The Crosstalk between Tissue Engineering and Pharmaceutical Biotechnology: Recent Advances and Future Directions

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1. INTRODUCTION

Every living cell is affected by the surrounding microenvironment [1]. Cell microenvironment is driven by biophysical and biochemical factors, including the extracellular matrix (ECM) nano/microscale topography [2-4] and composition [5], as well as soluble factors both resulting from cell-ECM and cell-cell signaling [6], that directly or indirectly affect cellular conditions.

Upon aging, injury or disease, tissue repair/regeneration demands the precise orchestration of complex signaling cascades to coordinate the replacement/improvement of the lost/damaged tissues, as well as their physiological roles [7]. The precise coordination of those signals perceived by the cells is essential for the successful regeneration of tissues [8]. Thus, physical, chemical, and biochemical recapitulation of ECM microenvironment is a fundamental aspect of tissue engineering aiming to direct and control cell behavior in three-dimensional (3D) scaffolds with multiple dimension ranges [9]. Langer and Vacanti defined tissue engineering, in 1993, as "an interdisciplinary field of research that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function" [10]. The main elements required to engineer tissues are the cells, the materials used to design the 3D biodegradable supporting structures, and the signaling molecules. Considerable efforts to design artificial matrices have evolved from simple 3D supporting scaffold to more complex tissue-*like* microenvironments. Both biologically-derived and synthetic materials have been extensively exploited to produce a wide range of scaffolds, whose properties determine the ultimate cellular response [11]. In general, materials from natural sources present advantages over synthetic, mainly due to their inherent biologically recognized features, such as presentation of cell receptor-binding ligands and vulnerability to cell-triggered proteolytic degradation [12]. The recapitulation of cellular microenvironment remains nevertheless one of the major challenges in TE, given the complexity of cell–ECM interactions as well as multicellular architectural features such as repeating tissue units and proper vascular structure [13]. In recent years, the combination of those 3D structures with signaling molecules known to be involved in different cellular processes such as activation, proliferation, migration, and differentiation, has

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been in focus aiming at further targeting specific healing events [14]. Among the most studied molecules are multifunctional proteins like growth factors or cytokines.

Biotechnology was defined as "the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services" [15]. Biotechnology has evolved over the years, developing new tools, including recombinant, fermentation and/or genetic engineering technologies that address current tissue engineering issues. Moreover, the field of pharmaceutical biotechnology represents one of the most rapidly advancing areas of science. Today, the shape and vision of pharmaceutical aspects and challenges are much more demanding, either by the necessity of new signaling molecules [16], or of improved vehicles to enhance efficacy and effectiveness.

The crosstalk between tissue engineering and pharmaceutical biotechnology has led to major improvements both in tissue regeneration and drug discovery (Fig. **1**). Pharmaceutical biotechnology is contributing, at the physical and biochemical levels, to the development of new strategies to engineer cellular microenvironments. In fact, the need to create materials that closely mimic the native ECM biochemistry urged for recombinant and fermentation technologies. Proteins are major cellular and extracellular players in native tissues [17] thus, the pursuing of tissue regeneration driven by protein-derived scaffolds or spatial and temporal control release of relevant signaling molecules is being more and more relevant. Tissue-engineered constructs made of biotechnology-derived materials have been given important insights into cellular gene expression and behavior when in the presence of specific genetic sequences. In opposition, more complex, reliable and accurate 3D tissue-*like* structures are expected to be provided by tissue engineers to improve drug discovery. Through tissue engineering, superior 3D *in vitro* models are being developed, which allow better and faster drug screening. In a different strategy, cell behavior has been manipulated, either as a therapeutic tool or as part of drug screening platforms, by means of gene delivery.

In this review, a critical overview of the recent achievements and approaches that highlight the interplay between the tissue engineering and pharmaceutical biotechnology fields is provided. The use of biotechnology-derived materials to generate 3D tissue-analogues and their major breakthroughs in tissue regeneration and drug discovery is discussed. Moreover, the crosstalk between tissue engineering and pharmaceutical biotechnology is also stressed by discussing how the outcomes of tissue engineering approaches have been enhanced through the use of biotechnology-derived signaling molecules. The interplay between these areas is also emphasized by discussing the gene delivery/therapy as another cross point.

2. PHARMACEUTICAL BIOTECHNOLOGY IN TISSUE ENGINEERING: FROM REPAIR TO REGENERATION

2.1. Biotechnology-Derived Materials to Engineer the ECM

Native tissues comprise a 3D viscoelastic milieu - ECM, within which cells interact constantly, and which guides their development or homeostasis. Under suitable conditions, cells are able to remodel their immediate microenvironment and form functional tissue units [18]. Thus, one of the most important ambitions in designing support structures for tissue engineering is the importance of mimicking the natural tissue microenvironment. The tissue engineering "biomimetic" approach combines biological principles with engineering design, to direct metabolically active cells in 3D artificial matrices that, in response to environmental signals, gradually form tissue-*like* structures [19, 20]. The development of these matrices has generated knowledge that allows tissue engineers, selectively and modularly, refining their materials in view of providing an appropriate environment that dictate and guide tissue regeneration.

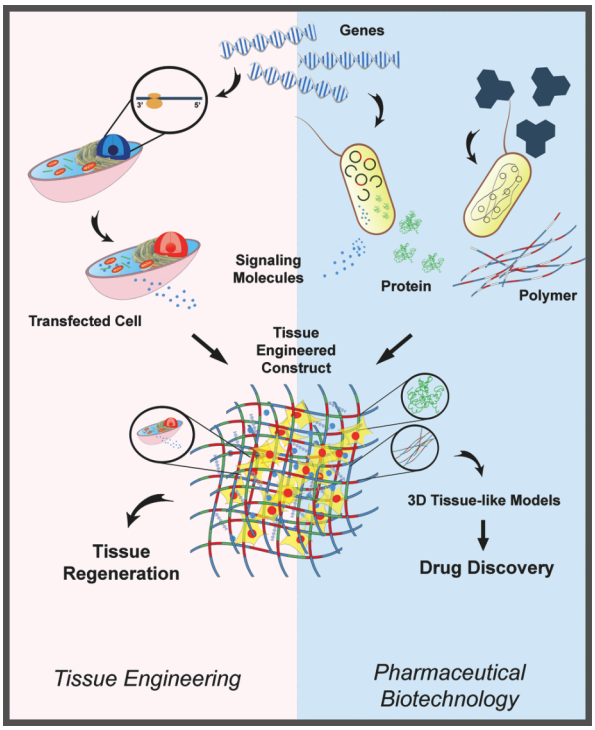

Fig. (1). The Crosstalk between tissue engineering and pharmaceutical biotechnology.

Biotechnology tools such as recombinant technologies and fermentation offered a mean to engineer new and modified molecules. Recombinant technology has enabled the development of proteins by the introduction of tailored synthetic genes into the genetic sequence of microorganisms [21, 22]. Recombinant technologies, bacterial and non-bacterial transformation or phage introduction have been widely explored. However, each one of them presents cons and pros, complexity of gene design, as well as high cost and time-consumption [21] are among the most common limitations, while the superior structural complexity and functionalization, as well as high stability of the obtained protein-based polymers has been of particular interest for tissue engineering. Thus, the symbiosis of biotechnology and biomaterials has set the stage for systematic advances in tissue engineering [10, 23, 24]. Examples of recombinant proteins include collagen, silk, silk-elastin like proteins (SELPs). Furthermore, much effort has been devoted to the development

of cost-effective and environmentally friendly production processes by exploring cheaper fermentation products, such as gellan gum (GG) and polyhydroxyalkanoates (PHAs) [25].

Collagen has inspired tissue engineers and the design of biomaterials since it primarily regulates and defines most tissues. Common sources of collagen for tissue engineering applications include bovine skin and tendons, porcine skin and rat-tail, among others [26-28]. The development of recombinant collagens provides the promise of a safe, predictable and chemically defined source [29, 30], identical in composition for different production lots [30], while overcoming the variability of the native tissue-based sources [31]. Recombinant collagens produced by yeast *Pichia pastoris*, is currently the most used strategy for effective commercial production [32]. Like collagen, recombinant silk proteins have been used in a variety of tissue engineering applications, particularly due to the outstanding mechanical toughness of silk fibers associated to their crystalline units [33-36]. The crystalline region of silk fibroins contains repetitive alanine or alanine–glycine rich sequences, which have been used as the basis for genetically engineer silk fibroin-like polymers in host systems like *Escherichia coli* [37], yeast [38], mammalian cells [39], and plants [40, 41]. Another example of a genetically engineered protein is elastin, which is found in tissues/organs where elasticity is of major importance [42-44]. *Escherichia coli* has typically been the expression system of choice for elastin production [45], but expression systems using plants [46] and *Pichia pastoris* [47] have also been effective. A major advantage of recombinant technology is that the variation of the peptide combinations allows the design of polypeptides with a range of different biological and physical properties. For example, SELPs composed of amino acid sequence motifs from *Bombyx mori* (silkworm) silk and mammalian elastin [48] combine the high tensile strength of silk with the resilience of elastin [49, 50].

GG is a polysaccharide derived from the microbial fermentation product of *Sphingomonas elodea* [51], which repeat unit resembles the glycosaminoglycans present in the native ECM (Fig. **2A**) [52, 53]. Its versatile features have lead tissue engineers to purpose it for a wide range of applications [54-58]. PHAs produced by fermentation using improved bacterial strains, such as *Cupriavidus necator*, several species of *Pseudomonas*, *Bacillus*, *Azotobacter* and also recombinant *Escherichia coli* [59], have also been intensively investigated as a family of natural-origin materials for tissue engineering (Fig. **2B**) [60]. The possibility of tuning their degradation profile by varying the bacterial source and fermentation conditions [61], their intrinsic piezoelectric nature, a featured exhibited in the majority of the living tissues [62], as well as its by-products, including D-3-hydroxybutyric acid, a normal constituent of human blood [63], are some of the PHAs properties relevant for tissue engineering.

2.1.1. ECM-Like 3D Structures

Hydrogels are 3D cross-linked hydrophilic polymeric networks with a high water content, facilitating free diffusion of oxygen, nutrients and growth factors, that partially mimic the physical characteristics of many native soft ECM [13, 64, 65]. Although hydrogels became the biomaterials of choice for artificial ECM recreation, hydrogels formed by those biotechnology-derived materials are still poorly explored. In 2010, Pulkkinen *et al.* proposed the use of human recombinant type II collagen (rhCII) for cartilage tissue regeneration, being incited by previous *in vitro* results [66]. The rhCII-chondrocytes construct led to subcutaneous neotissue formation with an abundant presence of type I and type II collagen, and proteoglycans [67], but further studies are required to infer about the potential of this strategy to regenerate fully functional articular cartilage. Despite the properties of SELPs, hydrogels encapsulating human bone marrow-derived mesenchymal stem cells (BM-MSCs) were shown to

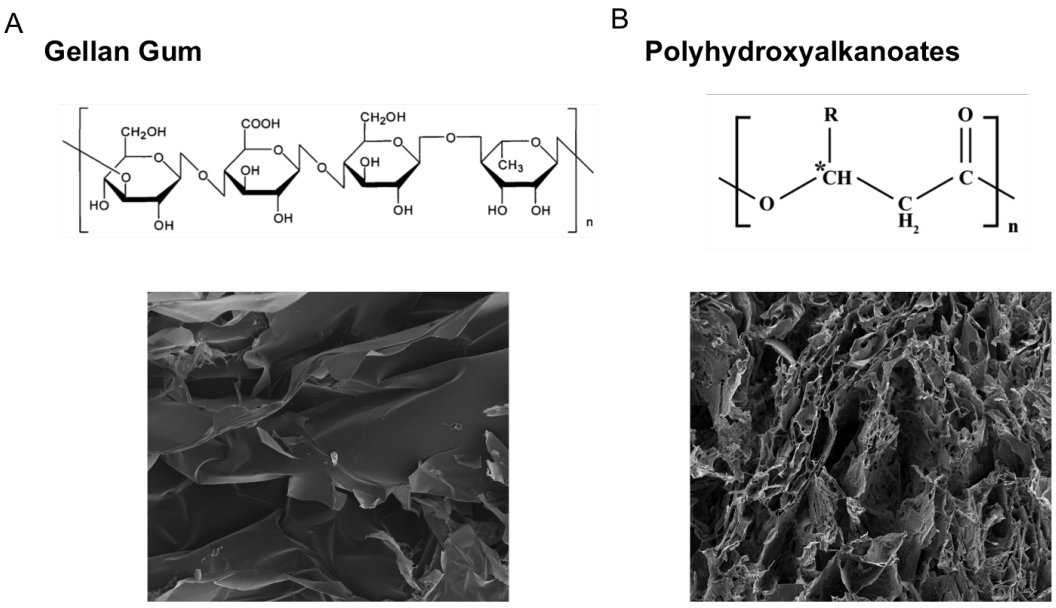

Fig. (2). Chemical structure of two bacteria fermentation derived polymers, (**A**) gellan gum and (**B**) polyhydroxyalkanoates. These can be processed using different methodologies to obtain 3D porous scaffolding structures relevant for tissue engineering applications.

support their chondrogenic differentiation and the deposition of cartilage-specific matrix *in vitro* only in the presence of transforming growth factor beta 3 (TGF-β3) [68]. Interestingly, among the described biotechnology-derived materials, GG hydrogels have been the most explored and shown to be a suitable platform to support and/or deliver cells in different TE applications [54-58]. Injectable GG hydrogels were initially aimed at cartilage regeneration. Human chondrocytes were shown to remain viable when encapsulated in GG hydrogels [54] and exhibited an upregulation of type II collagen and aggrecan, as well as downregulation of type I collagen when subcutaneously implanted in nude mice [69]. Later on, similar evidences were also observed after injecting GG hydrogels encapsulating pre-differentiated rabbit adipose stem cells (ASCs) into full-thickness knee cartilage defects in rabbits [70]. An improved processing methodology allowed attaining GG-based spongy-*like* hydrogels that depict features of both sponges and hydrogels, but superior physical properties in relation to the hydrogels and potentiated cell-ECM-*like* interactions [71]. GG/hyaluronic acid (GG-HA) spongy-*like* hydrogels entrapping human dermal/epidermal cell fractions directly from isolation, although not capable of sustaining the self-organization of the entrapped cells in the respective skin layers, were able to accelerate full-thickness mice wound closure rate and re-epithelialization, as well as tissue neovascularization at early stages of wound healing [56]. Likewise, synergized with cells obtained from adipose tissue, ASCs and microvascular endothelial cells (ECs), to promote superior skin wound neovascularization [57]. Recently, GG spongy-*like* hydrogels reinforced with bioactive-glass were proposed for bone tissue engineering [58]. This first assessment allowed confirming that hASCs were able to adhere and spread within GG spongy-*like* hydrogels reinforced with the bioactive glass without compromising its viability.

In addition to the ECM biochemical features, tissue engineers have also been focusing on emulating ECM topography as well as the structural diversity of each tissue using biotechnology-derived materials. The nanosized and fibrilar nature of ECM components has been widely mimicked with electrospun nanofibers. Despite the wide exploitation of the electrospinning method in the field of tissue engineering, its processing particularities have limited its use with the majority of the biotechnology-derived materials [72, 73]. Zonari *et al*. proposed electrospun polyhydroxybutyrate (PHB) and its copolymer poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) fiber meshes (diameters ranging 300 nm to 1.3 μm) to promote bone regeneration. Considering the required neotissue vascularization during bone regeneration, the developed structures were evaluated as supports for hASCs as well as their differentiation into the endothelial lineage [74]. A similar approach was adopted envisioning the regeneration of the myelinic membrane [75]. Different formulations based on electrospun PHB/PHBV fiber meshes with or without type I collagen were proposed to sustain Schwann cell (SCs) organization and function. SCs grown on aligned PHB/PHBV/collagen fibers exhibited a bipolar morphology and an orientation along the fiber direction, while SCs grown on the randomly oriented fibers presented multipolar morphology. Thus, aligned PHB/PHBV electrospun nanofibers provided a positive environment for SCs growth, which, together with the collagen, can contribute to improved cell phenotype and nerve tissue engineering. In a different approach PHBV bilayer microscale constructs were proposed aiming at mimicking the bilayer skin structure [76]. The independently designed structures depicted properties that respectively favored human dermal fibroblasts and human keratinocytes (hKC) performance under heterotypic culture conditions that led to the particular rearrangement of hKC (Fig. **3C**). As in native tissue, proliferative cells were present in the basal layer, and cells expressing a terminal differentiation marker in the upper layer.

2.2. Release of Relevant Signaling Molecules

Aiming at achieving full tissue regeneration, and a functionality equivalent to the native tissue, exogenous instructive signaling molecules have been widely used to recreate, at the spatial and temporal levels, the specificities of the regenerative pathway and ultimately the native microenvironments [14]. Numerous families of growth factors have already been identified and remarkable progresses have been made in understanding the pathways involved in the activation/regulation of regenerative processes [77]. Though, long is the path to be crossed to meet the precise mechanisms by which signaling molecules gradients lead tissue formation and development.

Explosive developments in recombinant technology have spawned the production of different proteins, including growth and differentiation factors. Moreover, drug delivery, a field of pharmaceutical technology, is one of the most rapidly advancing areas that combined with biotechnology techniques is a driving force not only of modern drug discovery but also of advanced therapies. In fact, the identification and production of relevant recombinant growth factors have generated much enthusiasm and numerous clinical trials, but the results of many of these trials have been largely disappointing [78–80]. The outcomes revealed limited clinical benefit, which can be related to both the mode of delivery and the requirement for multiple signals at different times [81]. Thus, the exogenous administration of signaling molecules has demonstrated that novel drug delivery systems are necessary to more accurately achieve specific spatial and temporal delivery control. So far, growth factors-based therapies that are currently approved by the US Food and Drug Administration use some form of delivery system to instigate the activity, as well as recruitment of undifferentiated host progenitor cells. Examples of such delivery systems include InFUSE™ Bone Graft/LT-CAGE™ (Medtronic, USA) a collagen sponge impregnated with human recombinant bone morphogenetic protein (BMP)-2 for the treatment of bone fractures and for replacement of certain interbody spinal fusion procedures [82, 83]. Osteogenic Protein-1® is also indicated for the treatment of bone fractures and is composed of BMP-7/collagen paste from Stryker Biotech (Hopkinton, MA, USA) [84], whilst REGRANEX® Gel (OMJ Pharmaceuticals, Puerto Rico), human recombinant platelet-derived growth factor (PDGF) sequestered in a hydrogel is approved for the treatment of diabetic foot ulcers [85]. These further strengthen the notion that the spatiotemporal control of growth factor delivery is important for the clinical success of growth factors as regenerative therapeutics [86].

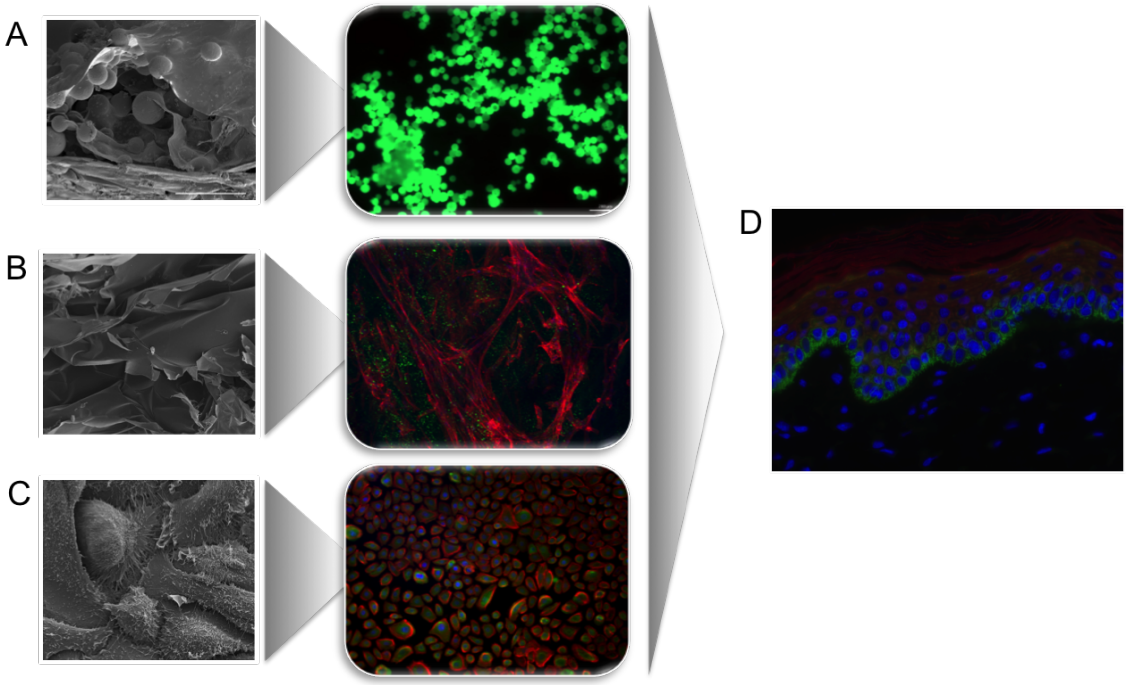

Fig. (3). Bacteria fermentation derived polymers as part of the tissue engineering triad. (**A**) signalling molecules loaded poly-3-hydroxybutyrate-co-3-hydroxyvalerate particles embedded in gellan gum hydrogel; (**B**) human dermal fibroblasts entrapped in gellan gum spongy-*like* hydrogel; (**C**) human keratinocytes organized on a poly-3-hydroxybutyrate-co-3-hydroxyvalerate membrane to target (**D**) fully functional skin regeneration.

Various approaches, to deliver a therapeutic substance to the target site, in a sustained and controlled release fashion, have been addressed for an equally wide range of tissue engineering applications. The random incorporation of BMP-2 in an alginate gel, used to increase the MSCs seeding efficiency in b-tricalcium phosphate structures and allow for its sustained release, resulted in greatest osteocalcin and osteoid deposition thus potentiating bone formation in an ectopic model [87]. Likewise, a combined action of PDGF-BB and canine ASCs incorporated in an heparin/fibrin-based gel embedding a poly(lactic-co-glycolic acid) (PLGA) nanofiber mat [88] lead to clinically relevant tendon healing in dogs. In a different perspective, TGF-β1 was tethered to poly (ethylene glycol) norbornene hydrogels to locally influence and promote cartilage matrix production by co-encapsulated chondrocytes and MSCs over a short period [89]. In opposition to the random incorporation of growth factors that aimed at controlled/sustained release, the chemical conjugation of the growth factors to the support polymeric structure allows overcoming the limitation of uncontrolled displacement that can be associated with the first approach. Nonetheless, short biological half-life *in vivo*, and vulnerability to structure disruption or hydrolyzation, leading to loss of bioactivity are issues to which both strategies are associated. In this sense, the exploitation of particulate systems that provide protection from enzymatic degradation and hydrolysis has been considered advantageous [90]. Thus, the incorporation of these particulate systems into 3D scaffolds has been hinted [91-93]. Oligo(poly(ethylene glycol) fumarate) hydrogels containing TGF-β3-loaded gelatin microparticles and rabbit marrow MSCs were used to fabricate the chondrogenic layer of an osteochondral construct. While at the chrondogenic layer, TGF-β3 proved to highly stimulate chondrogenic differentiation of MSCs, its effect over the osteogenic layer comprising pre-differentiated osteogenic cells, encapsulated in the same gel without the particles, resulted in delayed mineralization [91]. The potential feasibility of using a Neuregulin-1-loaded PLGA particles scaffold combined with ASCs for cardiac regeneration after myocardial infarction was evaluated [93]. Nonetheless, this work represents a proof-of-concept and further studies are needed to effectively assess the therapeutic potential of this growth factor to guide cardiac regeneration. In another embodiment, the combination of TGF-β3-loaded alginate microspheres and human MSCs in HA hydrogels, aimed at cartilage regeneration showed improved cartilage-*like* matrix deposition [92]. However, significant calcification was observed after 8 weeks of subcutaneous implantation. The requirement for the coupling of a parathyroid hormone-related protein to reduce calcification was demonstrated, which seems to further reinforce the need for a more complex signaling.

Although the sequential release of growth factors taking advantage of different release kinetics has proven beneficial it is still weakly explored in tissue regeneration. A dual release, with different kinetics, of vascular endothelial growth factor (VEGF) and monocyte chemotatic protein-1 from alginate microparticles embedded in a collagen/ fibronectin hydrogel was proposed in order to respectively improve the survival of transplanted ECs and to induce mural cell recruitment [94]. Increased functional vessel formation from transplanted ECs and a higher number of

smooth muscle cell-invested vessels after subcutaneous implantation confirmed the posed hypothesis. In a different approach neurotrophin-3 and PDGF were incorporated in a fibrin hydrogel containing an heparin-binding delivery system to increase mouse embryonic stem cell-derived neural progenitor cell (ESNPCs) survival and direct their differentiation Johnson and co-workers [95, 96]. This approach enhanced the total number of ESNPCs present in the spinal cord lesion 2 weeks after injury, as well as the number of ESNPC-derived neurons. Polyelectrolyte multilayer (PEM) films consisting of poly(b-amino ester)/polyanion/ growth factor/polyanion were proposed to deliver recombinant human BMP-2 and recombinant human $VEGF_{165}$, incorporated in amounts linearly proportional to the number of layers [97]. The BMP-2/$VEGF_{165}$ PEM films were shown, in comparison to BMP-2-loaded films, to increase in 33% the mineral density of ectopic bone formed *de novo* with a higher trabecular thickness. Additionally, bone formed throughout the scaffold when both growth factors were added, which suggesting more complete remodeling owing to an increased local vascular network.

2.3. Delivery of Genes to Modulate Cellular Behavior

Gene therapy strategies attempt to control cellular behavior through an ‘‘inside-out’’ approach by directly delivering nucleic acid molecules (i.e. DNA, siRNA, miRNA, and antisense oligonucleotides) into cells to trigger or stall gene expression [98, 99]. Thus, these nucleic acids can be considered a “super pharmaceutical” due to its potential to alter cell function for an extended period of time in relation to more established therapeutic agents [100]. Under the tissue engineering context, the introduction of exogenous genes inside cells has shown the ability to control several cellular events, including apoptosis, proliferation, migration, differentiation, cell-cell and cell-matrix interactions, as well as the secretion of relevant growth factors and cytokines [101]. Likewise, the endogenous production of signaling molecules can be triggered upon the delivery of specific genes, and then maintained in a tailored manner for specific periods of time. On the other hand, oligomeric genetic material, such as siRNA or microRNA, has been used to control the target activity inhibiting undesirable mRNA expression and/or protein synthesis.

The advantages of using transfected cells to target tissue formation or maintenance/survival of transplanted cells have been confirmed in different reports. Stably transfected rat MSCs with BMP-2 and basic fibroblast growth factor (bFGF)-encoding plasmids carried in cationic liposomes, showed improved proliferation and osteogenic differentiation *in vitro* and promoted faster and more active ectopic bone formation with high capillary density *in vivo* [102]. PLGA nanoparticles loaded with proangiogenic microRNA-132 were able to enhance growth factors-induced proliferation and migration of transfected human umbilical vein endothelial cells (HUVECs) that resulted in increased number of microvessels upon transplantation into immunodeficient mice [103], thus comprising a safe and efficient strategy to improve EC transplantation and vascularization.

From a different perspective, cell-selectivity and vector localization has been addressed by incorporating genetic material within engineered scaffolds to transiently express encoded proteins from infiltrating cells, the gene activated matrix (GAM) technology [104]. In general, GAM systems comprise DNA condensation with cationic transfection reagents such as polycations and liposomes, followed by DNA polyplex encapsulation into or onto the scaffolds, which upon implantation is released from the matrix to transfect the host cells and thereby trigger the desired effect [105, 106]. Acting as a localized storage of genes, GAM systems can maintain an elevated concentration of DNA within the cellular microenvironment and achieve localized and sustained transgene expression. Recently, poly (caprolactone-co-ethylene) nanofibers containing complexes of siRNA implanted in rats were shown to lead to thinner fibrous capsule formation than plain nanofibers [107]. Similarly, trimetylchitosan (TMC)/TGF-β-siRNA complexes were doped onto collagen-chitosan/silicone bilayer membranes seeded with human dermal fibroblasts forming a dermal equivalent [108]. Cells were able to internalize the TMC/TGF-β-siRNA and to induce a constant inhibition of TGF-β1 expression *in vitro* and *in vivo*, downregulating the levels of scar-related factors during the healing of pig full-thickness skin wounds. In addition to preventing/suppressing some undesirable signaling, GAMs have also been proposed to promote tissue formation. Pre-osteoblasts encapsulated in alginate hydrogel containing nanoparticles of calcium phosphate–DNA encoding for BMP-2 exhibited improved capacity to ectopically form bone-*like* tissue in mice [109]. Likewise, Runx2 or Osterix-transfected ASCs-PLGA hybrid scaffolds promoted ectopic bone formation in nude mice after 6 weeks of *in vivo* implantation [110].

3. TISSUE ENGINEERING IN PHARMACEUTICAL BIOTECHNOLOGY: FROM 2D TO 3D *IN VITRO* MODELS

3.1. Drug Screening in Two Dimensions

Pharmaceutical industry has continuously strived to find new, safer and more efficacious drugs for unmet clinical needs, while also meeting commercial objectives. According to the US Food and Drug Administration, it takes, on average, 12-15 years for an experimental drug to progress from bench to market [111]. The expected cost of developing a drug is estimated at $2.6 billion per new molecular entity that enters in clinical trials [112], which reflects high failure rate.

In general, the process of generating new drugs comprehends two stages: drug discovery and drug development. Drug discovery typically investigates interactions between a lead biomolecule and a target [113], whilst the development stage includes clinical trials, manufacturing and product lifecycle management [114]. A lead biomolecule is obtained after early *in vitro* screening of toxicity and efficacy of many similar compounds using specific cell lines, and further evaluated for efficacy and safety in animal studies.

Although recognized as highly reproducible and reliable conditions, *in vitro* screening using standard two-dimensional (2D) culture platforms do not assist high-throughput (HT) analysis, which significantly delays and adds significant costs to the all process. Miniaturized cell-based assays with HT capability have been gradually integrated as part of drug

discovery at the earlier stage of screening [115]. Examples of such systems include a multilayer elastomeric microfluidic cell-based HT platform that enables the simultaneous toxicity evaluation of different molecules at different concentrations using various cell types, namely murine embryonic fibroblasts, HeLa cells and bovine ECs [116]. An improved platform combining a microfluidic endothelial-*like* barrier with mass transport properties resembling the liver acinus and primary human hepatocytes was used to assess the hepatoxicity of diclofenac [117]. Recently, the simultaneous toxicity screening and drug absorption evaluation was validated, using methotrexate as a model drug, in an integrated system with two functional parts composed by liver hepatocellular carcinoma and human epithelial colorectal adenocarcinoma cell lines [118].

The need to study different cell interactions to address the biological complexity behind cell-cell, cell-ECM signaling and tissue morphogenesis and regeneration, have also led tissue engineers to focus on miniaturized HT screening (HTS) systems. Some of these platforms are also explored to predict drugs toxicity and effectiveness [119]. The toxic effect of many drugs in a target tissue often depends on its metabolization by another tissue, in particular the liver. In such situations, the independent tests of a drug on two cell lines would not necessarily reveal any toxicity. The development of a microsystem with interconnected channels and chambers was proposed to address this limitation, each of which containing different cell types representative of a particular tissue [120]. This on-chip system was used to evaluate the toxicity of naphthalene in an integrated system comprising liver, lung and fat cell lines contained in each chamber in order to assess their interplay. A similar approach was followed by Mahler and co-workers, to evaluate the transport of acetaminophen by liver, kidney, fat, and bone marrow cells independently grown in five communicating compartments allowing a more accurate prediction of the toxicity [121]. Analogous designs were further used for screening and optimizing combinatorial drug treatments for cancer therapy [122, 123]. The platform comprises a multidrug resistant (MDR) variant of uterine cancer cell line plus three others in different chambers representing the liver, bone marrow and uterine cancer allowed predicting the combination of drugs that may be advantageous *in vivo* by specifically targeting MDR cancer with acceptable side-effects [122]. Likewise, a device with 64 individually addressable cell culture chambers in which cells can be cultured and exposed either sequentially or simultaneously to 64 pairwise concentration combinations of two drugs allowed detecting the synergy between different sensitizer drugs and apoptotic agents used in cancer treatments [123].

Stem cells are a major element in tissue engineering and pivotal players not only in tissue regeneration but also in many pathological events. Thus, a 3120 minicompartmented chambers, recently proposed to monitor mesenchymal cell migration, represents an outstanding tool to screen anti-metastatic drugs that specifically inhibit mesenchymal migration [124]. So far, nine small-molecule compounds known for their ability to inhibit specific chemokines, growth factors, and kinases related to breast cancer metastasis were screened. Moreover, an HTS device with an array of chronic myeloid leukemia stem/progenitor cells was proved suitable to study chemo-resistance mechanisms against drugs such as dasatinib [125].

3.2. Engineering Three-dimensional Models for Drug Screening

Cells are accustomed to a 3D network environment of cell-cell and cell-ECM communication. These cellular settings are lost in monolayer cultures, and create a gap between *in vitro* cell-based assays and *in vivo* animal studies during drug development [126, 127]. Culture of tissue explants arises as a method to reduce this gap, exhibiting the appropriate cellular phenotype and 3D network environment of the pretended modeled tissue. For now, it seems that these 3D *ex vivo* models might be the most reliable approach, however drawbacks such as availability, poor tissue viability, and inconsistency due to high variability are present [128].

Cellular spheroids, essentially cell aggregates that can be manufactured as standardized "living materials", constitute the firstly developed 3D structures that better mimic natural cell arrangement within a tissue. From the tissue engineers/cancer biologists point of view, interested in studying specific cellular interactions, spheroids faithfully mimic the tumor microenvironment; after reaching a critical diameter, they are composed by an outer proliferating zone, an inner quiescent zone, and a central necrotic core. From the drug development perspective, spheroids recreate the mass transport limitations that an active pharmaceutical ingredient and carrier system are likely to encounter *in vivo* [129]. Thus, these generated 3D models have been further explored as a tool for drug development. A tumor spheroid model assembled as a HT platform composed by breast carcinoma cells allowed to screen a focused library of marine natural products and the identification of four drug candidates to target metastatic breast cancer [130]. In another approach, monkey kidney fibroblasts, murine embryonic stem cells, and human epithelial carcinoma cells spheroids were assembled into a HT system to screen two drugs with different modes of action, the anti-proliferative 5-fluorouracil and the hypoxia activated drug tirapazamine [131]. 3D spheroids-based HTS systems have been also applied to define appropriate chemotherapy schemes as effective drug sensitivity test platform to support individualized treatments. A 3D microfluidic HTS platform, which enable the generation of gradient concentrations of any drug, and comprising spheroids formed by lung cancer and stromal cell lines allowed to accurately screen appropriate-dose, single and combined-drug chemotherapy schemes for eight patients [132]. In a step further, a microfluidic device containing heterotypic spheroids of HUVECs and hepatocellular carcinoma cells was developed aiming at closely mimic vascularized liver as a way to improve the reliability of the screening of chemotherapeutic drugs [133].

A major barrier in the use of 3D spheroid models for HT drug screening has been the standardization of the structures and the reproducibility of the methodologies used to create the spheroids [134, 135]. Gelatin microparticles were introduced as artificial supports for the formation of 3D cellular aggregates in a sophisticated multi-channel 3D microfluidic system that attempted to represent multiple organs [136] in a similar concept to the use of spheroids for drug screening [132]. However, apart from this system, no further

developments have been reported. Tissue engineers have contributed to the evolution of constructs from simple and random 3D cell-containing supports to tissue-*like* structures that can be used as a valuable tool in the field of drug discovery and pharmaceutical research. Tough, so far, the miniaturization of those tissue analogues has been hampering their use and the efforts have moved toward the development of physiologically relevant 3D microenvironments without much thought to tissues architectural features [137–140]. Hydrogel-based systems have been adopted mainly due to facilitated free diffusion of oxygen, nutrients and growth factors, characteristic of native ECM. A 3D microfluidic HT system based on human neural stem cell encapsulated within alginate hydrogels was validated, using a control drug (acetaminophen), for assessing the toxicity of neurotoxicants (cadmium chloride, retinoic acid and dexamethasone) and an antiproliferative anti-cancer agent (5-fluorouracil) [141]. Mouse embryonic stem cells derived cardiomyocytes-based embedded within collagen/fibrin hydrogel were also assembled into a HT platform aiming to assess the effect of different compounds over cell contraction and beating frequency [142]. Cell pharmacological responses were evaluated using digoxin and isoproterenol, which showed typical toxicity in the case of digoxin and undesired inotropical reactions when the cells were exposed to isoproterenol.

4. CONCLUDING REMARKS AND FUTURE DIRECTIONS

When the human body fails to regenerate, tissue engineers can stand up and define the most suitable strategy by combining its main players to restore and/or improve tissue functions. The need for a suitable 3D structure that is bioinstructive is mandatory. So far, the mimicking of the natural ECM is seen as the most promising approach to achieve this requirement. However, both materials and processing methodologies limitations have constrained the attainment of the native ECM biochemical, mechanical and morphological/architectural features as a whole. The combination of biotechnology and materials science within genetic engineering has driven the improvement of biotechnology-derived biomaterials. By enhancing the expression of biological components in nature and further modify or bioengineer them, holds the potential to develop multifunctional biomaterial systems, including the generation of new protein sequences, new self-assembling peptides or fusions of different bioactive domains or protein motifs.

So far tissue engineers have not learned to mimic complex architectures, as vascular networks, which are essential for natural tissue function. Advances on methods to produce artificial structures, such as 3D printing have led to evolve from a simple supporting scaffold to a more complex and native tissue-*like* structure. Furthermore, the combination of smart materials that possess time-sensitive mechanisms may provide paths forward, by mimicking endogenous biomechanical a biochemical stimulus to encrypt biomolecular signals that govern regeneration.

Despite those promises, the lack of biological cues to effectively regulate cellular behavior and consequently tissue regeneration is often an issue. Signaling molecules, secreted either by exogenous or endogenous cells are critical to modulate the healing environment and consequently the outcome of the strategy. The discovery of naturally occurring molecules and respective mechanisms of action can further help in the reproduction, by tissue engineers, of tissue formation. Developments on recombinant technology have spawned the area of biotechnology of growth and differentiation factors. The administration of multiple growth factors in a timely mode has been recognized as requirement to maximize their potential towards tissue regeneration. Nonetheless, the ideal combination and/or gradients that better guide tissue regeneration is still to be unraveled. Moreover, key challenges associated with short half-lives, denaturation prior release, displacement and rapid body-clearance are yet recognized. A biotechnological approach using fusion proteins that preserve the active domains of the growth factors to stimulate regenerative cells, as well as containing matrix-specific binding motifs to promote cell-ECM-*like* interactions, offers an exciting addition to the current arsenal of growth factor-based therapies for tissue regeneration.

From a different perspective, the combination of gene therapy and tissue engineering strategies by directly deliver nucleic acid molecules that from inside the cells control *in situ* the cellular microenvironment also constitutes a promising approach to lead tissue regeneration. To date, the majority of these approaches has taken advantage of particulate systems to intracellularly deliver the genetic material of interest as an attempt to tackle some of the limitations commonly associated to the delivery of other molecules. Interestingly, the coupling of the genetic material with tissue-engineered matrices - GAMs - has been showing superior results for different tissue engineering applications. Whether the upregulation of the expression of a specific gene expression or the modulation of a particular signaling pathway through interfering molecules should be considered when designing gene therapy strategies certainly depends on the pathophysiology of the tissue to be regenerated.

Understanding and control all these components would provide tissue engineers the capability to overcome clinical challenges as well as to develop technologies for high fidelity tissue models to serve as drug screening platforms. The miniaturization of cell-based assays promises to have a profound impact on HTS of signaling molecules by minimizing the consumption of reagents and cells. Besides, HTS have evolved from simple cell on-chip to integrated systems that better predict the drug effectiveness and toxicity towards surrounding tissues, being largely catalyzed by the advances observed in tissue engineering. To achieve routine adoption of HT cellular microscale platforms, future directions will most certainly be focused on HT methods for the study of cellular microenvironments and growth conditions in 3D tissue-*like* environments. Improved cellular microarrays have been attained by embedding sensing elements alongside the cell culture for local detection of secreted cellular products. Rapid prototyping technologies that have shown promise outcomes in reproducing tissue-engineered constructs with native-structural fidelity are also expected to improve existent HTS, mainly to surpass the miniaturization problem.

Personalized drug screening has also gained more attention as the future of drug discovery, due to the well-known differences between individual patients. In this sense, more accurate evaluation is expected to enhance drug efficacy with reduced toxicity by simply developing patient on-chip. Accordingly, the developments of these organs-on-chips technologies aspire towards a major breakthrough in personalized drug screening.

LIST OF ABBREVIATIONS

ASCs	=	Adipose Stem Cells
bFGF	=	Basic Fibroblast Growth Factor
BM-MSCs	=	Bone Marrow-derived Mesenchymal Stem Cells
BMP	=	Bone Morphogenetic Protein
2D	=	Two-Dimensional
3D	=	Three-Dimensional
ECM	=	Extracellular Matrix
ECs	=	Endothelial Cells
ESNPCs	=	Embryonic Stem Cell-Derived Neural Progenitor Cells
GAM	=	Gene Activated Matrix
GG	=	Gellan Gum
GG-HA	=	Gellan Gum/Hyaluronic Acid
hKC	=	Human Keratinocytes
HT	=	High-Throughput
HTS	=	High-Throughput Screening
HUVECs	=	Human Umbilical Vein Endothelial Cells
MDR	=	Multidrug Resistant
PDGF	=	Platelet-Derived Growth Factor
PEM	=	Polyelectrolyte Multilayer
PHAs	=	Polyhydroxyalkanoates
PHB	=	Polyhydroxybutyrate
PHBV	=	Poly-3-Hydroxybutyrate-co-3-Hydroxyvalerate
PLGA	=	Poly(Lactic-co-Glycolic Acid)
rhCII	=	Human Recombinant Type II Collagen
SCs	=	Schwann Cells
SELPs	=	Silk-Elastin Like Proteins
TGF-β3	=	Transforming Growth Factor Beta 3
TMC	=	Trimetylchitosan
VEGF	=	Vascular Endothelial Growth Factor

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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