold pore size and pore interconnectivity in the TCP scaffolds. For instance, a recent study showed tailored pore geometry of β-TCP scaffolds using 3D wax printing and a slip casting fabrication technique depicted in **Figure 2,** to study the behavior bone marrow metastasized neuroblastoma cells [18]. Another

approach employed to improve the porosity of β-TCP scaffolds is 3D printing technology. In a study, PCL/ β-TCP scaffolds were fabricated by 3D printing technology to generate a bone substitute for osteosarcoma. The outcomes of the study suggest a significant improvement in limb function with a gradual increase in weight distribution and decrease asymmetry over time [16].

Biobased scaffolds:

Alginates are biobased materials obtained from extracts of brown algae that are block copolymers of β-D-mannuronate (M) and α-L-guluronate (G) linked via (1,4) linkage. The ratio of M/G plays a critical role in tuning the mechanical properties of alginate-based hydrogels. Alginate gels with high G-block concentration are usually stiffer and exhibit little or no immune response *in vivo* than high M-block alginates. Various studies have attempted to illustrate a pattern between substrate stiffness and cancer cell viability [19,20]. These scaffolds are often investigated to recapitulate the primary site breast cancer and also evaluate drug efficacies under 3D conditions[21].

Alginate has various advantages over other polymeric materials that include low cost and ease in achieving tailored mechanical properties by varying the crosslinker concentration and crosslinking time. However, an increase in crosslinker concentration of alginate scaffolds also enhances cellular toxicity that limits their application as tissue-engineered constructs. The other major limitation includes a lack of surface ligands on alginate scaffolds for cell attachment. Thus, the alginate scaffold surface is commonly covalently modified with adhesion peptides to improve cell-scaffold interactions such as incorporation of arginine-glycine-aspartic acid (RGD) peptide sequence on alginate scaffold surface [19].

To further overcome these limitations, researchers have also investigated the various combination of alginate with other polymeric materials (e.g., matrigel, gelatin) [22,23]. These studies develop

alginate-Matrigel and alginate-gelatin based 3D in-vitro models for a highly metastatic breast cancer cell line (MDA-MB-231).

Although significant studies have been conducted for the use of chitosan (a shrimp shell derived polymer) as drug delivery agents [24,25], recent studies also involve the use of chitosan-based scaffolds for bone tissue engineering for the development of cancer models [26,27]. Many efforts often use nano-hydroxyapatite to enable bone mineralization. Since the extracellular matrix of normal prostate tissue and prostate cancer both have chondroitin sulfate, this additive is also included in recent efforts to design prostate cancer *in vitro* models[28].

Silk based scaffolds:

Silk obtained from various sources such as Bombyx mori silkworm and Antheraea pernyi silkworm is a natural biodegradable material that consists of two major proteins, fibroin and sericin. The purification of silk to regenerated silk fibroin (RSF) involves the removal of the sericin layer that is believed to elicit prompt immune response during *in-vivo* applications. RSF based scaffolds have been extensively investigated for tissue engineering applications due to its fast biodegradation, excellent water holding capacity, and good mechanical properties [29]. The mechanical properties of silk fibroin are mainly attributed to a large number of β-sheets domains, which in turn influences the crystallinity of RSF. Recently, an increased interest has been developed to tailor the mechanical properties of RSF to create improved 3D *in vitro* cancer models. A recent study [30] suggested the effect of increasing mechanical properties of chemically crosslinked silk scaffolds on cancer migration rate. The results showed enhanced migration of cancer cells on 2% eSF hydrogels over 3%, where no cell migration was observed. The primary

cause of such variation in-migration rate is attributed to high matrix stiffness (1136 ± 94 Pa) of 3% eSF hydrogels compared to lower stiffness of the 2% eSF hydrogels (488 ± 72 Pa).

Decellularized biological scaffolds:

Decellularized extracellular matrix (dECM) based scaffolds have been developed an immense interest in the field of tissue engineering. The objective of decellularization involves complete elimination of cells (<50 ng dsDNA per mg ECM dry weight) from the harvested tissue/organ using various physical, chemical, and enzymatic methods while preserving composition and ultrastructural architecture of native extracellular matrix. dECM approach has been widely to construct a bioengineered organ or tissue such as kidney [31], liver [32], blood vessels [33], and trachea [34]. However, in the case of generating 3D in-vitro tumor models, dECM techniques have four approaches. The first approach of dECM includes decellularization of harvested tissue and use it "*in-situ*" to create *in-vitro* tumor models that precisely mimics complex ECM niche. For instance, a 3D *in-vitro* cancer model of colon cancer was developed using scaffolds derived from decellularized porcine jejunum. The study showed coculturing of two different human colon cancer cell lines (Caco2, SW480) with fibroblast grown on dECM resulted in mesenchymal to epithelial transition (MET) and tumor-like aggregates formation of SW480. In contrast, Caco2 cells were grown as a monolayer and made a separate compartment from fibroblasts [35]. The next approach of dECM is to ameliorate the biological activity of natural or synthetic materials. A recent study utilized a blend of alginate gel beads and decellularized liver matrix (DLM) powder to create a 3D in-vitro model of hepatocellular carcinoma. Alginate hydrogels do not possess receptors that are vital for cell adhesion; hence blending with ECM powder could overcome bio-inertness of alginate gels. The study evaluated a range of DLM powder concentrations on the mechanical and structural stability of the in-vitro model. The outcomes suggested that alginate beads with DLM

concentration ≤1% were mechanically more stable and had intact spherical geometry [36]. The third approach is to employ dECM as soft hydrogels for constructing the *in-vitro* tumor model. In a current study, the patient's brain tissue-derived ECM (pdECM) was employed to create 3D hydrogels as an *in-vitro* model of glioblastoma. The pdECM based hydrogel scaffolds showed an average elastic modulus of 78.09 ± 29.22 Pa that was comparable to brain tissue modulus [37].

The most recent and advanced application of dECM is to create a layered tissue matrix scaffold (TMS). Li and group have created a 3D-invitro model of MM231 breast cancer cells by coculturing MM231 with GM637 (fibroblast cells) in a separate hydrogel layer of dECM. The schematic of the scaffold formation is given in **Figure 3**. Briefly, decellularized breast tissue (DBT) of mice was utilized to fabricate porous DBT-TMS with a porosity of 100 μm. MM231 cells were seeded on this porous DBT-TMS followed by a coating of blank TMS layer and finally coated with a second hydrogel layer seeded with GM637 cells to create a 3D in-vitro model. The major advantage of such a multilayered system is to mimic layered tissue structures in-vivo and to evaluate the interactions of cancer cells with different cell lines using a single system [38]. Although various approaches of dECM have been defined yet designing *in-vitro* cancer models is always challenging due to variation in native ECM; thus, intensive optimization is required to evade batch to batch variation. Also, employing dECM as scaffolds is always relatively more expensive than other polymeric materials and sometimes needs strict adherence to the ethical procedure.

Nanoclay-based *In vitro* Models of Bone Metastasis

Clay minerals have been extensively used in the biomedical industry for a variety of applications, including wound healing, drug delivery, and tissue regeneration [39,40]. Nanoclays are

nanoparticles of layered silicates with one octahedral alumina sheet sandwiched between two tetrahedral silica sheets [41]. Nanoclays have previously been used as filler materials to improve the mechanical properties of polymeric materials when added in small quantities, and the altered phase model describes the mechanisms of property enhancement by nanoclays[42]. While commercial clays are investigated for biomedical applications, Katti & Katti group pioneered the use of engineered nanoclays with tailored clay modifications in tissue engineering scaffolds[43]. Further, nanoclay modified with amino acids was developed to mineralize hydroxyapatite (HAP) mimicking biomineralization in human bone [44]. The modified nanoclay was used to develop nanocomposite scaffolds with polymers, and hMSCs were cultured to investigate cellular response. Results indicated the formation of mineralized bone-like ECM via vesicular delivery by osteogenically differentiated MSCs on PCL/*in situ* HAPclay without the use of osteogenic supplements [45,46]. The sequential culture of prostate and breast cancer cells on the bone mimetic scaffolds nanoclay-based system indicates mesenchymal to the epithelial transition of prostate and breast cancer cells [47-50]. Further, the impact of cancer cells at the bone site was shown to impact and influence the osteogenesis through the role of Wnt/β-catenin signaling in breast cancer mediated osteogenesis at the metastatic bone site [51]. We observed that cancer-derived factors such as dickkopf-1 (DKK-1) and endothelin-1 (ET-1) were involved modulating bone mineralization via Wnt/β-catenin pathway. In another study, the prostate cancer phenotype was observed to influence bone mineralization at metastases [52]. Just as the evaluation of cancer at metastasis significant, so also is the impact of cancer cells on the bone site, as mortalities due to prostate and breast cancer result from skeletal failures due to metastasis. Further, the bone microenvironment is observed to confer drug resistance in breast cancer cells at metastases. Results showed bone-microenvironment secreted interleukin-6 (IL-6) activated signal transducer

and activator of transcription 3 (STAT3) in breast cancer cells, which conferred chemoresistance by inhibiting apoptosis and promoting efflux of drugs [53].

Conclusion

In recent years, studies towards building realistic 3D *in vitro* models of cancer are extensively popular. Although the pharmaceutical and drug delivery space is enthused towards the design of 3D systems for evaluation of drug penetration and efficacies, the development of 3D systems by itself is insufficient for recapitulating the metastasis environment. Hence, efforts towards the use of hypoxia chambers to study 3D cultures are also limiting, since the true nature of hypoxia, is an inherent characteristic of cancer tumors and can be captured accurately in the *in vitro* 3D models are truly replicative of the biological environment.

Here, we describe the efforts in the literature that use various novel bone tissue engineering approaches to develop bone-like environments using advanced biomaterials based on tissue engineering. While therapeutic strategies ranging from bone stabilizing drugs and tissue-engineered bone replacement are currently administered, recent advances in the evaluation of bone metastasis through *in vitro* models are suggestive of transformative approaches in the future. One such study proposes inducing tumor dormancy in bone microenvironment at metastasis as a new therapeutic strategy for bone metastasis[54]. Indeed, the mortality of prostate cancer and breast cancer results from skeletal defects. The role of newly arrived cancer cells at bone site dramatically affects osteogenesis [51], causing disruption in bone formation, and hence the skeletal defects, as also observed is the concurrent effect bone on breast cancer [55]. Thus both the role of the bone microenvironment in causing metastasis, as well as the role of cancer cells on bone remodeling, are essential. In addition, the use of patient-derived xenografts and organoid cultures over the

cancer cell lines are also being used [56] that further help duplicates the complexity of tumor-stroma interactions. Overall, the advanced manufacturing studies in tissue engineering, as well as the use of advanced nanobiomaterials, are leading to the development of realistic 3D models of metastasis of cancer and it is expected that these studies will lead to new therapies resulting from advanced knowledge of the microenvironments at metastasis in the future that would reduce the substantial cancer burden on humanity.

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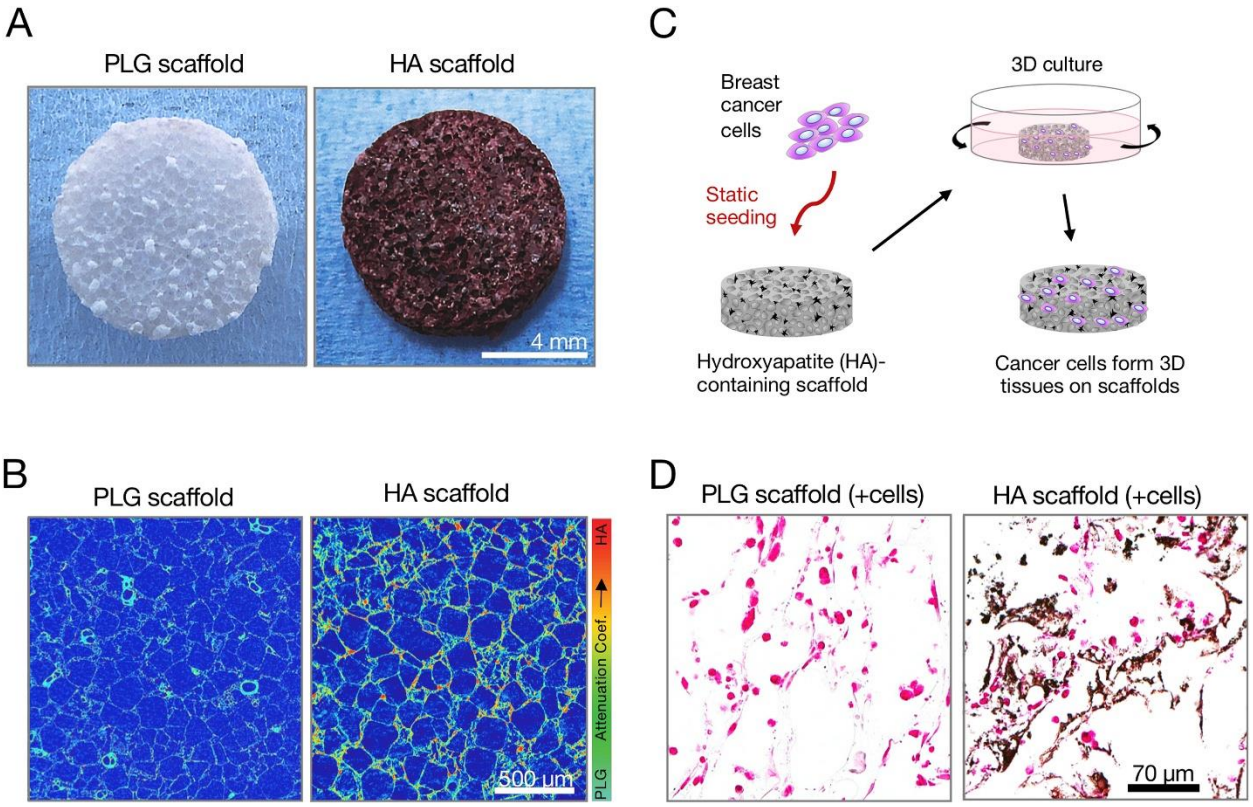

Figure 1. Hydroxyapatite (HA) containing poly(lactide-co-glycolide) (PLG) scaffolds stimulates the malignancy of ductal breast carcinoma cells (A) Surface mineralization shown by Alizarin Red S staining in PLG scaffold (left) and HA containing PLG scaffold (right). (B) MicroCT cross-sections are displaying mineral distribution as a function of the attenuation coefficient, which increases with the atomic number. (C) Schematic showing breast cancer cells seeding on the scaffold and then dynamically cultured. (D) Enhanced calcium phosphate mineralization (black) measured by Von Kossa-staining in breast cancer seeded (cell nuclei stained with pink) HA scaffolds (right) compared to PLG control scaffolds (left). Reprinted from [11] with permission from Elsevier.

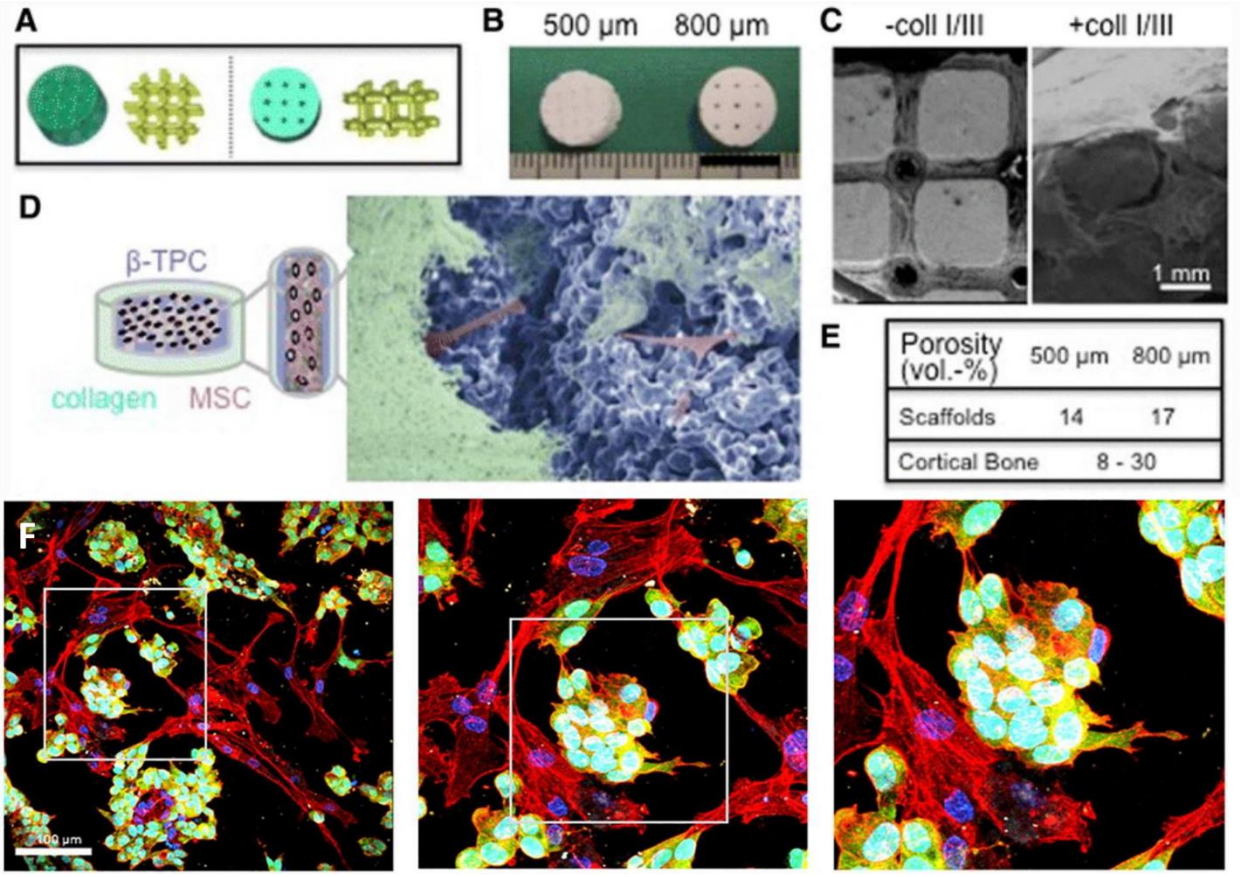

Figure 2. 3D wax printing and a slip casting fabrication technique utilized to tailor the pore geometry of β-TCP scaffolds (A) Fabrication of different β-TCP scaffold geometries using a wax casting mold. (B) Representation of the β-TCP scaffolds. (C) SEM representation of the β-TCP scaffolds. (D) Schematic showing mesenchymal stromal cells (MSCs) culturing on β-TCP scaffolds; MSCs (pink), β-TCP (blue), and collagen I/III (green). (E) Table showing porosities of scaffolds. (F) Immunostaining of neuroblastoma cells (GFP tagged -green) and stromal cells (F actin-red) showing their interactions. Adapted from [57] Copyright (2016) Springer Nature. Reprinted from [18] with permission from Elsevier.

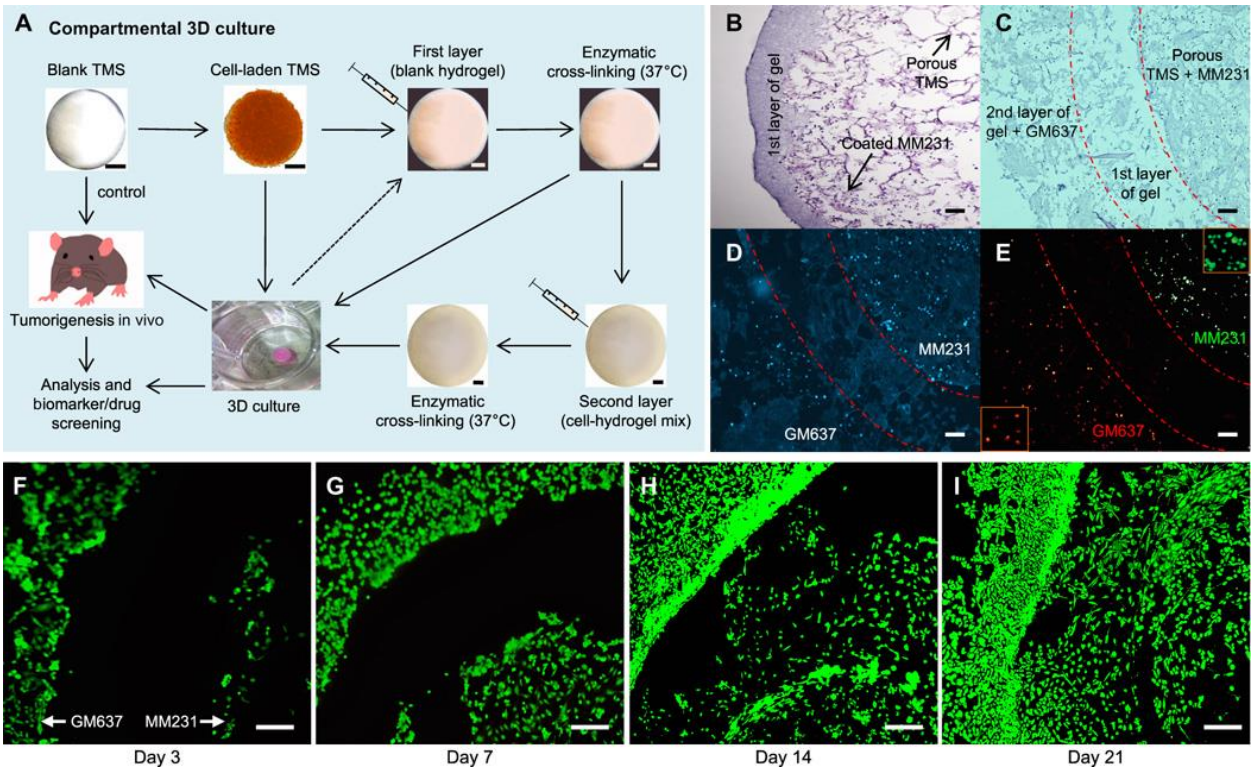

Figure 3. Decellularized extracellular matrix (dECM) based multilayered hydrogel scaffolds (A) Fabrication steps of layered tissue matrix scaffold (TMS) system and culturing of breast cancer cells (MM231) and fibroblast cells (GM637) in separate layers of a hydrogel. (B) Hematoxylin and eosin (H&E) staining of MM231 and the first layer of hydrogel. (C) H&E staining of MM231 cells, a middle blank layer, and second GM637 cells loaded hydrogel layer. (D) Cell distribution analyzed after 3 days of culture using DAPI staining. (E) Immunostaining of Ki-67 (green, MM231 cells) and HER2 (red, GM637 cells) representing the location of two cell types. (F to I) Cell migration between different hydrogel layers was analyzed by Live/Dead Cell staining. Scale bars, 100 μm. Adapted from [38] Copyright (2017) American Association for the Advancement of Science.

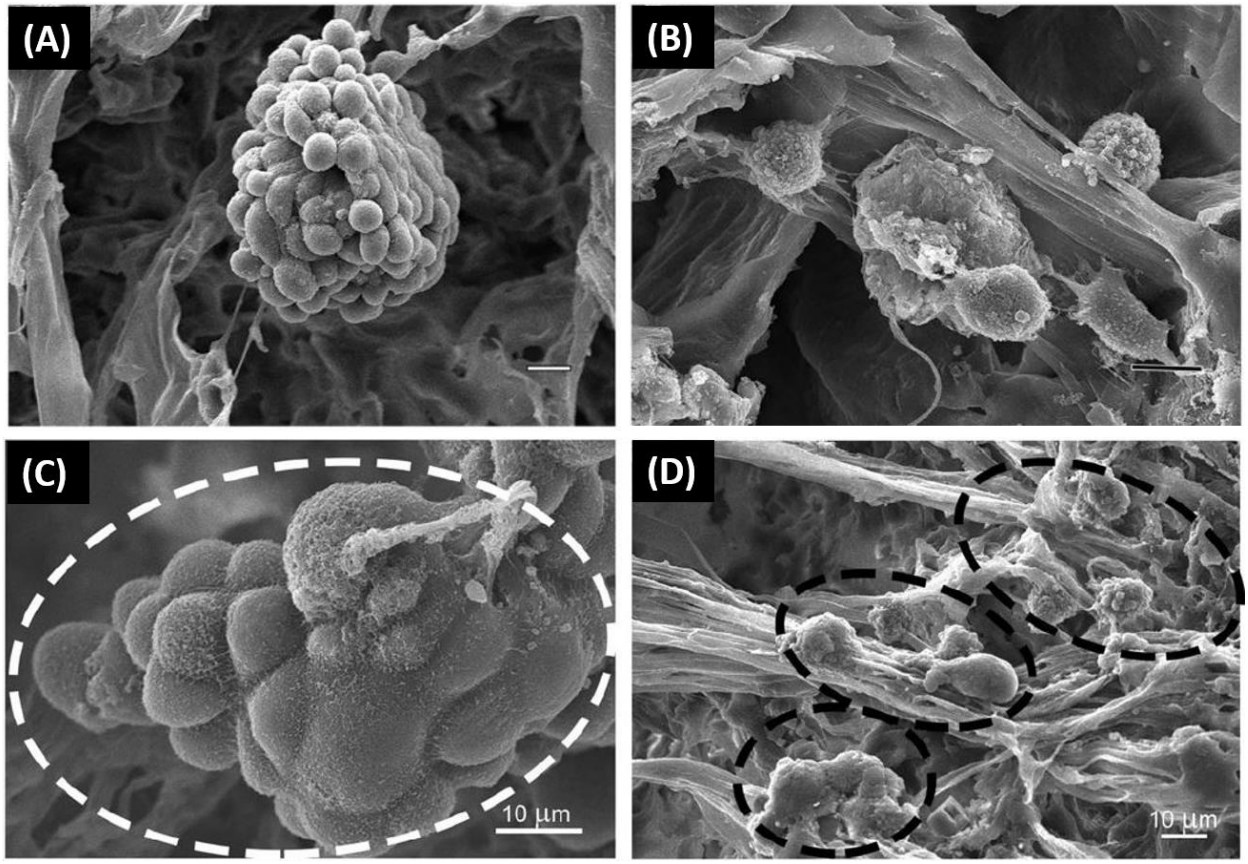

Figure 4. Nanoclay based scaffolds showing bone metastasis of prostate and breast cancer (A-B) Prostate cancer cells MDAPCa2b (left) and PC3 (right) grown on human mesenchymal stem cells (hMSCs) derived bone-mimetic nanoclay based scaffold. Adapted from [52] Copyright (2019) Wiley Periodicals, Inc. (C-D) breast cancer cells MCF 7 (left) and MDAMB 231 (right) grown on hMSCs derived bone-mimetic nanoclay based scaffold. Scale bars, 10 µm. Reprinted from [50] with permission from Elsevier.