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ENGINEERING PRINCIPLES FOR GUIDING SPHEROID FUNCTION IN THE REGENERATION OF BONE, CARTILAGE, AND SKIN

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Abstract

There is a critical need for strategies that effectively enhance cell viability and post-implantation performance in order to advance cell-based therapies. Spheroids, which are dense cellular aggregates, overcome many current limitations with transplanting individual cells. Compared to individual cells, the aggregation of cells into spheroids results in increased cell viability, together with enhanced proangiogenic, anti-inflammatory, and tissue-forming potential. Furthermore, the transplantation of cells using engineered materials enables localized delivery to the target site while providing an opportunity to guide cell fate *in situ*, resulting in improved therapeutic outcomes compared to systemic or localized injection. Despite promising early results achieved by freely injecting spheroids into damaged tissues, growing evidence demonstrates the advantages of entrapping spheroids within a biomaterial prior to implantation. This review will highlight the basic characteristics and qualities of spheroids, describe the underlying principles for how biomaterials influence spheroid behavior, with an emphasis on hydrogels, and provide examples of synergistic approaches using spheroids and biomaterials for tissue engineering applications.

Keywords

spheroid; aggregate; biomaterials; hydrogel; tissue engineering

1. INTRODUCTION

Cell-based therapies are a promising therapeutic alternative to tissue grafts or pharmacological approaches for treating tissue loss due to trauma, disease, or congenital malformation. Indeed, the cell therapy market is projected to grow to nearly $330 million by 2020.(1) Several cell-based therapies are already used as effective treatments, including bone marrow (2) and umbilical cord stem cell transplants (3) to treat cancer and anemia. Adipose tissue is another readily accessible tissue compartment to harvest cells for regenerative therapies. Adipose stromal cells can be isolated in large numbers from the donor for autologous use, possess multilineage potential, and may be used with minimal manipulation,

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(4) potentially reducing the oversight required by regulatory bodies such as the United States Food and Drug Administration (FDA). In cases where it is difficult to procure sufficient quantities of cells, investigators have used *ex vivo* expansion prior to reinjection into the body. For example, chimeric antigen receptor T cell (CAR-T) therapy utilizes T cells harvested from the patient, which are genetically engineered to recognize and attack tumor cells and expanded *ex vivo* for infusion to the patient. This therapy achieved successful outcomes in clinical trials with more than 75% of B cell acute lymphoblastic leukemia patients treated with CAR-T classified as minimal residual disease negative(5), leading to FDA approval in 2017 for treatment of acute lymphoblastic leukemia and relapsed large B-cell lymphoma.

Mesenchymal stem cells (MSCs) are a widely studied candidate for cell-based therapies and tissue engineering. MSCs possess multilineage potential and a potent secretome that promotes tissue repair and modulates the local inflammatory microenvironment. More than 600 clinical trials are ongoing that utilize MSCs as an intervention for numerous diseases including arthritis, diabetes, cardiovascular disease, and lung disease (www.clinicaltrials.gov; accessed 11/3/2017). Despite exciting results when transplanting somatic or stem and progenitor cells into damaged tissues, numerous challenges remain for cell-based therapies to achieve their full clinical potential. The vast majority of cells transplanted into an injury site are no longer viable within days due to the harsh microenvironment and limited cell-cell and cell-matrix interactions.(6-8) While short-term cell survival has resulted in detectable improvements, these effects may be insufficient when considering the costs associated with cell collection, expansion, and ensuring the purity and safety prior to transplantation. The therapeutic benefits of transplanting cells into damaged tissues will no doubt be enhanced by prolonging their survival and guiding their activity *in situ*.

Cellular spheroids represent one approach to address the shortcomings of individual cells freely injected or transplanted into the body (Fig. 1). Spheroids are dense aggregates formed when cells exhibit preferential cohesion to other cells over adhesion to the underlying matrix. Cells within spheroids are exposed to physical interactions that more closely reflect behavior in three-dimensional (3-D) native tissue.(9) Unlike individual cells liberated from the culture dish, spheroids retain their endogenous extracellular matrix (ECM), which has instructive potential to promote physiologically accurate connections with their environment, prolong cell survival, and direct differentiation.(10-13) Beyond cellular aggregation, the use of engineered carriers as cell delivery vehicles provides additional means to instruct cell function and enhance the effect of cell therapies. Cells that are intravenously or locally injected are rapidly cleared from the body or fail to remain at the intended target site following migration or death.(14, 15) Biomaterials present instructive cues to entrapped cells to potentiate their survival and guide cell fate over predetermined spatial and temporal time scales. The contribution of spheroids towards tissue repair, once entrapped and transplanted in engineered materials, is under investigation to pursue new opportunities and propel their therapeutic benefit in cell-based therapies. This review will describe the fundamental principles and strategies of spheroid formation, articulate the benefits of spheroids over individual cells, and highlight recent examples using spheroids with engineered materials, particularly hydrogels, for tissue engineering applications.

2. SPHEROIDS FOR DEVELOPMENT AND DRUG DISCOVERY

Individual cells, whether in monolayer culture or distributed in a 3-D environment, fail to mimic the complex cell-cell and cell-matrix interactions present within a tissue. The limited interaction between individual cells in culture hinders efforts to model early events in development, accurately test the potency of numerous chemotherapeutic molecules, and prepare cells for their intended use in cell therapies. Spheroids directly address these concerns by promoting cell-to-cell contact and presenting more physiologically relevant characteristics. In 1944, Holtfreter pioneered the use of spheroids as a morphogenic model in his investigation of ectoderm and endoderm behavior during development.(16) Embryonic stem cell spatial positioning in a spheroid approximates that of a dividing fertilized egg by maintaining spherical geometry for several developmental stages.

Spheroids are also valuable tools in the understanding and experimental modeling of cancer.(17) Tumor cells within the spheroid central core experience reduced oxygen and nutrient availability, reflecting a hypoxic and nutrient-starved core evident in tumors.(18) Tumor cell spheroids are widely used as models to test the efficacy of antineoplastic drugs, while mathematical models devised to predict tumor response have further improved the design and dosing of these drugs.

Spheroids formed of other cell types are used to model cell function in various tissues, although with alternate rationales from studying embryological development or cancer. Spheroids are not normally found under physiological conditions in the postnatal organism, yet aggregates of neural cells and cardiomyocytes have revealed new findings in the behavior of these cell populations.

Neurospheres are commonly used for modeling neural tissues to study brain tumors and developmental neurotoxicity. Neurospheres exhibit many processes observed in brain development including cell proliferation, migration, and apoptosis.(19) Neurospheres can be used to model adverse chemical effects in a developing brain and the impact of the Zika virus.(20, 21) Following brain tumor excision, the incidence of tumor reoccurrence is extremely high, likely due to the retention of a subset of the tumor cell population that can regenerate the tumor after removal.(22) Neurospheres were employed as a model system to evaluate the behavior and function of these tumor cells and to assess lineage commitment and mitogenic potency.(23) Neurospheres have also been directly used for tissue engineering applications. Neural stem cells maintained as neurospheres in culture were transplanted into the brains of neonatal mice where they engrafted and differentiated *in vivo*.(24) Cardiospheres of cardiac progenitor cells exhibit enhanced differentiation, increased ECM secretion, and in some cases, can functionally beat.(25, 26) Cardiomyocytes derived from cardiospheres can be directly grafted into infarcted cardiac muscle(27) and form new cardiac tissue in mice.(28) Although spheroids can accurately recapitulate some aspects of the physiological environment, they alone are still imperfect models. When multiple cell types are present, as occurs *in vivo*, cellular organization within a spheroid may not mimic physiological conditions. Furthermore the proper management of the physiochemical conditions for each individual spheroid can be challenging, often requiring the incorporation of more complex systems such as bioreactors or microfluidic devices.(29)

Spheroids are increasingly pursued as therapeutic agents in tissue engineering, due to their capacity to outperform individual cells in monolayer culture or suspended in a 3-D environment. Chondrocytes undergo rapid dedifferentiation in culture, and the micromass assay is a standard approach for generating small cartilaginous pellets.(30) Chondrogenic differentiation is enhanced in spheroids, possibly due to the activation of the Rho/Rho-associated kinase (ROCK) pathway.(31) Spheroids mimic mesenchymal condensation, an embryonic event that is prerequisite to chondrogenic differentiation.(32) Mesenchymal stem/stromal cells (MSCs) from bone marrow, adipose tissue (ASCs), and other tissue compartments are used in tissue engineering due to their multilineage potential and their potent secretome that stimulates angiogenesis and suppresses inflammation.(33-35) MSCs stimulated with transforming growth factor-β (TGF-β) formed aggregates and produced cartilage that was within range of physiological stiffness.(36) ASC spheroids stimulated the regeneration of cartilage and subchondral bone in microminipigs after one year of implantation.(37) MSC spheroids increased angiogenesis by enhanced secretion of endogenous proangiogenic growth factors, and injection of spheroids into ischemic hind limbs accelerated neovascularization in rodents.(38) Spheroids also upregulate production of prostaglandin E2 (PGE_2)(39, 40), which polarizes pro-inflammatory M1 macrophages toward a more anti-inflammatory, regenerative M2 phenotype.(41) Spheroids formed of post-thawed human ASCs possessed greater cell viability compared to cells in monolayer, highlighting the clinical potential of spheroids in cell therapies.(42) Furthermore, cells within spheroids decrease their expression of several surface markers, making them less likely to trigger an immune response compared to individual cells.(43) Overall, spheroids possess many desirable qualities for tissue engineering applications that will be further discussed in subsequent sections.

3. CONSIDERATIONS FOR SPHEROID FORMATION

Spheroid formation requires compaction and protein accumulation that occur through self-assembly without the need for additional stimuli (Fig. 2).(44-47) While the precise sequence of events is likely related to the preparation and identity of selected cells, spheroid formation is dependent upon increased cadherin expression and the production and engagement of an endogenous ECM. Beyond cohesion, adhesion, and compaction, spheroids depict characteristics of self-organization by partitioning themselves based on cell type and adhesive strength. Cellular self-organization has been described by Foty and Steinberg using the differential adhesion hypothesis (DAH), demonstrating that cells of higher adhesive strength aggregate in the center, while less adhesive cells are displaced and surround them.(48, 49) Although the DAH seems to predict the behavior of embryonic tissues, interactions among other more differentiated cell populations such as non-random cell motion, cell traction, and excessive cell compaction may dictate organization and merit further consideration.(50)

Spheroids are a powerful tool for research and clinical application, and thus, reliable and cost-effective means are necessary for their rapid and reproducible production. The hanging drop method remains one of the most commonly used techniques for spheroid formation due to its relative ease and lack of required specialized equipment.(51) This gentle, gravity-driven approach is unlikely to adversely affect cells. However, the utility of this method is

confined to smaller spheroids, as larger aggregates fall from the droplet. Furthermore, the hanging drop method is labor intensive with low throughput capacity, limiting the numbers of spheroids that can be produced for therapeutic purposes. Micromolded pellet culture was developed to address throughput limitations of the hanging drop method. In this approach, non-adhesive culture surfaces, commonly prepared from agarose, are generated from a mold.(52, 53) Upon addition of a cell suspension, cells are forced to aggregate, and this process can be accelerated by centrifugation. Although this strategy eliminates restrictions on spheroid size and increases production throughput, high or prolonged centrifugation can disrupt the spheroids and potentially alter their function. Spheroids have also been made in even greater quantities using spinner flasks, wherein cells are maintained in media with continual stirring. Since cells are continually moving, they cannot adhere to the surface and may only aggregate with other cells. Stirring speed is a key optimization parameter, as cells exposed to excessive shear may die. Insufficient shear allows multiple spheroids to coalesce into larger aggregates, resulting in irregular spheroids or large nutrient gradients that reduce cell viability.

The surface properties of biomaterials, namely surface tension and adhesivity, may also be manipulated to guide spheroid formation. Stromal vascular fraction (SVF) derived from lipoaspirate was formed into spheroids by seeding on a hydrophobic/hydrophilic patterned surface using a bioprinter technology similar to extrusion 3D printing.(54) Compared to self-assembly based methods, bioprinting was faster, produced spheroids of similar size and shape, and enabled the precise positioning and patterning of cells. However, bioprinting requires specialized equipment and exposes cells to potentially harmful shear forces that must be optimized for each cell type. Chitosan, a material derived from crustaceans, presents no human cell-binding domains and encourages cell-cell binding, making chitosan membranes a popular material for spheroid formation.(55, 56) Spheroid diameter can be controlled by cell seeding density on the membrane and peptide modification of the surface. ASC spheroids formed on chitosan membranes exhibited enhanced pluripotent gene expression compared to cells in monolayer culture, representing a strategy to impair undesired differentiation during culture expansion.(57) While the use of chitosan to form spheroids is relatively inexpensive and facilitates high throughput spheroid production, the size of resulting spheroids is irregular, leading to batch inconsistencies.

Spheroid diameter is a key variable to consider when translating this approach into clinical use, as spheroids may be delivered to ischemic tissues. While MSC spheroids can be formed with diameters as large as 600 μm without a hypoxic core(58), spheroids formed of other cells may be more vulnerable to limitations in nutrient transport. Importantly, spheroid diameter did not correlate with the number of cells per spheroid, suggesting that cells adapt their packing density during spheroid formation. When evaluating a comparable total number of cells, MSC spheroids composed of 40,000 cells secreted more PGE_2 and VEGF compared to more numerous, yet smaller spheroids made from 25,000 or 10,000 cells.(40) Thus, the number of cells per spheroid represents an important element in the design of cell-based approaches using spheroids.

4. FABRICATION OF ENGINEERED TISSUES USING SPHEROIDS

Over the last two decades, spheroids have become increasingly relevant for tissue engineering applications. Papas *et al.* initially evaluated the insulin-secreting characteristics of AtT-20 spheroids for their potential as bioartificial endocrine organs.(59) Since that report, the use of spheroids in tissue engineering applications has expanded. Spheroids have been used to engineer bone(60-63), cartilage(64, 65), skin(66), heart(67), liver(68, 69), brain(24), and various other tissues or tissue mimetics.

As previously stated, spheroids may contribute indirectly or directly to tissue formation. Compared to individual cells, spheroids secrete increased concentrations of trophic factors that speed angiogenesis, promote cell migration, and modulate the local inflammatory microenvironment, making them ideal for tissue engineering.(39, 40) Beyond secretion of endogenous factors, spheroids are promising building blocks for fabricating engineered tissues, as they can be further aggregated into larger tissue constructs. The use of spheroids as building blocks is motivated by eliminating the interruptions in cell-cell interactions that may occur when cells are seeded in scaffolds, a challenge identified in vascular tissue engineering.(70) Spheroids formed of human pluripotent stem cells (hPSCs) exhibited higher fusion capacity than aggregates of differentiated cells, resulting in successful post-fusion differentiation into neural tissue.(71) These data suggest an optimal protocol exists regarding the sequence of fusion and differentiation for each cell source and engineered tissue.

The positioning of spheroids may be manipulated through magnetic forces. Magnetoferritin nanoparticles, a less toxic alternative to iron oxide nanoparticles, were successfully internalized by cells that were then incorporated into spheroids. Nanoparticle-loaded spheroids were then magnetically patterned and fused into rings for vascular tissue engineering, resulting in rings with a 250 μm inner diameter after 156 hours of fusion.(72, 73) Alternatively, microtissues of various geometries have been generated directly from cell suspensions using polydimethylsiloxane (PDMS) molds through cell self-assembly methods, reducing delays associated with spheroid formation.(74)

The emerging field of 3D printing leverages spheroids as building blocks to fabricate engineered tissues. To make larger aggregate structures for tissues such as liver, the Bio Pick, Place and Perfuse (Bio-3P) was engineered to precisely place cell aggregates together into a tissue while maintaining perfusion. Aggregates were formed as spheroids, toroids, or honeycombs and then oriented into a large-scale tissue.(75) Alternatively, the Kenzan printing method, under investigation for robotic spheroid-based 3-D printing, forms spheroids within stainless steel microneedles and can spatially position the aggregates in the desired orientation.(76) Endothelial colony forming cells (ECFCs) and smooth-muscle forming cells (SMFCs) derived from induced pluripotent stem cells (iPSCs) were formed into spheroids and printed using the Kenzan method. These spheroids fused within 7 days, and spheroids derived from SMFCs yielded tubular structures with apparent ECM deposition.(77) Heterogeneous cell spheroids composed of umbilical vein endothelial cells, aortic smooth muscle cells, and dermal fibroblasts were similarly printed into a tubular shape to engineer 1.5 mm inner diameter blood vessels that underwent maturation when

cultured in an *ex vivo* perfusion system.(78) After 5 days in the rat aorta, these engineered vessels remained patent and exhibited remodeling and endothelialization of the tube. However, the time required for vessel formation remains a caveat of this approach, requiring 4 days for fusion and another 4 days for maturation in the bioreactor. Spheroids have also been used to bioprint engineered blood vessels with complex geometry and multiple layers. (79) Investigators reported that bioprinting cylinders rather than spheroids was more efficient at increasing structure homogeneity, reducing the fusion time from 5-7 days to as little as 2-4 days.

The assembly of spheroids into engineered tissues provides an exciting strategy to build densely cellularized tissues from the bottom up. However, numerous limitations remain unsolved. Significant delays are often incurred when expanding biopsies to clinically relevant numbers in culture. A massive number of cells is needed to construct a tissue, potentially eliminating the possibility for using autologous cells. When incorporating undifferentiated stem and progenitor cells, it may be necessary to provide prolonged instructional cues, whether as soluble growth factors or mechanical stimulation, to ensure proper differentiation and avoid aberrant tissue formation. The resulting tissues lack mechanical integrity until sufficient ECM is deposited to bridge the spheroids or adequate fusion occurs. Collectively, these challenges necessitate extended culture durations to make desired tissues, combined with costly recombinant growth factors and unique bioreactors, which may limit the translation of this approach to the clinical setting.

5. BIOMATERIALS TO INFLUENCE SPHEROID FUNCTION

The clinical use of spheroids for tissue regeneration and repair is primarily restricted to two approaches: 1) maintaining spheroids in culture to promote fusion and formation into a more coherent structure; or 2) transplanting the cell aggregates to the target site immediately after formation. The former requires extended *ex vivo* culture time, hence delaying delivery to the patient, while the latter relinquishes control of spheroid function to the surrounding environment that may be damaged or inhospitable to transplanted cells. As an alternative approach, the entrapment of spheroids into biomaterials is a promising strategy that can address many of these challenges while heightening the therapeutic potential of spheroids in tissue repair. Biomaterials can be engineered with target ligands to engage neighboring cells and possessing desired mechanical properties including stiffness, porosity, and degradation. (80) Spheroids may also be implanted at a lower cell density when delivered in a biomaterial in anticipation that cells in the construct will proliferate and host cells will infiltrate the material. This can eliminate the need for costly growth factors to induce differentiation while reducing the time before implantation. However, the introduction of a biomaterial represents another level of complexity to the implant that requires careful consideration.

Hydrogels formed of alginate, fibrin, hyaluronic acid (HA), gelatin, and polyethylene glycol (PEG) have many favorable characteristics for cell entrapment and delivery, and they have been broadly used in cell-based therapies for tissue engineering.(81) Their gelation characteristics enable facile entrapment of cell aggregates and direct injection to the target site. Spheroids may be entrapped in large numbers or as individual aggregates. Additionally, many characteristics of the native ECM can be recapitulated in engineered hydrogels

including the presentation of native adhesive ligands, cell-responsive linkages that promote hydrogel remodeling and degradation, and known mechanical properties. These properties can then be manipulated to influence cell growth, differentiation and behavior.(81)

The vast majority of cells used in tissue engineering are anchorage-dependent, requiring adhesion to the surrounding ECM to remain viable and undergo instruction toward the desired lineage. Cells transplanted in biomaterials consistently outperform those simply injected into the damaged tissue site. Biomaterials facilitate the localized delivery of cells and provide adhesive cues to promote cell survival, differentiation, or increase trophic factor secretion.(82, 83) Similarly, spheroids entrapped in biomaterials possessing engineered biophysical properties may enhance their therapeutic potential. For example, spheroids entrapped in biomaterials engineered to control adhesion exhibited increased secretion of many proangiogenic growth factors, as well as high cell viability and differentiation potential.(57, 84) To engineer specific adhesive properties into the material's bulk, one strategy is to covalently couple ECM proteins or peptide sequences to the polymer backbone that engage cellular receptors. The most common peptide used in this approach is arginine-glycine-aspartic acid (Arg-Gly-Asp, RGD), present in numerous ECM proteins such as fibronectin and collagen that enable cell adhesion. Most cell types are able to bind to RGD, making this a widely employed peptide to provide adhesivity to alginate and other polymers. (83, 85)

Ho *et al.* demonstrated the importance of adhesion to MSC spheroids by presenting RGD ligands to cells entrapped in alginate hydrogels (Fig. 3A).(84) Compared to spheroids in unmodified alginate, which preserved the spherical structure due to its non-fouling nature, the presentation of RGD significantly increased both cell viability and VEGF secretion by entrapped spheroids. MSC spheroids entrapped in RGD-modified alginate gels exhibited increased osteogenic markers such as alkaline phosphatase (ALP) activity and calcium deposition compared to spheroids entrapped in unmodified gels, translating to increased bone formation *in vivo* in the absence of additional cues. More recently, the effect of RGD density on entrapped osteogenically induced MSC spheroids was reported (Fig. 3B), with increased ligand density translating to improved maintenance of the osteoblastic phenotype and increased bone formation *in vivo*.(86) Conversely, poly-L-lysine coated alginate beads possessing no cell adhesion sites impaired cell spreading and promoted embryonic stem cell (ESC) spheroid pluripotency.(87) These reports demonstrate that engineering the adhesive nature of biomaterials is a key parameter for instructing spheroid survival and function.

The density and spatial positioning of adhesive ligands within a biomaterial are important properties to regulate cell outgrowth from spheroids. 3D patterning of RGD in a collagenase-sensitive poly(ethylene glycol) diacrylate (PEGDA) hydrogel was achieved using a two-photon laser scanning (TPLS) photolithography technique.(88) TPLS immobilized RGD ligands on the gel in prescribed 3D patterns and gradients. Fibroblast clusters encapsulated in these gels were able to successfully migrate only into the patterned regions of the gel. By controlling the total area of pathways radiating from the spheroid, one could maintain aggregate formation for a known duration to sustain the desired therapeutic effect, while still allowing cells to migrate into the native tissue where they could further enhance repair.

Implantable scaffolds formed of synthetic polymers are commonly used in bone tissue engineering due to their tailorability (*e.g.*, shape, porosity, and mechanical properties), ease of synthesis, stability, and predictable resorption profile. Poly(lactic-co-glycolic acid) (PLGA) is an FDA-approved biodegradable polyester widely used for drug delivery that can be easily fashioned into porous biomaterial scaffolds.(12, 89-91) Poly(ε-caprolactone) (PCL) is another FDA-approved polyester possessing biodegradability and tailorability. PCL is commonly fabricated into scaffolds *via* electrospinning to mimic the fibrous structure of the native ECM.(92) Although spheroids cannot be formed *a priori* and entrapped in these dry scaffolds like hydrogels, the biophysical properties of the material can induce spheroid formation of dissociated cells within the scaffolds.(82) ASCs seeded into dried porous PLGA scaffolds exhibited spheroid formation and enhanced adipogenic differentiation and vascularization after subcutaneous implantation into mice.(93) This group pursued a similar approach using ASC spheroids in poly(L-glutamic acid)/chitosan scaffolds for cartilage repair.(94)

The encapsulation of therapeutic cells has been widely investigated for endocrine cells such as hepatocytes.(95, 96) Similar to many other cell types, hepatocyte function is enhanced when formed into spheroids versus cells in monolayer(97), motivating the exploration of hepatocyte spheroids for studies *in vitro* and upon implantation.(98) The immune response to transplanted cells can be suppressed by entrapping spheroids in a biomaterial before implantation to the patient, thereby addressing a primary challenge in cell transplantation. The semipermeable biomaterial enveloping the spheroids enables necessary exchange of small molecules between hepatocytes and the surrounding environment, allowing for continued albumin secretion and oxygen diffusion without the risk of immune response. Without immunoisolation of the hepatocytes, functional cells could only be maintained using long-term immunosuppression, which has numerous drawbacks.(99) Therefore, spheroid encapsulation offers a promising solution.(96) Advanced methods have emerged to efficiently entrap hepatocytes by using microfluidic devices that combine aggregation and encapsulation in a PEG hydrogel in a single step. PEG-entrapped hepatocyte spheroids exhibited greater albumin secretion compared to spheroids in AggreWells™, with only a 17% loss in viability.(100) This study demonstrated that hepatocyte spheroids could achieve enhanced function when encapsulated, emphasizing the potential for other applications of this approach.

Although hydrogels are intuitive biomaterials for spheroid entrapment, microporous scaffolds such as PLGA have also been used for endocrine aggregate cell delivery and allow for better host tissue integration. Islet cells, spheroid-like clusters of endocrine cells that produce insulin, have been delivered via porous PLGA scaffolds, with properties of the scaffold having a direct effect on islet behavior *in vivo*. Greater pore interconnectivity of the scaffold showed faster reversal of diabetes when islet cells were implanted into the epidermal fat pad of mice.(101) This further enforces the conclusion that proper biomaterial manipulation has a profound effect on spheroids potential for tissue engineering. The potential of these scaffolds for use as drug delivery vehicles was demonstrated by sustained release of transforming growth factor-beta1 (TGF-β1). Islet cells face similar hurdles as hepatic transplants due to concerns regarding the immune response, and TGF-β1 is a potent growth factor that can combat the inflammation response occurring after implantation.

Scaffolds delivering islet cells and releasing TGF-β1 exhibited improved graft survival and decreased leukocyte infiltration, thus reducing the immune response.(102)

Nanofibers have also been pursued as a favorable biomaterial for spheroid delivery due to their similar nanotopography to native ECM. Fibroblast spheroids were deployed for diabetic wound healing on 0.4 mm-(2D) or 4 mm-thick (3D) porous nanofiber mesh scaffolds made of PCL and gelatin.(103) Compared to 2D nanofiber scaffolds, fibroblasts had greater interaction with the pores in 3D scaffolds, suggesting an opportunity to improve cellularization of wound dressings. PLGA nanofibers coated with fibrin, collagen or no protein were placed on top of fibroblast spheroids for gingiva connective tissue engineering.(104) Fibers with collagen and fibrin prompted cell extensions protruding out of the spheroid, while uncoated fibers induced no spheroid migration. Further experiments showed collagen coated nanofibers allowed fibroblasts to migrate deeper into the scaffold and fuse into larger microtissues. Meanwhile, fibrin coated nanofibers promoted the disassembly of fibroblast spheroids, leading to scaffolds with highly dispersed fibroblasts Collectively, these examples of engineering nanofiber scaffolds for spheroid delivery exhibit the profound effect a properly designed scaffold may have on spheroid function. Through manipulation of fiber composition and structure, spheroids can remain as aggregates or become dissociated, ultimately dictating their therapeutic function.

Chitosan has been used to leverage the multilineage potential of MSCs for tissue engineering. Adipogenesis was enhanced in MSC spheroids after incubation on a chitosan coated amyloid fibril network for 7 days before induction(105), an effect potentially resulting from the nanotopography of the fibrils. Furthermore, ASC spheroids cultured on chitosan exhibited cardiac marker gene expression without additional inductive cues, potentially expanding the use of this population in cardiac repair.(55) In another example, the differentiation capacity of ASCs was significantly enhanced after spheroid formation on chitosan membranes, pluripotency markers were upregulated, and transdifferentiation into neuronal and hepatocyte-like cells was reported.(57) Collectively, these reports demonstrate that the characteristics of the biomaterials have a profound effect on spheroid differentiation and function.

6. SPHEROIDS IN BIOMATERIALS FOR CELL-BASED TISSUE ENGINEERING

The entrapment and transplantation of spheroids in engineered materials with preferred adhesivity, stiffness, and degradation enables *in situ* control over spheroid function. The transplantation of MSCs in biomaterials has consistently yielded improved tissue formation compared to systemic or localized injection of cells, providing a strong motivation to study the therapeutic promise of entrapped MSC spheroids for tissue engineering. We provide some recent examples of this approach when applied toward bone and cartilage tissue engineering and wound healing.

Bone tissue engineering

Bone formation and repair is a delicate interplay between angiogenesis, driven by local VEGF signaling, and the local availability of bone-forming cells, which may be achieved by transplanting cells of the osteoblastic lineage or stimulating differentiation of host cells.(106) Cell-based approaches are widely studied for bone tissue engineering as viable alternatives to bone grafts, synthetic materials, and pharmacological approaches. Osteogenically-induced MSC spheroids entrapped in fibrin hydrogels exhibited enhanced osteogenesis, improved survival, and increased angiogenic potential compared to individual MSCs.(107) Platelet-rich plasma (PRP) is another promising cell carrier for use in bone tissue engineering, buoyed by its long-term use and safety profile in applications of wound healing.(108) PRP may be isolated for autologous use and formation into hydrogels that retain pro-regenerative growth factors or cytokines that suppress local inflammation. Compared to individual MSCs on a ceramic construct, MSC spheroids within a PRP gel containing ceramic particles generated more bone when implanted in a murine ectopic site.(61) However, the variability of these naturally-derived hydrogels and challenges associated with independently modulating the mechanical properties limit their use as a research platform to explore the effect of various stimuli on spheroid function.

Adhesivity is a key design parameter to instruct the function of entrapped, anchorage-dependent cells, and this is commonly manipulated by the incorporation of adhesive proteins or peptides onto the polymer backbone. When entrapping osteogenically-induced MSC spheroids in alginate gels, the density of the adhesive peptide was a crucial aspect to maintain their osteogenic phenotype.(86) Bone formation was increased in gels that limited MSC migration from the spheroids, whether unmodified gels or alginate gels with high RGD density, suggesting that restricting the migration of cells from the spheroidal structure is a viable strategy to enhance bone formation with MSCs.

Pullulan and dextran are naturally-derived polysaccharides that can form hydrogels or solid scaffolds upon crosslinking under aqueous conditions. The incorporation of hydroxyapatite enhanced the osteogenic potential of such composite hydrogels when freeze-dried into macroporous scaffolds, thus recapitulating the nanostructure and mineralized environment of bone tissue.(62) These scaffolds successfully induced cell aggregation and spheroid formation of implanted human MSCs, improving bone formation in the rat femoral condyles. However, these materials require modification to present adhesion sites necessary for spreading, proliferation, and osteogenic differentiation.(109, 110) Additionally, the commercial production of dextran remains inefficient, presently limiting its widespread use as a biomaterial for bone tissue engineering.(111)

In addition to the materials already discussed, nanofibrous mesh scaffolds formed of PCL have been used as a carrier for osteoblast spheroids. Compared to scaffolds seeded with individual human primary osteoblasts, calvarial bone defects treated with spheroid-loaded scaffolds exhibited enhanced bone regeneration, particularly within the core region of the scaffold.(112)

Cartilage tissue engineering

In light of the self-assembling nature of cartilage during development (113), cartilage tissue engineering is being pursued by entrapping spheroids in biomaterials that promote signaling present during these events.(114) Biomaterials can potentiate the condensation processes that promote cartilage formation while providing an effective delivery method of spheroids to damaged tissue. Preferred biomaterials are sufficiently non-adhesive to inhibit cell spreading and dedifferentiation from the chondrogenic phenotype, yet also possess sufficient porosity to enable diffusive nutrient transport to entrapped spheroids.

Hydrophilic, non-fouling biomaterials provide characteristics ideal for retaining spheroid morphology and preventing adhesion and migration from the aggregate. Among these materials, alginate has been used due to its hydrophilic nature and lack of native cell binding sites. Spheroid formation was achieved by entrapping ASCs in unmodified alginate and culturing in chondrogenic media, which maintained expression of chondrogenic markers after subcutaneous implantation in mice.(115) By 12 weeks, entrapped spheroids produced substantial cartilaginous ECM. Polyurethane (PU) and hyaluronic acid (HA) are other biomaterials that have been investigated for cartilage tissue engineering. Using a water-based 3-D printing technique, PU-HA scaffolds were seeded with MSCs that aggregated into spheroids within the construct and underwent chondrogenesis. When implanted into rabbit chondral defects, the PU-HA scaffolds induced significantly more cartilage regeneration compared to PLGA scaffolds.(116)

PLGA and chitosan have been used to deliver spheroids for cartilage engineering despite their tendency to adsorb plasma proteins that provide adhesion sites for associated cells. Both PLGA and chitosan can crosslink *via* amide bonds, forming a hydrophilic network that can then be dried into a porous scaffold. Individual ASCs were seeded into PLGA-chitosan hybrid scaffolds, resulting in spheroids with a diameter of 80-110 μm.(94) When compared to scaffolds that failed to sustain spheroid morphology, leporine cartilage defects treated with spheroid-maintaining scaffolds exhibited better organized repair tissue with perpendicularly aligned cells similar to neocartilage. Conversely, PLGA-chitosan scaffolds made using solid freeform fabrication failed to induce differences in cartilage repair within rabbit chondral defects when seeded with MSC spheroids over individual MSCs.(65)

Chitosan has been used in combination with other biomaterials such as silk fibroin, a fibrous protein found in silk. Biphasic scaffolds were successfully engineered with silk fibroin and chitosan for cartilage tissue engineering. The top of the scaffolds was composed of a silk fibroin film to prevent cells from leaving the defect site, while the bottom half of the scaffold was made of a silk fibroin and chitosan sponge that could be seeded with cells. Composite materials that induced spheroid formation by MSCs, attained by manipulating the ratio of silk fibroin to chitosan, resulted in enhanced cell survival and glycosaminoglycan secretion. (117)

As an alternative to entrapping spheroids in the material itself, chondrogenic differentiation of MSCs can be enhanced by presenting chondroinductive cues to responsive cells. For example, MSC chondrogenic differentiation was enhanced by delivering TGF-β to cells within the aggregate using gelatin microspheres, resulting in enhanced chondrogenic

differentiation of the MSCs.(64) MSC spheroids were then formed into a tube structure, resulting in cartilage possessing similar mechanical properties to native trachea.

Wound healing

Chronic or non-healing wounds of the skin are a significant clinical problem, occurring in 7 million patients each year in the United States alone.(118) Chronic wounds have been treated using recombinant growth factors such as VEGF, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and others, to speed neovascularization and epithelialization.(119) Due to the short half-lives of these molecules, cell-based therapies are under investigation to provide cells that jumpstart wound closure or secrete the numerous signals necessary for coordinated healing.(120) MSCs can significantly enhance granulation tissue formation, angiogenesis, and reduce inflammation through their potent secretome.(121, 122) Moreover, the quantity of endogenous factors secreted by MSCs increases when formed into spheroids compared to an equivalent number of individual cells.(40, 84) The composition of the MSC secretome can be tailored by the culture conditions employed during spheroid formation. VEGF(38, 123) and PGE_2 (41) are present within the MSC secretome and signal resident endothelial cells and macrophages to initiate wound repair. Importantly, both VEGF and PGE_2 promote re-epithelialization by stimulating keratinocyte migration and proliferation.(35, 124) To sustain the advantages of spheroids over individual cells *in situ*, the transplantation of spheroids within biomaterials that potentiate growth factor secretion will advance their therapeutic potential for wound healing.

The entrapment of spheroids in engineered biomaterials represents an exciting approach to regulate and even potentiate spheroid function in wound healing and tissue regeneration. Fibrin gels are FDA-approved wound dressings that can be engineered by modulating the composition of clotting proteins and other additives. Murphy *et al.* demonstrated that the biophysical properties of fibrin gels, modulated by altering composition, can guide the simultaneous secretion of robust concentrations of VEGF and PGE_2 (Fig. 4).(125) Importantly, fibrin gels could be tuned to independently or simultaneously enhance secretion of VEGF and PGE_2 from entrapped MSC spheroids, providing a tailorable platform for use in specific applications of wound healing and tissue repair. Stiffer gels induced secretion of VEGF by entrapped spheroids, while more compliant gels preferentially stimulated PGE_2 secretion. The mechanical properties and degradation rate can be further tuned by addition of crosslinking agents or inhibitors of degradative enzymes. As another example, ASC spheroids entrapped in composite hydrogels of chitosan and HA yielded more vascularized tissue compared to spheroids exposed to HA alone.(126)

In clinical applications, transplantation of spheroids using biomaterials improves critical aspects of handling and localizing cells at the target site. When properly designed, biomaterials can promote regenerative properties in the cells and enhance repair of the surrounding tissue.

7. FUTURE OUTLOOK

Spheroids formed of somatic or stem and progenitor cells are a promising tool to propel the therapeutic efficacy of cell-based therapies and enhance our understanding of morphological

development. Numerous examples can be found in the literature which demonstrate the benefits of spheroids over individual cells, yet there is no consensus on the ideal density of cells per spheroid to achieve a desired outcome. We anticipate that new techniques to form spheroids will yield higher throughput technologies to accommodate the vast number of cells required for clinical use. Similar to individual cells, the transplantation of spheroids in biomaterials localizes cells at the target site and facilitates *in situ* instruction. Despite early successes reported when simply injecting spheroids into damaged tissues,(38) the engineering of materials that direct the behavior of entrapped spheroids and respond to the localized environment is an exciting strategy to potentiate the efficacy of spheroids.

The advancement of spheroids for clinical use will be realized through advancements on several fronts including 1) reducing the time required for formation; 2) transplantation of co-culture spheroids; and 3) engineering materials that respond to changes in aggregate size or metabolic activity. To reduce the time required for spheroid formation, high density aliquots of cells could be entrapped in non-adhesive materials that promote cell-cell adhesion and degrade in a few hours. Various microfluidic approaches have been used to successfully entrap individual cells(127, 128), opening the door to larger payloads for encapsulation. Hydrogels formed of low molecular weight alginate, HA, or PEG may be acceptable platforms, provided they contain enzymatically responsive linkages or are not heavily crosslinked. Secondly, the transplantation of a heterogeneous cell population or co-cultures may provide valuable contributions to promote tissue formation.(129-131) Accessory cells such as endothelial and hematopoietic cells secrete bioactive factors to support parenchymal cells and form nascent capillaries to enhance nutrient delivery. Finally, analyte-responsive materials may be used to capitalize on the changing metabolic profile of entrapped spheroids.(132) As spheroids undergo differentiation, the surrounding material may degrade or alter its biophysical properties upon secretion of new biomacromolecules by entrapped cells. The use of such materials as cell delivery vehicles would provide untapped potential to instruct spheroid fate upon implantation (Fig. 5).

This review highlights recent efforts to develop and apply biomaterials to guide spheroid function and their contributions to tissue repair. To extend the use of spheroids beyond preclinical studies, their advancement into clinical use will require additional testing to demonstrate safety and efficacy and to determine the minimum number of required cells. Since cells within spheroids exhibit improved cell viability, it is possible that spheroid-based therapies may be available to patients directly off-the-shelf. This concept is not so far from reality, as MSC spheroids have retained their function in ambient conditions for up to 7 days.(133) Among their promising characteristics, the improved cell survival observed when cells are formed into spheroids is one of the most exciting qualities for translation into the clinic, as this addresses a major hurdle of many cell based approaches. Numerous studies have recognized the fact that a high percentage of cells implanted do not survive, severely diluting the regeneration potential of the implant.(134) Spheroids could result in marked improvements in potency for many cell-based approaches, making them more clinically feasible by requiring fewer cells for similar or improved therapeutic outcomes.

High throughput methods of spheroid formation are ideal for use in a clinical setting, as they require less specialized equipment and skilled labor. Future applications that require

spheroids of autologous cells will be facilitated by improving the capacity to quickly and easily produce them. Moreover, hydrogels are commonly used in tissue engineering approaches. They are easily tailorable, can be delivered in a minimally invasive manner, and there are FDA-approved hydrogels for use in the clinic. Therefore, pursuing these promising results with spheroids entrapped in engineered hydrogels could further accelerate the translational use of spheroids. The value of biomaterials-based delivery of spheroids over free injection or pharmacological approaches of delivering recombinant growth factors will be established by minimizing costs associated with this cell-based approach and demonstrating reproducibility of their application in blinded studies. By combining such strategies with existing knowledge of biomaterial properties to instruct cell phenotype, spheroids may become an even more powerful tool in advancing our knowledge of morphogenesis and the repair and regeneration of damaged tissues.

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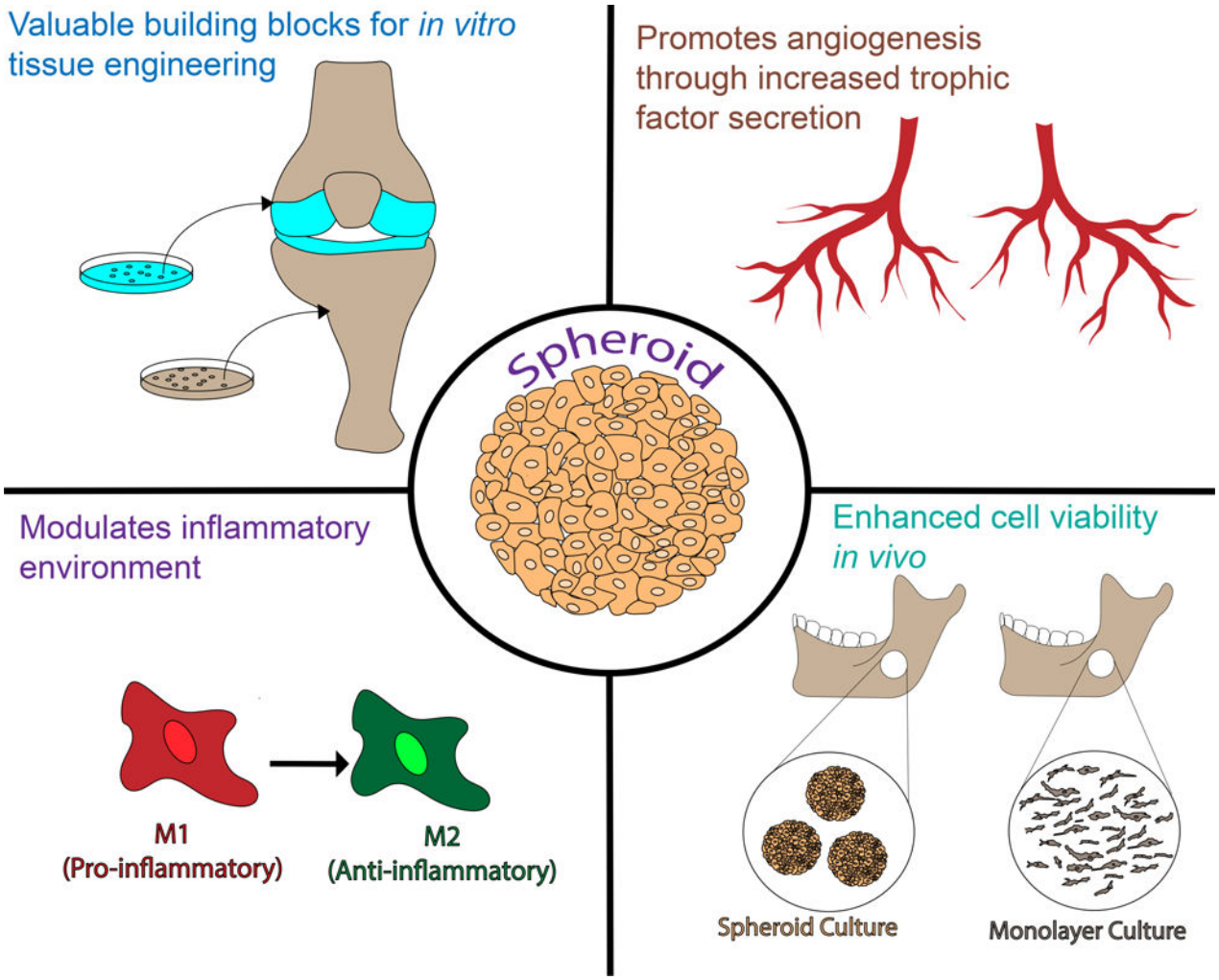

Figure 1.

Spheroids overcome many shortcomings inherent with individual cells when used in cell-based therapies and tissue engineering. Compared to individual cells, spheroids secrete increased levels of trophic factors with proangiogenic and immunomodulatory potential, exhibit enhanced cell viability and persistence, and are valuable building blocks for tissue formation.

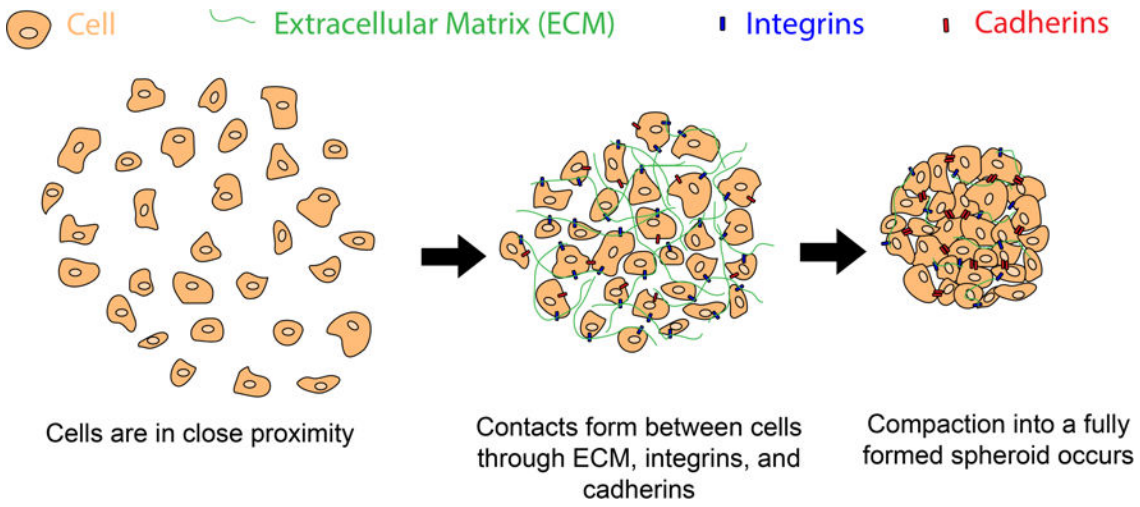

Figure 2.

Cell suspensions assemble into spheroids upon cell-to-cell binding and establishing ligand-receptor interactions *via* cadherins and integrins, respectively. The cellular aggregate compacts into a fully formed spheroid over time.

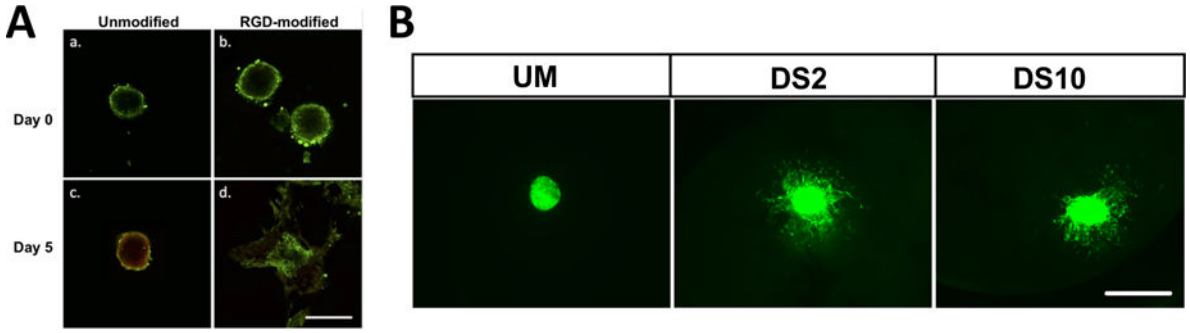

Figure 3. The presence and density of RGD in alginate hydrogels influence MSC spheroid viability and migration

(**A**) Live/dead stain reveals comparable viability and spherical morphology for spheroids at Day 0 in (**a**) unmodified and (**b**) RGD-modified gels when visualized with confocal microscopy. (**c**) Live/dead stain demonstrates increase in dead cells and retained spherical morphology for spheroids at Day 5 in unmodified alginate, while (**d**) spheroids in RGD-modified alginate gels have increased viability and migration from the aggregate. Scale bar = 200 μm. Reprinted with permission from (84), Copyright 2016 John Wiley and Sons. **(B)** Representative fluorescent images of spheroids in unmodified, DS2, and DS10 RGD-modified alginate gels at Day 10. Scale bar = 500 μm. Reprinted with permission from (86), Copyright 2017 American Chemical Society.

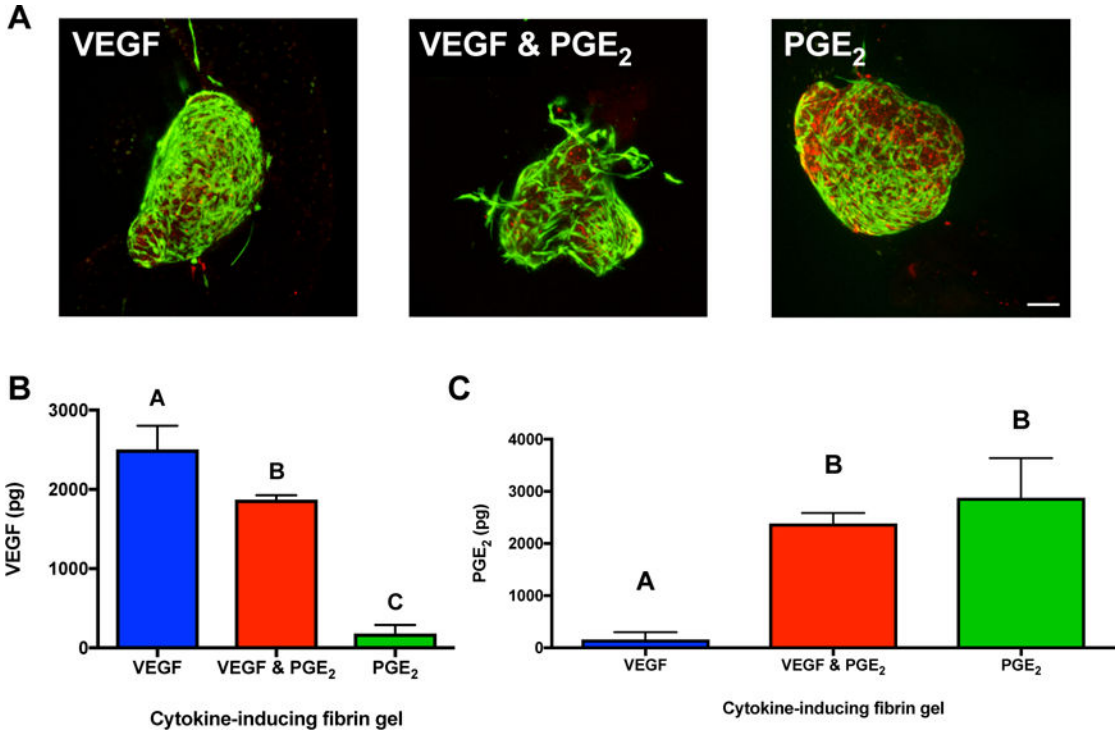

Figure 4. The biophysical properties of fibrin gels can be tailored to promote the wound healing potential of entrapped MSC spheroids

(**A**) Representative confocal microscopy images of live (green)/dead (red) assay revealing MSC spheroid viability when entrapped in a fibrin gel optimized for VEGF secretion, VEGF & PGE_2 secretion, and PGE_2 secretion after 7 days of culture. Scale bar is 100 μm. **(B)** Proangiogenic potential as measured by VEGF secretion by MSC spheroids entrapped in engineered fibrin gels designed to promote growth factor secretion. **(C)** Anti-inflammatory potential as measured by PGE_2 secretion by MSC spheroids entrapped in fibrin gels designed to promote growth factor secretion. Reprinted from (125) Copyright 2017 with permission from Elsevier.

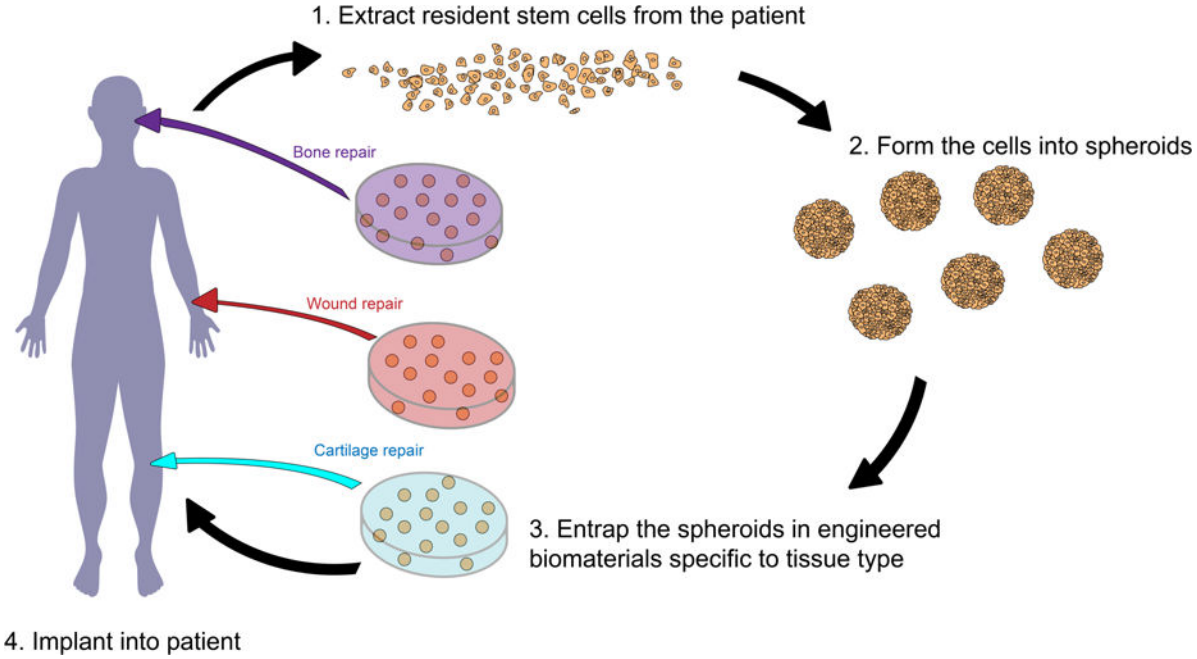

Figure 5.

Advanced spheroid-based therapies may benefit from entrapping spheroids derived from the patient's stem cells into biomaterials known to promote a specific lineage. This approach would increase the off-the-shelf potential for using spheroids in tissue regeneration.