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Strategies to develop endogenous stem cell recruiting bioactive materials for tissue repair and regeneration

Settimio Pacelli[a,1], **Sayantani Basu**[a,1], **Jonathan Whitlow**[a], **Aparna R. Chakravarti**[a], **Francisca Acosta**[a], **Arushi Varshney**[b], **Saman Modaresi**[a], **Cory Berkland**[a,c], and **Arghya Paul**[a]

[a]Department of Chemical and Petroleum Engineering, Bioengineering Graduate Program, University of Kansas, Lawrence, KS, USA

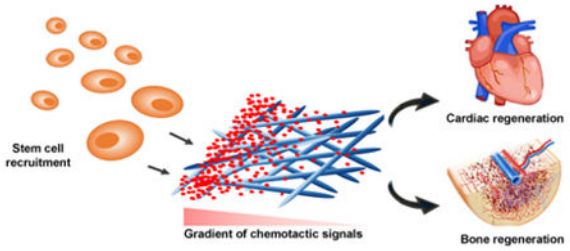
[b]Department of Human Genetics, University of Michigan, Ann Arbor, MI, USA

[c]Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS, USA

Abstract

A leading strategy in tissue engineering is the design of biomimetic scaffolds that stimulate the body's repair mechanisms through the recruitment of endogenous stem cells to sites of injury. Approaches that employ the use of chemoattractant gradients to guide tissue regeneration without external cell sources are favored over traditional cell-based therapies that have limited potential for clinical translation. Following this concept, bioactive scaffolds can be engineered to provide a temporally and spatially controlled release of biological cues, with the possibility to mimic the complex signaling patterns of endogenous tissue regeneration. Another effective way to regulate stem cell activity is to leverage the inherent chemotactic properties of extracellular matrix (ECM)-based materials to build versatile cell-instructive platforms. This review introduces the concept of endogenous stem cell recruitment, and provides a comprehensive overview of the strategies available to achieve effective cardiovascular and bone tissue regeneration.

Graphical abstract



Corresponding author: Arghya Paul (arghyapaul@ku.edu), Department of Chemical and Petroleum Engineering, University of Kansas, 1530 W. 15th Street, Lawrence, KS 66045 Tel. 785-864-6544.

[1]These authors contributed equally to the work.

Keywords

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Introduction

Stem cell-based therapies hold great promise in regenerative medicine for the treatment of a variety of diseases in which the body's ability to repair tissue is hampered or irreversibly compromised [1]. To name a few, myocardial infarction, osteoarthritis or bone fractures, and nonunions represent such medical conditions. In these pathological cases, stem cells treatment can potentially restore the lost tissue function [2-4]. The basic rationale behind the therapeutic use of stem cells in the clinic relies on their ability to differentiate and maintain homeostasis in healthy tissues. This vital role is guaranteed by the presence of stem cells in many adult organs and tissues such as the bone marrow, which represents their main reservoir in the body [5]. These cell niches provide a source of quiescent stem cells that can be activated as a result of normal tissue homeostasis or as a reparative response to pathological conditions [6]. Specifically, after injury or ischemia, a cascade of events is generally observed, including stem cell migration into the site of inflammation, differentiation into the desired cell type, and secretion of various biological factors such as chemokines and growth factors to promote tissue repair [6, 7].

However, this endogenous healing process is not always capable of restoring normal tissue homeostasis especially when the damage is too extensive and irreversible. In this scenario, a possible solution is to administer *ex vivo* cultured stem cells at the site of injury for their reconstructive action.

Stem cell can be harvested from different sources including bone marrow, adipose tissue, umbilical cord, lung, and muscle. These tissue-specific stem cells are able to regenerate the tissue from which they are isolated and they do not have the ability to "trans-differentiate" outside their lineage as suggested by recent studies [8]. In addition, it is becoming evident how their origin is not embryonic, and although they present similar surface markers, they cannot be classified as a unique cell line. For these reasons, the term mesenchymal stem cells (MSCs) is not adequate and should be avoided when referring to tissue-specific stem cells [9].

The isolated stem cells can be expanded *in vitro* to reach clinically relevant cell number, and locally administered alone or in combination with natural or synthetic scaffolds [10, 11]. However, to achieve significant functional benefits, the strategy requires a defined selection of several variables including the number and type of stem cells delivered, and the time of administration. All of these parameters have a profound effect on the final clinical outcome, which can vary according to the type of disease that needs to be treated. Moreover, this tissue engineering method involves invasive donor biopsies, labor-intensive, time-consuming and costly cell culture steps, which can also adversely affect stem cell behavior and phenotype [12, 13]. Finally, another risk is the possible malignant transformation of *in vitro* cultured stem cell commonly used for clinical cell-based therapies [14]. Overall, the

technical issues, logistics, and safety concerns have posed impediments in the successful clinical translation of stem cell treatments. In fact, although many studies have confirmed their therapeutic effects in animal models, the majority of clinical trials are now in Phase I and II and only a very few have reached Phase III [15, 16]. For these reasons, a simpler approach is to use the body's own resources, by augmenting the healing and remodeling mechanisms of endogenous stem cells. Developing *in situ* strategies towards this end requires a better understanding of the underlying biology for stem cell recruitment. This can be supported by the design of novel bioactive materials to bolster stem cell survival, signaling, and function at the target site [17-22]

The process of cell recruitment can be controlled using a variety of biological tools, such as cell-adhesive peptides, antibodies, aptamers, genes or biocompatible nanoactive materials or by engineering selective chemoattractant gradients of growth factors [23-27]. These biomolecules can be chemically or physically conjugated to a scaffold and delivered to an injured site in order to promote stem cell migration. In addition to designing biomimetic scaffolds with synthetic materials, naturally derived ECM, which is rich in chemokines and other bioactive molecules, presents an alternative solution for creating acellular scaffolds that actively recruit stem cells.

In the first part of this review, we will focus on strategies for host stem cell recruitment and provide a comprehensive overview of the different techniques and bioactive materials used to achieve this process. Additionally, approaches to engineer chemoattractant materials will be discussed. These include surface modification of scaffolds, sustained delivery of entrapped growth factors from hydrogels and the use of decellularized ECM-based scaffolds. In the second part of the review, we will define the role of stem cell recruitment in cardiac and bone tissue engineering. Specifically, the emerging trends in the cardiovascular field will be highlighted with particular attention to techniques that aim to promote endothelialization of stents and vascular grafts. Moreover, strategies for myocardial regeneration using ECM-based scaffolds will also be examined with the goal of defining their potential as stem cell recruiting agents. Finally, we will conclude with an overview of several emerging approaches for stem cell recruitment to repair and regenerate osteochondral defects.

2. Strategies to achieve stem and progenitor cell recruitment

Cell homing can be defined as the migration of stem cells into a target location as a response to multiple biological signals including soluble chemokines, growth factors, cytokines and cell-adhesive molecules. This process is fundamental in the regulation of organogenesis and is essential for maintaining homeostasis in healthy tissues [28] . Adult stem cells are dynamic entities that can be recruited and differentiated into almost any type of tissue. After the migration of stem cells through the bloodstream to a target organ, their successful integration occurs following a cascade of events involving the close interaction between flowing cells and the vascular endothelium. Specifically, the first step in this cascade is defined as rolling, where the stem cells establish low-affinity interactions through transmembrane receptors with endothelial cells. This phase is fundamental for the capture of fast-flowing stem cells within the vascular bed of tissues. Subsequently, stem cells adhere and transmigrate through a layer of endothelial cells and into the targeted tissue [29]. In

addition, stem cells can migrate from the tissue niches in the surrounding areas through interstitial migration induced by chemokines and other chemoattractant factors without any involvement of the vascular system [30, 31] . Mimicking these complex signaling events is then a crucial step for the design of novel tissue engineering strategies that can enhance the recruitment of endogenous stem and progenitor cells into damaged tissues. To achieve this goal, a scaffold or implant must be engineered by tuning its physical and biological properties to generate a suitable microenvironment that can control stem cell behavior in terms of adhesion, proliferation, and differentiation into the desired lineage. Following this concept, many strategies have been proposed to control host stem cell recruitment, and we will divide this broad subject into three main areas of interest: (i) integration of cell-adhesive molecules or antibodies to ensure selective stem cell adhesion, (ii) design of carriers for growth factor delivery to attract stem cells *in vitro* and *in vivo*, (iii) use of decellularized ECM-based scaffolds to instruct stem cell migration and tissue repair *in vivo*.

2.1 Modulation of surface adhesion properties for efficient stem cell recruitment

In this section, an overview of the strategies for promoting adhesion of stem cells on the surface of scaffolds or biomedical implants will be examined. Specifically, the discussion is subdivided according to the type of biological molecules selected for stem cell homing and the approaches for their efficient binding.

2.1.1. Cell-adhesive proteins and peptides—Regulating cellular adhesion is crucial for the successful control of stem cell recruitment. This challenge can be addressed by modulating the surface properties of implants and scaffolds with cell-signaling biomolecules that can serve as a biological interface between the implant and the surrounding cells [32] . To this end, the design requisites for stem-cell adhesive biomaterials include the proper selection of the adhesive signal as well as a suitable linking strategy that preserves the biochemical functionality of the selected signaling molecule. Among the variety of biological chemoattractants, proteins that are abundant in native ECM represent a popular choice to enhance the cell-adhesive properties of biomaterial surfaces [33] . Fibronectin and vitronectin are thoroughly researched proteins that promote cell attachment due to the presence of the ‘RGD’ (Arg-Gly-Asp) amino acid sequence, which is selectively recognized by cell transmembrane receptors [34]. These integrins are heterodimeric proteins composed of α and β subunits that contain ligand-specific domains. For instance, integrins αvβ1 and αvβ3 are both receptors for fibronectin, but αvβ3 has a lower specificity and can be bound with several other ECM proteins including vitronectin and fibrinogen. Aside from RGD, researchers have discovered and isolated some other integrin-activating domains that function cooperatively to elicit an amplified integrin activation response [35]. This network among functional domains vastly increases the magnitude of the chemotactic response in comparison with that of single peptide domains. For example, fibronectin presents the ‘PHSRN’ amino acid sequence, which is activated alongside with the RGD domain generating an enhanced signaling response [36]. Despite the advantages of ECM proteins, several drawbacks have limited their clinical use including the risk of pathogen transfer, adverse immune response to cadaveric or xenogenic proteins, and a loss of biological activity due to conformational changes resulting from chemical conjugation to biomaterials. Adhesive peptides, on the other hand, which can be easily linked with scaffolds without any

reductions in bioactivity, currently represent a much more favorable option over ECM proteins for controlling stem cell adhesion with scaffolds. Furthermore, as opposed to laborious recombinant protein production processes, peptides can be synthesized in bulk in a short time, which is favorable for their clinical translation.

The RGD sequence is one of the most widely investigated adhesive peptides, and furthermore studies have shown that the spatial orientation of the RGD peptide within a scaffold can have a profound effect on the extent of stem cell adhesion. Specifically, strong evidence suggests that peptide density and the degree of spacing between clusters of RGD peptides can dictate integrin receptor activation and influence focal adhesion of stem cells [37] (Figure 1A). The conformational structure of this peptide can also affect its biological activity. The synthetic cyclic forms of RGD (cRGD) generally present an increased stability compared to linear RGD sequences associated with a higher affinity towards the specific integrin isoform αvβ3 [38] . cRGD can be chemically conjugated within the structure of synthetic scaffolds and the specific conformation of the peptide has a direct role in modulating stem cell adhesion and differentiation [39] (Figure 1B). Furthermore, to elicit an enhanced response of stem cell adhesion, the RGD sequence can be combined with other motifs, such as the YIGSR sequence or the triple-helical GFOGER peptide, which bind to laminin receptors or collagen integrins, respectively [40-42]. This combination of signals can be a practical strategy to mimic the complex extracellular microenvironment that regulates stem cell behavior. Aside from selecting specific amino acid domains that have been discovered in natural ECM proteins, synthetic peptides with cell-mediating functions have also been identified through phage display technology [43] . Phage display is a powerful tool that enables the high throughput identification of peptides, activate specific receptors that regulate cell adhesion, differentiation as well as those that bind to specific cell types. This technology allows the discovery of new adhesive peptides that can show increased selectivity towards specific stem cell populations. While the RGD peptide has a high cell-binding affinity, it lacks the ability to bind to only one type of stem cell, which is the main drive for the discovery of novel peptide sequences. Following this concept, Shao *et al.* utilized phage display to identify the cell-adhesive peptide E7, which possesses a high affinity for human bone marrow-derived mesenchymal stem cells [44]. The presence of the peptide increased the efficiency of stem cell recruitment into polycaprolactone (PCL) meshes both *in vitro* and *in vivo* in a rat model in contrast to RGD peptide (Figure 1C-E). Similarly, the E7 peptide was recently combined with a collagen-binding domain and was able to enhance mesenchymal stem cell adhesion and infiltration in the collagen scaffolds *in vivo* in a porcine model [45] .

Once the suitable adhesive molecule has been selected, it is also important to choose the appropriate method for immobilizing the molecule on the cell-scaffold interface. The binding efficiency is determined by the surface properties of the scaffold and can vary based on the material's hydrophilicity, hydrophobicity, and the presence of reactive functional groups. Two main possibilities are available for linking peptides and proteins including physical and chemical strategies. Physical linking involves the reversible physisorption of a peptide or protein to a selected surface by hydrogen bonds, electrostatic forces, and hydrophobic interactions. Physical linking techniques are advantageous in that they can be applied to a large variety of both natural and synthetic materials and they do not require

treatment of the surface prior to linking. However, the binding stability of an adsorbed peptide or protein can be dramatically reduced by fluctuations in environmental conditions including pH, ionic strength, and temperature [46] . To overcome this issue, chemical conjugation is generally favored to ensure increased stability after peptide or protein linking with less risk of detachment. To incorporate peptides or proteins into unreactive materials, UV irradiation, plasma treatment, or chemical modification has to be performed to introduce reactive sites to the material surface [47]. Plasma treatment utilizes high energy irradiation to modify the surface of a material using plasma from a gas phase mixture [48]. This method is more efficient than UV irradiation and allows surface modification without altering the bulk properties of the material.

Once the desired functional group is introduced, peptides or proteins can be linked using a variety of chemical crosslinkers. Among them, ethyl carbodiimide and N-hydroxysuccinimide (EDC/NHS) chemistry is a popular choice as the waste products of the reaction are water-soluble and can be easily washed after the process is complete. Alternatively, physical and chemical strategies can be combined by creating reactive coatings on material surfaces to which biomolecules can be physically and reversibly linked. This strategy is demonstrated in the example of coating material surfaces with a layer of polydopamine (pDA) that can be formed by exposing the surface of synthetic or natural scaffolds to slightly alkaline condition (pH 8.5). These pDA coatings allow both the physical adsorption and the chemical conjugation of peptides or proteins through Michael addition with thiol groups (-SH) or base Schiff reactions with amine ($-NH_2$) groups [49]. This strategy enables the efficient and reproducible adsorption of large quantities of signaling molecules without altering their bioactivity.

2.1.2. Antibodies—In addition to cell adhesive peptides or proteins, antibodies can be utilized in surface coatings to bind with antigens displayed by targeted cells [50]. Antibody coating of biomaterial surfaces has emerged as a promising strategy for promoting recruitment of stem cells to scaffolds or biomedical implants [51, 52]. Antibodies can be immobilized onto the surfaces of scaffolds by chemical or physical interactions and the choice of a particular approach depends on the original properties of the material. For instance, naturally derived scaffolds composed of collagen and hyaluronic acid inherently possess reactive groups that enable the covalent attachment of antibodies [53]. Shi *et al.* investigated this concept by covalently conjugating the anti-stem cell antigen-1 (Sca-1) monoclonal antibody on the surface of collagen scaffolds [54]. This antibody was selected since Sca-1 protein is the most common surface marker expressed in hematopoietic, cardiac, and skeletal muscle stem cells [55]. The functionalized collagen scaffolds demonstrated efficient recruitment of autologous Sca-1 positive progenitor cells by antibody-mediated capture, which induced cardiomyocyte regeneration *in vivo* in a mouse model.

In contrast to naturally derived scaffolds, synthetic constructs often do not inherently possess reactive surface groups that enable direct attachment of antibodies. A possible route to enhance their reactivity is the introduction of an intermediate polymer layer to enable subsequent anchorage of antibodies. For example, titanium surfaces can be modified with carboxyl-terminated polyethyleneglycol (PEG) to chemically graft anti-CD34 antibody resulting in selective adhesion of endothelial progenitor stem cells (EPCs) (Figure 2A) [56].

In addition to PEG, other classes of biomolecules such as hydrophobins can be used as reactive intermediates to link unreactive surfaces with antibodies. Hydrophobins are a subset of cysteine-rich peptides that can form a hydrophobic coating capable of immobilizing antibodies [57]. Recently, hydrophobins were used to coat the surface of polycaprolactone (PCL) scaffolds to enable the adsorption of anti-CD31 to the surface, leading to increased adhesion and retention of endothelial cells [58]. Aside from hydrophobic interactions, the intermediate layer can also establish electrostatic forces as in the case of charged proteins.

Heparin is generally used to form polyelectrolyte multilayers on the surface of chitosan [59] and collagen [60] scaffolds for the immobilization of anti-CD34 and anti-CD133 respectively.

One common problem with all the aforementioned strategies is the random orientation of the antigen binding sites and the resulting steric hindrance which causes a loss in cell binding activity [61, 62]. A possible solution is to link the antibodies in a particular orientation, specifically with their fragment antigen-binding (Fab) sites distal from the surface of a scaffold [61, 63]. This goal can be achieved using several bacterial proteins (e.g., protein G and protein A) as intermediate linkers to the fragment crystallizable (Fc) region of the antibody [64, 65]. In a study by Markway *et al.*, protein G was used to precisely orient the Fab domain of the anti-kinase insert domain receptor (KDR) antibody distal from the surface of the scaffold [66]. The antibody was selected as KDR can be found on the membranes of both EPCs and endothelial cells [67]. The presence of the protein G increased the capture selectivity of KDR-positive cells compared to the groups where the antibody was randomly attached because of the absence of the protein G (Figure 2B).

A similar approach was proposed by Li *et al.* where the biotinylated protein A was linked to avidin-modified titanium surfaces to spatially orient the anti-CD34 antibody with its Fab regions distal from the surface of the implant [68]. The desired antibody orientation was possible due to the presence of four Fc binding regions, which are inherently present in the structure of protein A. The introduction of protein A enhanced the attachment of EPCs on the coated titanium surfaces inducing rapid and complete endothelialization of the implant surface *in vivo* in canine femoral arteries.

An alternative idea for spatially controlling the orientation of linked antibodies is the oxidation of the Fc domains using periodate to introduce reactive aldehyde groups before chemical conjugation. For instance, this periodate-oxidation strategy has been used to immobilize anti-CD34 antibodies on the surface of stainless steel implants modified with amine groups that can establish imine bonds with the oxidized Fc domains [69]. The process did not alter the immunoactivity of the antibody and the achieved spatial orientation enabled high cell capturing efficiency.

Finally, a more sophisticated material-based approach to control antibody immobilization for the recruitment of particular cell populations is by the photolithographic micropatterning of biomaterials [70]. Custodio *et al.* achieved successful attachment of endothelial cells by immobilizing antibodies to chitosan gels with micropatterning techniques [71]. Chitosan films were chemically modified with a UV photocleavable biotin derivative, which was

coupled with streptavidin and biotinylated CD31 antibody. Although the study was mainly focused on the attachment of endothelial cells on the micropatterned films, the same strategy could be extended to promote adhesion of EPCs using the same antibody (Figure 2C).

2.1.3 Aptamers—Aptamers are synthetic, single-stranded oligonucleotides which can act as targeting ligands for a variety of cells [72]. Immobilization of aptamers on the surface of scaffolds and implants represents a valid approach with numerous advantages such as low cost, low immunogenicity, and high affinity to the target cell when compared to other classes of ligands including antibodies and peptides [73, 74]. To this date, aptamers have been primarily used for identifying and capturing cancer cells [75-77], but they can also be applied for targeted isolation and recruitment of stem cells [78, 79].

For instance, aptamers can be selected to isolate EPCs from the bloodstream and create autologous functional endothelium surfaces. To achieve this goal, several immobilization strategies have been designed. A common solution consists in the deposition of a polymeric layer which can establish covalent bonds with the aptamers. This approach has been used to chemically graft EPC-binding aptamers onto polytetrafluoroethylene and polydimethylsiloxane surfaces using a blood compatible star-PEG coating as an intermediate covalent binding layer [80]. Additionally, strong physical electrostatic interactions between the single strands of oligonucleotides and chemically modified surfaces is utilized for the immobilization of aptamers to create implants with cell-adhesive properties. Following this approach, Qi *et al* modified the surface of stainless steel substrates by deposition of plasma polymerized allylamine (PPAam), which enabled the electrostatic adsorption of single stranded DNA [81]. Specifically, the EPC binding aptamers were immobilized onto the PPAam film substrates through the electrostatic interaction between positively charged PPAam and negatively charged DNA.

Aside from isolating stem cells, another interesting application of aptamers is their use as a biological tracking device to monitor stem cell migration in a tissue [82]. This concept is particularly relevant to evaluate the stem cell's interactions with the extracellular environment and to discover new signaling pathways responsible for their recruitment. For instance, aptamers displaying fluorescent dyes can be covalently linked to the surface of stem cells to produce a fluorescent signal when exposed to chemoattractant signals. Based on this concept Zhao *et al.*, modified the surface of stem cells with a fluorescent aptamer capable of binding to platelet-derived growth factor (PDGF), which is a potent chemoattractant that directs cells to inflamed tissues [83]. The stem cells displaying the aptamers on their membranes were able to produce a fluorescent signal after binding to PDGF due to a change in the aptamers' conformation and they could be tracked in mouse bone marrow.

2.2 Delivery of growth factors for stem cell recruitment

A valid approach towards controlling stem cell recruitment is the design of growth factor releasing- platforms. Growth factors are soluble signaling proteins that control a wide variety of cellular responses through specific binding of transmembrane receptors on target cells [84]. For decades these proteins have been administered for a variety of treatments;

however, therapies involving growth factors are costly because large quantities are necessary to achieve biological responses due to their short half-lives. To lower the required loading dose while maintaining the therapeutic effect, growth factors may be loaded on carriers to guarantee stability against chemical and enzymatic degradation [85]. They can be chemically or physically entrapped into polymeric scaffolds to prevent their degradation and to prolong their therapeutic activity. Growth factor release can be modulated in response to physical changes in the local microenvironment including variation in pH, temperature, or enzymatic degradation. In addition, novel substrates can be designed to mimic the interaction between the growth factors and ECM proteins as an alternative strategy to enhance their biological activity. The next section focuses on existing growth factor release strategies aimed towards controlling stem cell recruitment.

2.2.1 Carrier design for growth factor delivery and stem cell recruitment—The design of a growth factor delivery platform requires a detailed study of the physical properties of the scaffold used as a carrier. Specifically, the porosity, swelling, and degradability can influence the release kinetics, and these parameters dictate the availability of growth factors in the surrounding target site. Degradation can be controlled by the presence of cleavable groups in the scaffold such as ester or carbonates that can undergo hydrolysis under physiological conditions. Moreover, the molecular weight, type of polymer, and modality of crosslinking are other variables that need to be considered for the fabrication of the desired carrier to modulate the degradation rate and obtain a sustained release of growth factor over time.

To demonstrate this concept, He *et al.* chemically conjugated stromal derived factor-1α (SDF-1α) into poly(lactide ethylene oxide fumarate) (PLEOF) hydrogels for sustained release of SDF-1α in a manner that matched the human body's proliferative healing phase for recruiting bone marrow stromal cells (BMSCs) [86]. SDF-1α is one of the most thoroughly investigated chemoattractants for stem cell recruitment. The chemokine selectively binds to the surfaces of stem cells through CXCR4 receptors [87]. The PLEOF hydrogels were designed with different hydrolysis rates by varying the fraction of polylactic acid (PLA) in the scaffolds. The range of formulations resulted in either hydrophobic or hydrophilic gels displaying slow and fast degradation rates, respectively. Regardless of the formulation, the gels retained at least 30% of the loaded SDF-1α after three weeks as determined by *in vitro* cell studies. It was also demonstrated that the kinetic release profile of SDF-1α was tunable by adjusting the hydrogel properties, and the migration of BMSCs showed a direct correlation with the release kinetic of the chemokine. Similarly, another study showed efficient recruitment of BMSCs by the sustained release of SDF-1α from hyaluronic acid (HA) based hydrogels for the treatment of myocardial infarction. HA was modified with methacrylic groups, which were linked to the polymeric backbone using ester groups. The hydrolysis of these functionalities enabled the degradation of the gel and a sustained release of the growth factor for over one week in mice [88]. Other research groups have instead modified the physical properties of scaffolds by introducing charged groups in the polymeric backbone that can influence the water uptake and subsequently the scaffold degradability. Following this fundamental principle, Liang *et al.* synthesized poly(ethylene glycol) diacrylate (PEGDA)-poly(ethyleneimine) (PEI) hydrogels with controllable

degradation rates by varying the presence of protonated amine groups (Figure 3A) [89]. The injectable system delivered the granulocyte colony stimulate factor (GSCF) in a sustained manner both *in vitro* and *in vivo*. Moreover, a higher mobilization of EPCs was observed after a single intramuscular injection of the hydrogel in mice when compared to the bolus administration of GSCF for six days.

In addition to chemical hydrolysis, stem cell recruitment by sustained growth factor release can also be achieved by designing scaffolds that are enzymatically degradable. Based on this concept, Prokoph *et al.* designed PEG-heparin hydrogels presenting matrix metalloprotease (MMP)-cleavable peptides and used this carrier for the controlled release of SDF-1α [90]. The aim of the study was to generate a chemokine gradient to induce the local recruitment of early endothelial progenitor cells (eEPCs). The hydrogels allowed precise and long term delivery of SDF-1α, and the release profile of growth factor was primarily influenced by the enzymatic degradation of the scaffolds. Following the same concept, PEG-based hydrogels were fabricated introducing a cysteine flanked protease-degradable sequence as the crosslinker. The scaffolds were used for the dual release of both hepatocyte and vascular endothelial growth factors (HGF and VEGF) to the myocardium of rats (Figure 3B). It was observed that the scaffolds' degradation was strictly dependent on the amount of collagenase present. The corresponding release of the loaded growth factors caused an increase in angiogenic response *in vivo* as well as an increase in the recruitment of stem cells while reducing fibrosis in the myocardium of rats [91].

Another popular material-based solution used to control and sustain growth factor release is to fabricate polymeric matrices that can establish physical bonds with the growth factors. This design enables the efficient entrapment of growth factors within scaffolds without adversely affecting their biological activities, in contrast to chemical binding strategies that can hinder or reduce the bioactivity of bound biomolecules. Physical loading can be achieved using several techniques including solvent casting and particulate leaching, freeze-drying, phase separation, and gas foaming. For instance, Lee *et al.* evaluated the entrapment of SDF-1α using a coacervate between heparin and a synthetic polycation.. The resulting coacervate was then incorporated into a poly (glycerol sebacate) scaffold. The coacervate-laden scaffolds displayed sustained release of SDF-1α with minimal initial burst release and were effective in promoting recruitment of mesenchymal progenitor cells (Figure 3C). [92] In another study, SDF-1α was incorporated into polyelectrolyte multilayers film composed of chitosan and poly-(γ-glutamic acid) using a layer-by-layer synthesis technique. The system was able to act as a constant-activity reservoir of SDF-1α releasing the protein for five days, which promoted stem cell migration *in vitro* [93].

Aside from controlling the bulk properties of the carrier in terms of degradability and type of crosslinking, the design of the scaffold can be modified with impermeable layers to spatially control growth factor diffusion. This concept of oriented growth factor delivery is particularly important in applications that require recruitment of stem cell to a particular tissue or organ. For example, in cartilage defects, the migration and retention of a large population stem cells from the bone marrow into the defect site is a major key to promote successful tissue regeneration. To achieve this goal, Lee *et al.* designed a water-resistant catechol-conjugated chitosan (CHIeC)-adhesive gel patch [94]. The system was capable of

delivering platelet-derived growth factor-AA (PDGF-AA) with specificity to defects created in the bone marrow cavities of rats. The bioadhesive patch induced the release of growth factors specifically into the defect area, which directed the migration of stem cells in the marrow cavity, thus enhancing the regenerative effect of PDGF-AA (Figure 4).

Moreover, an alternative to polymer scaffolds is represented by self-assembled peptides that can found application as carriers of soluble factors and therapeutic biomolecules. Specifically, these systems are made of amphiphilic proteins, which present both hydrophilic and hydrophobic regions and are capable of self-assembling into stable β-sheets. After exposure to salt or changes in pH, self-assembled peptides can create flexible nanofibers with diameters ranging from 7 to 20 nm [95]. This type of carrier presents a high surface/volume ratio, which allows the adsorption of large amounts of growth factors. The release kinetics of the loaded biological molecule can be modulated by varying the concentration of the peptide or by covalently linking the growth factor at the N or C-terminus of the peptide prior to the process of self-assembly [96]. Following this strategy, it is possible to create chemoattractant gradients that induce recruitment of specific types of stem cells as reported by Segers *et al.* [97]. Specifically, SDF-1α was combined with a self-assembling peptide to form nanofibers, which were used to deliver the protein *in vivo* to treat myocardial infarction in a rat model. The sustained release of the protease-resistant form of SDF-1α enhanced recruitment of stem cells and improved the rats' cardiac function post-myocardial infarction.

Finally, aside from physical adsorption into self-assembly peptides, growth factors can also be chemically conjugated within ECM-based scaffolds [98]. This approach enables the possibility to design new systems that can improve the potency of growth factors due to the role of ECM proteins in modulating growth factor activity *in vivo*. Based on this concept Llopis-Hernandez *et al.*, developed a material ECM-based system to enhance BMP-2 activity [99]. Specifically, fibronectin was spontaneously adsorbed onto poly(ethyl acrylate) scaffolds in the fibrillary conformation which allowed the presentation of integrin binding sites for BMP-2. The conjugation of the growth factor on the fibronectin increased BMP-2 activity, which resulted in higher osteogenic differentiation of mesenchymal stem cells *in vitro* and full regeneration of bone defect *in vivo* with very low doses of BMP-2. This straightforward technology can be applied to unlock the full potential of growth factors and represent a valid alternative to classic strategies based mainly on their delivery.

2.3 Use of ECM-based scaffolds for stem cell recruitment

ECM-based materials from decellularized tissues hold great promise in the field of tissue engineering as a subset of naturally derived materials. ECM can induce the biological responses necessary to facilitate the spatiotemporally controlled recruitment and migration of stem cells. ECM is the acellular component of tissues which provides a suitable microenvironment for cells and offers the necessary cues for their proliferation, adhesion, differentiation, migration, and viability [100]. Additionally, acellular ECM materials retain the structure of their native tissue, which leaves these matrices with the chemical and mechanical cues that help to modulate stem cell behavior.

The extraction of intact ECM from human or animal tissue involves a process called decellularization, wherein all cellular and nuclear content from a tissue are removed while

retaining the structural integrity, composition, and biological activity [101]. Efficient decellularization methods include chemical, enzymatic, physical, or combinatorial approaches that have been applied to many types of tissues [102]. These decellularized tissues can then be combined with synthetic and/or natural biomaterials to create tissue scaffolds that possess bioactive properties. Once these ECM-based scaffolds are implanted into the body, the tissue remodeling is initiated by the degradation of the ECM scaffold, which in turn releases soluble matricryptic peptides that can attract stem cells. The degradation products of ECM function as chemo-attractants to local tissue progenitor and stem cells, though the underlying mechanism of action has not been determined [103-105].

To demonstrate these chemoattractive properties of ECM, Reing *et al.* tested the *in vitro* effects of enzymatically degraded ECM from porcine urinary bladder (UBM) tissue to isolate cultures of progenitor stem cells from differentiated endothelial cells [106]. Interestingly, the ECM degradation products showed chemoattractant and mitogenic properties when exposed to progenitor cells. On the other hand, an inhibition of cell migration and proliferation was observed when differentiated endothelial cells were exposed to the products of UBM digestion. A similar study analogously showed these chemoattractive properties *in vivo*, upon the administration of enzymatically degraded UBM-derived ECM to wound sites of mice. Specifically, the pedal digits of mice were amputated and subsequently ECM degradation products were injected into the injured sites. Histological analysis revealed the local accumulation of mononuclear cells distal to the wound sites only in the mice treated with ECM degradation products as compared to the untreated control groups. Cells extracted from these injured areas were found to be multipotent and able to differentiate to neuroectodermal, adipogenic, and osteogenic lineages [107].

One critical downside of ECM-based materials is the variability in the chemoattractive properties that arises due to differences in the species, age, gender, and physical characteristics of the subject from which the tissue is harvested. Brennan *et al.* investigated this concept by testing the stem cell recruitment abilities of enzymatically digested ECM from both adult and fetal human skins [108]. Degradation products of the ECM samples were assessed by their *in vitro* chemoattractivity towards human keratinocyte progenitor and stem cells (HEKn). The ECM degradation products derived from fetal samples as opposed to adult samples showed an enhanced ability to recruit HEKn cells. In the same study, intraspecific variability was also evaluated by comparing the chemoattractant activity of ECM products derived from porcine and human skin. Results indicated that adult porcine skin ECM displayed higher chemoattractivity towards HEKn cells than ECM from adult human skin.

Another crucial aspect to consider is that the process of decellularization must be optimized according to the type of tissue, which frequently varies in ECM content and cell density. Each technique of decellularization has also distinct drawbacks that are responsible for significant changes in the physical and chemical properties of the original tissue once decellularized [109]. For instance, freeze-thawing and pressure techniques can influence the initial structure of the ECM and alter the mechanical integrity of the ECM structure, respectively. Similarly, the use of acid or alkaline treatments may cause a degradation in the

major components of the ECM while ionic detergent may disrupt electrostatic interactions among proteins and be difficult to remove after the process is completed. All these aspects can affect the process of stem cell recruitment and differentiation toward a defined cell lineage [110]. A more detail discussion regarding the topic of decellularized ECM tissues for cardiac and bone tissue regeneration will be provided in the next sections.

3. Cardiovascular regeneration using stem cell recruitment strategies

We can infer from the previous sections that bioactive materials can be engineered to facilitate the selective recruitment of circulating stem cells from the blood or tissue niches. These cells are directed by the body's immune system to sites of injury to differentiate into either white blood cells to prevent infection or to other defined cell lineages to promote tissue regeneration. Stem cell recruitment has recently emerged as a potential solution to improve the clinical outcome of cardiovascular therapies based on stents or vascular grafts, which are commonly used to treat conditions such as coronary artery disease. The need for improved cardiovascular implant technology arises from several major drawbacks of these devices that frequently lead to further health complications, which can require follow up procedures. For instance, vascular grafts often have low patency after implantation due to their thrombogenic surfaces and additionally pose a high risk for intimal hyperplasia (IH) [111]. IH is a pathological condition characterized by the thickening of the intimal layer of a blood vessel in response to injury or vascular reconstruction and is a prevalent cause of graft thrombosis and failure.

In healthy blood vessels, the complications associated with grafts such as the thickening of the intimal layer is prevented by a protective layer of endothelial cells that surround the lumen of the vessel. In addition to serving as a physical barrier that preserves the vessel, the endothelial layer also provides a variety of biological functions that regulate vessel functions. For instance, endothelial membrane molecules such as thrombomodulin and heparin sulfate are responsible for regulating blood clot formation and thrombosis. Additionally, endothelial cells secrete regulatory signals such as prostacyclin and nitric oxide (NO) that mediate vasodilatation in healthy vessels. Given the vital functions that the endothelium provides to maintain blood vessel functions, developing therapeutic approaches to promote *in situ* endothelialization of cardiovascular implants is widely regarded as an essential step in improving stent and graft technology. Toward this aim, designing implants that actively recruit endothelial progenitor cells (EPCs) is a leading research strategy to extend the patency of cardiovascular implants and mimic the functionality of normal vessels.

Furthermore, the concept of stem cell homing holds great promise in the cardiovascular field also as an innovative approach to design effective therapies aimed to restore cardiac damage after myocardial infarction (MI). After this event, necrotic scar tissue is generally formed at the injured site post-MI replacing the functional cardiac tissue. Despite the large reserve of cardiac stem cells (CSCs) in the human heart, it is difficult to regenerate cardiac tissue at the injured region as the healing pathways are commonly expressed over a limited period of time after injury. This is the main reason why myocardial damage is mostly permanent [112-114]. The lack of endogenous regeneration after infarction has prompted a great deal of effort to establish new methods that can enhance or prolong the regenerative ability of heart

tissue. For instance, the use of hydrogels containing growth factors or ECM-derived scaffolds that inherently possess chemoattractant properties are growing areas of interest as suitable techniques to promote the recruitment of CSCs to restore myocardial function.

An overview of the possible strategies designed so far in the field of cardiovascular regeneration to promote endothelialization in cardiovascular implants and to restore myocardial function using ECM derived tissues will be discussed in the following sections.

3.1 Stem cell recruitment for the endothelialization of cardiovascular implants

In situ endothelialization of stents and vascular grafts can be realized by modifying the surfaces of these medical devices with various biomolecules that facilitate EPC recruitment. EPCs are bone marrow-derived cells, which can differentiate into endothelial cells (ECs). EPCs are also involved in re-endothelialization and neovascularization and therefore regulate the maintenance and remodeling of endothelial cells. The central strategy for selectively recruiting EPCs from the bloodstream is by binding to the specific surface markers uniquely expressed by these cells. The most commonly targeted surface markers for EPC recruitment include the following proteins: CD31, CD34, CD133, CD144 (vascular endothelial cadherin), and CD309 (vascular endothelial growth factor receptor-2) [115].

One possible strategy to target the surface markers expressed by EPCs is to modify the surfaces of cardiovascular implants with cell-adhesive molecules such as ECM proteins. Visscher *et al.* demonstrated that the commercially available polyester grafts Gelsoft™ and POLYMAILLE® C each displayed enhanced endothelialization when coated with fibronectin (FN) and SDF-1α [116]. FN+SDF-1α coatings, which were formed by the physisorption of both proteins to the grafts' surfaces, promoted the recruitment and adhesion of ($CD117^+$) hematopoietic stem cells (HSCs) and ($CD34^+$) EPCs. The FN served to enhance cell adhesion and SDF-1α directed the recruitment of autologous stem cells and modulated the inflammatory response of cells. Together the functions of both proteins provided synergistic effects to jumpstart stem cell-mediated healing at the implant interface. This finding is in accordance with earlier studies, which concluded that the combination of FN and SDF-1α provided an increase in cell recruitment in comparison to FN or SDF-1α individually. To evaluate the therapeutic efficacy and stem cell homing properties of the coating *in vivo*, FN+SDF-1α-coated and uncoated vascular grafts were implanted into resected carotid arteries in an ovine model. Upon analyzing the explanted grafts after 1 and 3 days, a higher quantity of $CD117^+$ and $CD34^+$ cells adhered to the walls of the coated grafts with respect to the uncoated surfaces. The increase in EPC adhesion resulted in the growth of a neointimal layer and overall coverage with endothelial cells in the coated grafts, and the selectivity of cell recruitment prevented adhesion of thrombotic tissue.

Aside from proteins, genes and adhesive peptides have been also been investigated for enhancing cell recruitment to cardiovascular implants [117-119]. The RGD peptide, which was previously discussed in section 2.1, is one of the most widely investigated binding motifs for promoting EPC adhesion. The RGD sequence, which binds to $\alpha_v\beta_3$ receptors expressed on the surface of EPCs, can be incorporated into biomedical devices resulting in the selective recruitment of EPCs to the site of the implanted material. This concept was applied to drug eluting stents for the controlled release of cyclic RGD (cRGD) peptide by

applying a cRGD-loaded synthetic polymer coating to commercially available 316L stainless steel stents (Figure 5A) [120]. When implanted in a porcine model *in vivo*, the coated stent exhibited a controlled release of the hydrophilic cRGD peptide that was localized to the tissue surrounding the stent, therefore directing a localized recruitment of EPCs into the implant site. At 4 weeks post-implantation in coronary arteries of pigs, the cRGD-loaded stents displayed an increase in the early recruitment of EPCs in comparison to unmodified bare metal stent (BMS). In addition, a greater endothelial surface coverage was detected on the modified grafts in comparison to that observed in the animals implanted with BMS (Figure 5B). As a result of the benefits introduced by loading grafts with cRGD, a reduction in neointimal hyperplasia was achieved through enhanced stent endothelialization and reduced SMC progenitor cell homing. Along with the RGD peptide, other motifs such as PHSRN [121] and GRGDSP [122] have been identified as essential binding domains of fibronectin. Recently discovered binding domains of laminin such as YIGSR [123] and IKLLI [124] have also been grafted to or released from cardiovascular implants with the same goal. However, one of the disadvantage of using the aforementioned peptides as recruiting and adhesive signaling factors is that they bind with a wide variety of cell types and therefore lack the specificity that is necessary for recruitment of EPCs alone. For this reason, the use of these peptides for therapeutic applications *in vivo* is limited due to the possibility of the recruitment of undesired cell types, which could cause further complications or undesired tissue growth in the site of injury. Therefore, an increasingly popular research focus is the identification of novel adhesive peptides that selectively bind to single cell types such as EPCs [125, 126].

A second strategy for enhancing endothelialization of cardiovascular implants involves the surface functionalization of stents and grafts with antibodies to enable specific binding with the unique surface markers expressed by stem and progenitor cells. Beusekom *et al.* developed Genous™, an EPC-capturing stent, by covalently attaching a murine anti-human monoclonal CD34 antibody to the stent to recruit $CD34^+$ EPCs [127]. The EPC-capturing stainless steel stents were fabricated by coating them with an intermediate polysaccharide covalently coupled with murine monoclonal anti-human CD34 antibodies. The antibody-coated stents were then implanted into a porcine coronary model and assessed for the extent of endothelialization and neointimal thickness (NIT) formation at 2 days post-implantation. Bare metal stents (BMS) were used as control groups. It was found that the Genous™ stent showed greater endothelialization but did not reduce NIT in comparison to BMS. The observed results could be attributed to the fact that the anti-CD34 can recognize other cell types apart from EPCs. Specifically, smooth muscle progenitor cells are also recruited which then differentiate into smooth muscle cells (SMCs) leading to an increase in NIT. This problem could be circumvented by using a different antibody, which can recruit EPCs with higher selectivity.

Following this concept, stainless steel stents can be coated with synthetic polymeric as a layer to promote the covalent conjugation of anti-CD133 antibody through EDC/NHS chemistry (Figure 5C). Alternatively, the same antibody can be loaded and linked on the surface of stainless steel stents using chitosan/hyaluronic acid (CS/HA) multilayers that can be assembled through electrostatic interactions (Figure 5D) [128]. These stents selectively captured HSCs since the CD133 marker is expressed only on HSCs. The exclusion of SMCs

prevented inflammation, thrombosis, and rejection of the stent. The captured HSCs differentiated into vascular ECs by CS/HA induction leading to successful endothelialization. The high molecular weight HA on the surface of the coating hydrolyzed to a low molecular weight that caused a controlled differentiation of HSCs into EPCs. Once differentiated, the matrix provided an ideal microenvironment for EPC proliferation. Thus, these stents were able to facilitate vascular repair while inhibiting hyperplasia and stent failure. A summary of other similar recent studies aimed to improve endothelialization of stents and vascular grafts are reported in Table 1.

Finally, an emerging technique for selective cell recruitment in cardiovascular devices is the use of aptamers, which are single-stranded oligonucleotides that can bind to specific target proteins or entire cells. Aptamers are identified *in vitro* using a procedure known as systematic evolution of ligands by exponential enrichment (SELEX) which allows high throughput screening and selective isolation of biofunctional oligonucleotides. This screening process is commonly achieved starting from large combinatorial libraries of DNA and RNA sequences [129]. Moreover, aptamers are nonimmunogenic and can be easily conjugated to polymer coatings that are commonly applied to vascular grafts and stents [130]. In a study by Hoffmann *et al.,* aptamers that can selectively bind to porcine EPCs were identified and conjugated with polymeric grafts using a star-PEG polymer coating [80]. The aptamer-coated polymeric surfaces were able to promote the selective adhesion of EPCs followed by their differentiation into endothelial cells after ten days. Similarly, in a more recent study, DNA oligonucleotides were immobilized on different vascular grafts made of expanded polytetrafluoroethylene (ePTFE) and polystyrene to promote *in situ* endothelialization [131]. The coated surfaces allowed adhesion of murine EPCs and showed low thrombogenicity and improved hemato-compatibility with human blood. These promising results suggest that the use of aptamer coatings can represent a valid alternative for the *in vivo* self-endothelialization of vascular implants, although further studies are necessary to prove the efficacy of this technology and evaluate its potential as a clinically available therapeutic tool.

3.2 ECM-based scaffolds for stem cell recruitment in myocardial regeneration

The heart possesses a pool of cardiac stem cells (CSCs) that are self-renewing, multipotent *in vitro*, and are involved in myocardial tissue homeostasis [132]. Heterogeneous populations of CSCs reside in stem cell niches and exist in a quiescent and proliferative state that are generally identified by the c-kit$^+$ receptor. Despite evidence of their presence, the stem cell compartment in the heart may not have the potential to restore myocardial function after injury. Specifically, during the early stage of myocardial infarction, the ECM of cardiomyocytes is degraded by matrix metalloproteinases, causing an adverse effect on the regenerative capacity of the stem cell niches [133]. In an attempt to repair the damage, CSCs tend to migrate in the ischemic periphery of the infarct area, but the absence of functional ECM negatively affects their functionality. In this second stage, CSCs start to proliferate into myofibroblasts and deposit collagen to form fibrotic scar tissue. This process is irreversible, and no treatment is currently capable of repairing the necrotic myocardium. Among the possible strategies, stem cell transplantation in the infarct area is a valid alternative. This therapy involves the local injection of stem cells to improve angiogenesis, promote

cardiomyocytes regeneration, and restore cardiac function. However, it is still not clear what is the most suitable source of stem cell for myocardial regeneration. In addition, cell isolation protocols and maintenance *in vitro* can have a profound effect on their performance after transplantation [134]. For this reason, alternative approaches have been investigated to treat myocardial infarction and a promising alternative is the use of acellular ECM-based scaffolds that can uncover the regenerative potential of the CSC niche.

The use of decellularized matrices offers many advantages as it avoids the problems associated with synthetic implants, which are prone to foreign-body reactions. In addition, since these matrices are stripped of most of their cellular and nuclear materials, the chance of any unwanted immunological responses to decellularized tissue is minimized [135].

Based on this concept, Sarig *et al.* developed a natural myocardial ECM patch for cardiac regeneration [136]. The bioactive patches, which were fabricated with decellularized porcine cardiac extracellular matrix (pcECM), were able to recruit cardiomyocyte progenitor cells ($GATA4^+$, $c\text{-}kit^+$) and myocytes ($MYLC^+$, $TRPI^+$) in both acute and chronic MI models. It was shown that the degradation of the pcECM material attracted cardiac progenitors which differentiate towards the cardiomyocyte lineage while, some of the $GATA4^+$ cells differentiated to $MYLC^+$ cells causing rapid vascularization of the patch, ultimately leading to cardiac function restoration.

Another group successfully developed an injectable myocardial matrix by decellularizing porcine myocardial tissue [137]. This approach offered a minimally invasive therapy wherein the injectable matrix was able to self-assemble to form a nanofibrous structure upon injection into rat myocardium. *In vivo* analysis revealed migration of endothelial cells and smooth muscle cells toward the matrix, and at 11 days post-injection arteriole (small diameter blood vessel) formation was observed. This naturally derived injectable matrix was able to mimic the natural myocardial ECM closely (Figure 6).

Similarly, a promising material for myocardial regeneration is the ECM derived from porcine small intestinal submucosa (SIS-ECM) which is commercially available as a patch (CorMatrix®) to enhance the repair of the pericardium after surgery and for other cardiovascular surgical applications [138]. SIS-ECM is biodegradable and is capable of inducing the recruitment of marrow-derived stem cells and proliferation of cardiomyocytes at the site of implantation. In a preclinical study in rats, SIS-ECM has also been tested for its ability to treat myocardial infarction [139]. Specifically, an injectable form of small intestine extracellular matrix emulsion (EMU) was introduced into the myocardium of rats to evaluate its effect after myocardial infarction. The injected EMU induced increased recruitment of $c\text{-}Kit^+$ (marrow-derived) stem cells, macrophages, and myofibroblasts in the myocardium of rats after infarction, which improved the left ventricular function.

Overall, these promising results suggest that ECM based materials represent a promising approach for the treatment of myocardial infarction. Currently, further preclinical studies and clinial trials are underway to test the efficacy and safety of these natural derived scaffolds. The desired goal is that ECM-based therapies alone or in combination with stem

cell-based strategies will restore the integrity and functionality of the myocardium after infarction by harnessing the regenerative potential of CSCs in the heart.

4. Endogenous cell recruitment strategies for bone regeneration

As opposed to cardiac injuries for which there is limited regenerative potential, the human body possesses intricate response mechanisms for musculoskeletal injuries that can facilitate the complete and functional restoration of hard tissue. Trauma to bone disrupts the local vasculature, nerves, muscles, and other tissues, creating an immune response that in turn triggers temporally overlapping phases of repair. These are the inflammatory phase, the reparative phase, and the remodeling phase [147]. The inflammatory phase involves the platelet-mediated recruitment of BMSCs and other precursor cells while blood coagulates to plug the fracture site with a hematoma. This hemostatic plug is the body's endogenous cell scaffold that provides mechanical support as well as the biological cues that recruit stem cells from surrounding tissues and circulating blood. During the reparative phase, the hematoma is gradually replaced with bone by the differentiation of progenitor cells, following two distinct pathways: intramembranous (IM) ossification and endochondral (EC) ossification. IM ossification is the direct differentiation of BMSCs into osteoblasts, which forms mineralized bone on the periosteum of the fractured site. On the contrary, the bulk space of the fracture is commonly repaired by EC ossification, which directs chondrogenic differentiation of stem cells to create an intermediate cartilage callus [148]. The hypertrophic cartilage creates a matrix that is populated by osteoblasts and blood vessels followed by a step of mineralization. Finally, the last phase consists in the remodeling of bone tissue to restore its native architecture.

Despite this efficient endogenous healing response, intervention by orthopedic surgery is still frequently required for defects above a certain critical size (3 mm for humans). In fact, these defects are not capable of spontaneously healing, due to the lack of vascularity or insufficient population of progenitor cells [148, 149]. The clinical gold standard for treating these fractures is autologous bone grafts (ABGs) which have historically been very successful. However, several drawbacks limit their use such as risk for infection, necrosis in the donor site, and primarily the limited source of autologous bone[147]. For these reasons, grafts made of xenogeneic or allogeneic sources and synthetic graft substitutes have been investigated as clinical alternatives to ABG over the past decades of research, but none have yet matched or surpassed ABGs [150]. Aside from these classical strategies, the field of bone tissue engineering is evolving to become more deeply rooted in the new concept of developmental biology. This trend is leading researchers to explore methods of stem cell recruitment with the goal of utilizing the body's native mechanisms to process the cells into functional bone tissue. To explain this concept of developmental engineering in regards to bone regeneration, this section provides an overview of several materials-based approaches for regulating chemoattractant properties. Finally, innovative strategies for stem cell recruitment are discussed by describing the inherent chemoattractant properties of native extracellular matrix (ECM) materials.

4.1 Strategies for successful stem cell mobilization to enhance bone formation

The cascade of events responsible for bone regeneration can be controlled by tuning the physical and biological properties of biomedical implants. Scaffolds made of natural and synthetic polymers have been designed to locally deliver a variety of growth factors and chemoattractant signals to recruit stem cells and accelerate the process of osseointegration (Figure 7).

The most investigated technique consists in the delivery of growth factors to locally provide instructive signals that can mimic the process of bone regeneration. It is well-established that angiogenic growth factors are only involved in the stage of new vasculature formation while osteogenic growth factors are present constantly throughout the whole process of bone regeneration and remodeling. It is then important to design systems that can provide this biological cues in time controlled manner to mimic closely the normal process of bone healing.

Considering this concept, several studies have focused their efforts in the design of sequential or dual delivery systems for both vascular endothelial growth factor (VEGF) and the bone morphogenetic protein-2 (BMP-2) [151, 152]. The combination of both growth factors enhances the chances of bone regeneration compared to scaffold loaded with a single growth factor. These results can be attributed to the synergic effect of growth factors on stem cell recruitment. Specifically, BMP-2 regulates the complete process of bone formation including the migration of mesenchymal stem and their differentiation into osteoblasts. Meanwhile, VEGF aside from orchestrating the fundamental process of angiogenesis is also responsible for the mediation of cell-cell interaction which directs stem cell recruitment in the fracture area. Support for this important role has been provided in a study by Zhou *et al.* where the secretion of VEGF-A was identified as the primary signal responsible for the recruitment of undifferentiated stem cells [153]. It was found that differentiated stem cells can secrete VEGF-A that in turn activates the CXCR4 receptor leading to the recruitment of other stem celland macrophages to the bone defect site. These findings suggest an active role of VEGF-A in the process of cell-cell interaction and modulation of local inflammatory response.

Growth factors can also be conjugated with ECM-mimetic scaffolds to unlock their full biological activity and increase their chemoattractant ability at lower doses. In a study by Garcia *et. al.*, this hypothesis wastested by conjugating VEGF into PEG hydrogels containing different integrin binding peptides [154]. When these scaffolds were integrated into radius critical size defects in mice, the VEGF-loaded RGD hydrogels showed increased vascularization compared to the free VEGF RGD scaffolds. Similarly, in another study by Martino *et al.*, the same idea was investigated using different growth factors [155]. Platelet-derived growth factor subunit B (PDGF-BB), and BMP-2 were linked to a multifunctional recombinant fragment of fibronectin (FN) scaffolds which contained integrins binding domains for all growth factors. The scaffolds were tested for bone repair in a rat model and recruitment of mesenchymal stem cells was higher due to an increased biological activity of both growth factors at doses that normally would have not elicit any response.

Aside from growth factors, another possibility to guide stem cell recruitment and promote bone regeneration is the use of peptides or drugs loaded within a scaffold that displays chemoattractant properties. For instance, the substance P is a neuropeptide that has been mainly studied as a recruiting agent of circulating stem cells to treat several cardiovascular diseases, although its use can also be extended for promoting bone repair. In a recent study by Kim *et al.*, the substance P was conjugated with a synthetic self-assembling peptide and combined with a polylactic acid/beta-tricalcium phosphate (PLA/β-TCP) porous scaffold [156]. The composite material was implanted in a rat calvarial defect model and was able to recruit circulating stem cellinto the defect site. The recruitment of stem cells accelerated the process of bone formation after 24 weeks post-surgery.

Another interesting approach is the use of statins that have been recently investigated as potential therapeutic agents to enhance bone fracture healing and for the recruitment of bone marrow-derived endothelial progenitor cells [157, 158]. Based on this concept, Yueyi *et al.* studied the mobilization of BMSCs and EPCs into polylactic acid (PLA) scaffolds loaded with simvastatin and implanted into rabbits' calvarial defects [159]. The local release of simvastatin was responsible for the increased bone formation in the defect site associated with higher expression of bone morphogenetic protein 2 (BMP-2) and hypoxia- inducible transcription factor-1α (HIF-1α). Both growth factors were responsible for the observed recruitment of BMSCs and EPCs in the defect area (Figure 8A). In addition to simvastatin, erythropoietin (Epo) has been evaluated as an alternative chemoattractant signal to promote mesenchymal stem cell migration in a murine calvarial model [160]. Gelatin-based scaffolds loaded with Epo showed the best results in terms of autologous stem cellrecruitment in the defect site when compared to scaffold delivering Epo in combination with BMP-2 or BMP-2 only. In addition, Epo-loaded scaffolds were also able to induce osteogenic differentiation of autologous stem cellwith higher efficacy compared to the other groups investigated (Figure 8B).

Finally, a crucial aspect to consider while designing new solutions to enhance bone formation is the role of the immune systems and its effect on the process of stem cell migration in the defect site. For this reason, several studies have focused their attention on this important aspect trying to understand the intricate cross-talk between the immune system and the pathways that mediate the process of stem cell recruitment [161, 162]. To prove this concept, in a recent study by Lv *et al.*, the pro-inflammatory signal high mobility group box 1 (HMGB1) was immobilized in a nanofiber scaffold of PCL/PLA coated with heparin [163]. HMGB1 can modulate local and systemic inflammation and is a chemoattractant agent that regulates the recruitment of inflammatory cells and mesenchymal stem cells [164]. The fibrous scaffold was implanted in a rat calvarial defect, and the presence of HMGB1 had multiple effects including stem cells migration, differentiation, and enhanced vascularization in the bone defect. In another strategy, the pro-inflammatory molecule fibrinogen (Fg) was adsorbed into chitosan films leading to the adhesion of peripheral blood human natural killer (Nk) cells [165]. These type of cells are the first population to migrate to the site of injury and play a fundamental role in regulating bone regeneration through stem cellss recruitment. The presence of Fg in the polymeric films was able to enhance the adhesion of Nk cells which in turn were responsible for the invasion ofstem cell. In addition, the expression of the differentiation marker alkaline phosphatase

(ALP) was not affected by the presence of the Nk cells. Overall, these findings suggest the possibility to modulate the properties of scaffolds taking in consideration the role of the inflammatory response as a key factor to recruit stem cells, which is necessary to initiate the process of bone regeneration.

4.2 ECM-based materials for stem cell recruitment

As opposed to fabricating biomimetic scaffolds with synthetic or natural polymers, many researchers are also using biomaterials derived from the ECM of native tissues as a leading strategy to promote bone regeneration. When the cellular and mineral component of bone is removed, the remaining matrices retain the native cell's biochemical and structural cues that are involved in the vast majority of cell signaling functions such as migration, proliferation, and differentiation.

To date, demineralized bone matrix (DBM) based materials represent a significant portion of the bone graft market. DBM was pioneered by Marshal Urst in 1965, who created the terms 'osteoinductive' and 'osteoconductive' to describe DBM's ability to recruit stem cells and promote osteogenesis [166]. Over a decade later in 1978, Tuli *et al.* demonstrated the successful healing of critical-sized osseous defects in rabbits using preserved decalcified bone and noted the recruitment of stem cells to the bone matrix leading to either IM or EC ossification [167].

In recent years, many DBM-based products for use as bone graft substitutes have been FDA approved as medical devices. These include DynaGraft-D™ (Keystone Dental), Puros® (Zimmer Dental), Grafton® (BioHorizons), and DBX® (Synthes), which are viscous bone pastes formulated with allograft-derived human DBM to deliver osteoinductive and osteoconductive DBM particles. These products have shown potential to heal critical-sized defects in animal models by inducing a rapid recruitment of osteoprogenitor cells followed by differentiation to osteoblasts. For instance, DBX®, a paste of DBM suspended in sodium hyaluronate was applied *in vivo* into segmented critical-sized defects in radial bones of rabbits. Osteoprogenitor cell recruitment along with the formation of new bone surrounding DBM particles were noted within 4 weeks after implantation. Effective BMSC recruitment was also evidenced by the presence of newly formed trabeculae within 8 weeks and full repair of mature bone by 12 weeks [168]. Clinical pilot studies have deemed DBM equivalent to iliac crest ABG for the treatment of non-unions or void spaces due to the removal of benign tumors [169] as well as the treatment of long bone fractures [170]. A more extensive evaluation of DBM as a clinical product for the treatment of bone disorders has been reviewed thoroughly by Drosos *et al.* [171].

Aside from these encouraging findings, DBM products present a series of limitations that is important to consider when used in clinical settings. For example, some reports have suggested inconsistencies in the osteoinductive and conductive properties of DBM materials and in some cases between samples of the same products [172, 173]. These issues are mainly due to variable concentration of BMPs and other growth factors as a result of the demineralization technique used and the variability in the donor tissue.

Therefore, DBM is typically only used to enhance autografts, allografts, or other osteoconductive scaffolds. Most DBM pastes are marketed as ‘bone graft extenders’ which indicates their intended use as filler to extend the volume of bone grafts. Common clinical practice by orthopedic surgeons is to implant DBM pastes into bone fractures or nonunions along with the patient’s bone marrow aspirate, which is rich in BMSCs and can be extracted from bone marrow and implanted using minimally invasive techniques [174, 175]. The osteoconductivity of DBM is often enhanced by coupling DBM with cancellous allograft bone chips or granules that serve as osteoconductive scaffolds to which the DBM-recruited osteoprogenitor cells can adhere and differentiate into osteoblasts. Kang *et al.* evaluated this technique by combining DBM with allogeneic cancellous bone granules and subsequently implanting the scaffolds *in vivo* in critical-sized radial defects in rabbits, comparing the effects to control groups implanted with only the cancellous bone granules [176]. Not only did the combined DBM and bone granule scaffolds induced a more rapid healing response by 6 weeks but also significantly higher levels of bone regeneration were observed after 12 weeks compared to treatment with DBM alone. Most commercial DBM products are offered as composite graft substitutes, such as DynaBlast™ (Keystone Dental), which is an injectable treatment combining human DBM with allogeneic cancellous bone chips. These type of grafts are particularly useful for fostering a sufficiently osteoconductive environment for endogenous repair of osseous defects devoid of healthy bone, such as in sinus augmentation procedures [177].

As mentioned above, the biological cues present in extracted DBM are also highly dependent on the tissue source. Rabie *et al.* discovered that DBM harvested from intramembraneous bone (DBM-IM) could be applied to IM autografts to induce healing by only the IM pathway *in vivo* in rabbits. Likewise, DBM from endochondral bone (DBM-EC) induced the EC ossification as the main mechanism of bone repair [178]. Interestingly, EC ossification was also initiated in IM autografts that were implanted with DBM-EC [179]. This strongly implies that the *in vivo* effects of ECM-based materials not only depend on the species or even the type of tissue from which the ECM originates, but also the developmental pathway by which they were formed. Further research has yet to answer the question of whether or not this engineered DBM can provide the biological cues necessary for EC ossification in large avascular defects while simultaneously recruiting stem cells. Aside from DBM-based products, researchers are currently investigating cartilage ECM as an acellular initiator of EC bone regeneration, since cartilage is produced during the early reparative development phases. A recently proposed hypothesis is that cartilage ECM, commonly formed by decellularizing cartilage (DCC) or devitalizing cartilage (DVC) is a favorable candidate for bone regeneration of avascular defects. In large defects that lack vascular networks, osteogenesis by IM ossification is not suitable as it is generally associated with the formation of scar tissue or nonunion within the defect site [180]. On the contrary, osteogenesis by the EC pathway is a favorable strategy for this type of defect since cartilage is an avascular tissue. Utilizing DCC or DVC would theoretically enable the ECM-guided differentiation of stem cells to chondrocytes [181]. These cells are then able to form a vascularized matrix during a process known as hypertrophy, effectively introducing vascularized tissue in previously avascular defects. Finally, the mineralization of the soft callus can lead to the formation of new bone.

Sutherland *et al*. reported that both DVC and DCC derived from articular cartilage displayed chondroinductive properties and both materials were found capable of recruiting stem cellss *in vitro* [182]. Furthermore, DCC demonstrated chondroinductive properties to the same extent as therapeutic doses of TGF-β3. In a different study, Beck *et al*. investigated the regenerative potential of photocrosslinked hydrogel pastes containing DVC or DCC particles, which were placed surgically into cartilage defects [183]. The study highlighted the *in vitro* stem cell recruitment's ability of these chondroinductive formulations, which hold potential as platforms for the regeneration of critical-sized bone defects by EC ossification of intermediate cartilage tissue.

The rapid expansion of this research field within the past two years has led to significant evidence that a crucial parameter for an ECM-based endochondral ossification template is the use of the appropriate cell source and extraction method. While previous work with DCC or DVC pastes may prove successful in generating hyaline cartilage in an osseous defect, the difficulty in regulating chondrocyte hypertrophy after chondrogenesis presents a significant hurdle for researchers. Thus, one potential solution may be the use of ECM derived from readily available allogeneic osteoarthritic (OA) cartilage, which undergoes hypertrophy according to the pathology of OA [184]. To demonstrate this concept, Bahney *et al*. implanted human osteoarthritic (OA) cartilage scaffolds *in vivo* into segmented tibial defects of mice [185]. OA articular cartilage was chosen as the tissue source since OA chondrocytes readily undergo hypertrophy, which is a necessary key to successful EC ossification. The OA cartilage explants formed bone *in vivo* by EC ossification within 4 weeks, while healthy articular chondrocytes did not undergo hypertrophy and retained their cartilaginous phenotype even after 4 weeks [185]. These promising results caution that the success of such a therapy strongly relies on controlling the process of hypertrophy.

In a comparative study between stem cell-derived decellularized cartilage (CT) and decellularized hypertrophic cartilage (HT), polymeric scaffolds fabricated with CT or HT were analyzed for their *in vitro* and *in vivo* osteogenic and angiogenic capacities [186]. Only the HT-derived scaffolds were able to promote vascularization and *de novo* mineralization when subcutaneously implanted in mice. Additionally, HT and CT-derived scaffolds were implanted into critical-sized femoral defects in rats, and after eight weeks, over half of the sample size of decellularized HT scaffolds displayed full bridging of the defects, whereas CT scaffolds displayed minimal bone regeneration (Figure 9). Histological analysis revealed significant recruitment and migration of host progenitor cells *in vivo* within the defect sites as a result of the chemoattractant properties of the HT scaffolds [186]. Notably, the results may be difficult to replicate with cartilage ECM that has been chemically decellularized, since the study only uses lyophilization for the decellularization protocol.

In fact, chemical decellularization techniques may be too harsh to preserve the chondroinductive and osteoinductive factors of cartilage ECM. A recent study assessed the formation of EC bone by scaffolds constructed with enzymatically decellularized cartilage (DCC). Cartilage was harvested from the femoral condyles of equine donors and subsequently decellularized by enzymatic degradation and lyophilization. The resulting DCC constructs were seeded with human stem cells and subcutaneously implanted *in vivo* in rats. The human stem cellss were primed in both chondrogenic medium as well as

hypertrophic medium prior to implantation. After 8 weeks, the unseeded DCC scaffolds showed no significant mineralization response, while the DCC scaffolds seeded with primed stem cells induced extensive endochondral bone formation. Interestingly, the newly formed bone was entirely of rat origin, though the scaffolds were of equine origin and the cells were human-derived. Overall, these results indicate host cell recruitment and infiltration during bone formation. The lack of osteoinductive properties of the unseeded scaffolds is likely due to the harsh enzymatic decellularization procedure, which the authors noted led to the nearly complete reduction in GAG content of the native tissue [187]. GAGs bind with and sequester growth factors in the native tissue, so removal of these polysaccharides during decellularization also results in removal of native growth factors. For this reason, as the authors noted, it is necessary to consider other decellularization or devitalization procedures that preserve the GAG content. With this approach, the DCC scaffolds could be used for bone regeneration without an exogenous cell source. It is expected that the chondro and osteo-inductive properties will be sufficient to facilitate endogenous repair.

Similarly, in another interesting study, Bourgine *et al.* demonstrated that cartilage ECM devitalized by standard freeze-thaw methods was not capable of forming bone due to the significant reduction of GAG and growth factor content during devitalization [188]. On the contrary, the same group demonstrated the possibility to generate hypertrophic cartilage ECM *in vitro* from human stem cells that was highly preserved after devitalization by using an ‘inducible-apoptosis’ technique. This method is minimally disruptive and, as opposed to freezethaw devitalization, enabled the preservation of essential GAGs and cytokines involved in the inflammatory phase (IL-8, M-CSF, and MCP-1), the angiogenic response (VEGF), the osteoinductive phase (BMP-2 and BMP-7), and the final bone remodeling stage (OPG and MMP-13) [188]. Specifically, hMSCs were transduced with a retrovirus expressing modified caspase 9 (Casp9), such that in the presence of a chemical activator, Casp9 can dimerize causing the cells to undergo induced apoptosis. The modified hMSCs were cultured *in vitro* in chondrogenic medium followed by hypertrophic medium, to generate hypertrophic cartilage. The resulting tissues could be devitalized by the addition of the chemical inducer for Casp9 dimerization to induce apoptosis, which removed cellular content to a similar extent as other common devitalization procedures. After 12 weeks *in vivo*, scaffolds fabricated with the hypertrophic cartilage ECM showed successful recruitment of host vasculature and significant stem cell recruitment was indicated by large quantities of newly formed cartilage, mineralized bone, and bone marrow cells. Formation of perichondral bone was noted to be absent from human cells, which indicates that the new bone was formed entirely by the host’s recruited cells [188]. Towards the clinical translation of hypertrophic cartilage ECM for bone regeneration platforms, the extensive focus needs to be applied on finding new effective decellularization or devitalization methods that preserve rather than destroy the acellular components of the tissue. Researchers must also strive to consider *in vitro* strategies for producing ECM that are scalable (i.e. grown in bioreactors) and consistent in composition.

5. Conclusions and future directions

Stem cell recruitment represents an innovative solution that can significantly improve the regenerative capabilities of existing therapies used so far in both fields of cardiac and bone

tissue engineering. We analyzed three main strategies to reach this goal including the use of adhesive biological molecules, delivery of growth factors, and the fabrication of decellularized material based scaffolds. Each one of these approaches has proven to be a valid alternative to instruct stem cell behavior in terms of adhesion, migration, and differentiation.

For instance, in the field of cardiovascular regeneration, a great deal of effort has been invested in the fabrication of new technologies aimed at the recruitment of EPCs to guarantee effective endothelialization of vascular grafts and stents. In addition, the use of injectable ECM-based scaffold for stem cell homing in injured myocardial tissue represents another promising area of future development. Specifically, ECM-derived from decellularized porcine myocardial tissue is one of the most investigated material as it can self-assemble into a porous and interconnected network upon injection. An example in this direction is represented by VentriGel™, which is anECM-based scaffold derived from porcine myocardial ECM that has reached in 2015 Phase I clinical trial for the repair of cardiac tissue after myocardial infarction [189]. This type of natural-derived matrix can be administered via a catheter to the damaged cardiac tissue in patients who have suffered a decline in cardiac function subsequent to a heart attack.

Additionally, attempts have been made to investigate alternative routes of injection for ECMbased material for myocardial regeneration. Specifically, a pre-clinical study in large animal models has shown the possibility to deliver porcine myocardial-based matrix via a transendocardial approach, which allows the material retention at the site and does not require the access to the coronary arteries using catheters [190]. The possibility to deliver the ECM-based scaffold by injection through this approach represents a great advantage for their translation into the clinic as it can reduce the associated risk of embolization when using catheters to access the coronary arteries. Moreover, this approach could be beneficial for patients that cannot be treated with catheters due to a compromised artery network.

Meanwhile, stem cell recruitment strategies aimed to regenerate bone tissue are mainly oriented in the discovery of new biological signals that can trigger the biological cascade of events involved in bone healing. Particularly encouraging are the studies that consider bone regeneration as an integrated process where the stem cell migration can be modulated by the inflammatory response. Furthermore, the use of engineered hypertrophic cartilage ECM may represent an innovative alternative material to autografts and allografts transplantation. In fact, these off-the-shelf scaffolds have shown the ability to recruit and guide resident stem cells toward the formation of new bone.

Overall, it is possible to forsee that all these active areas of research will greatly contribute to the discovery of new solutions for the treatment of cardiovascular and bone diseases simply by enhancing and instructing the body’s own ability to heal. However, several concerns are still unresolved and need to be addressed for the successful translation of these technologies into the clinic.

Firstly, although the delivery of growth factors represents a useful plan to create local chemoattractant environments around the implanted scaffold, issues regarding their efficacy

and safety are still an open challenge. To uncover the real potential of growth factors, further investigation is required on the molecular pathways that are behind the process of stem cell recruitment. To achieve this goal, studies aimed to track *in vivo* stem cell migration in response to local gradients of growth factors can help discover new biological signals and improve the selectivity of existing ones. Several steps have been undertaken following this idea such as, designing stem cell biological track devices using aptamers containing fluorescent dyes [83] or labeling stem cells with fluorescent nanoparticles [191].

Furthermore, the intricate signaling between the immune response and growth factors activityneeds to be further investigated as well. In fact, the process of stem cell recruitment is generally controlled by the inflammatory state of the injured tissue. This notion is particularly true in the case of bone regeneration where the inflammatory response plays a crucial role in stem cell recruitment and bone remodeling. Several examples reported in this review have started to consider this fundamental aspect by including in the scaffold signaling factors such as the protein HMGB1 or fibrinogen that can stimulate the recruitment of inflammatory cells and regulate stem cell migration in the bone defect. Further investigation and more efforts in this direction can pave the way for the development of more sophisticated designs where the presence of multiple inflammatory factors can precisely control the process of stem cell recruitment.

Finally, the limited clinical success of ECM-based scaffold for both cardiac and bone regeneration can be attributed to the variability in the ECM composition, which strictly depends on the process of decellularization. In fact, each technique of decellularization has precise limitations that can lead to significant changes in the physical and chemical composition of the original tissue once decellularized. Additionally, the lack of reproducibility in the process of decularization from one batch to another is another great drawback that can profoundly affect the safety and efficacy of ECM-based materials. To address these issues, much evidence from pre-clinical studies proving their safety needs to be gathered to further justify their use in clinical settings.

For instance, in the case of ECM-based materials for cardiac regeneration. it is important to investigate the hemocompatibility and thrombo-embolic potential of these bioactive materials, as well as the possible in site-specific inflammatory response. Studies investigating these aspects in large animal models are crucial to clarify their potential for clinical applications. Alongside, a better understanding of the key factors and the underlying mechanisms that regulate the process of stem cell homing in ECM-derived scaffolds is required to further advance this type of technology in the clinic [192].

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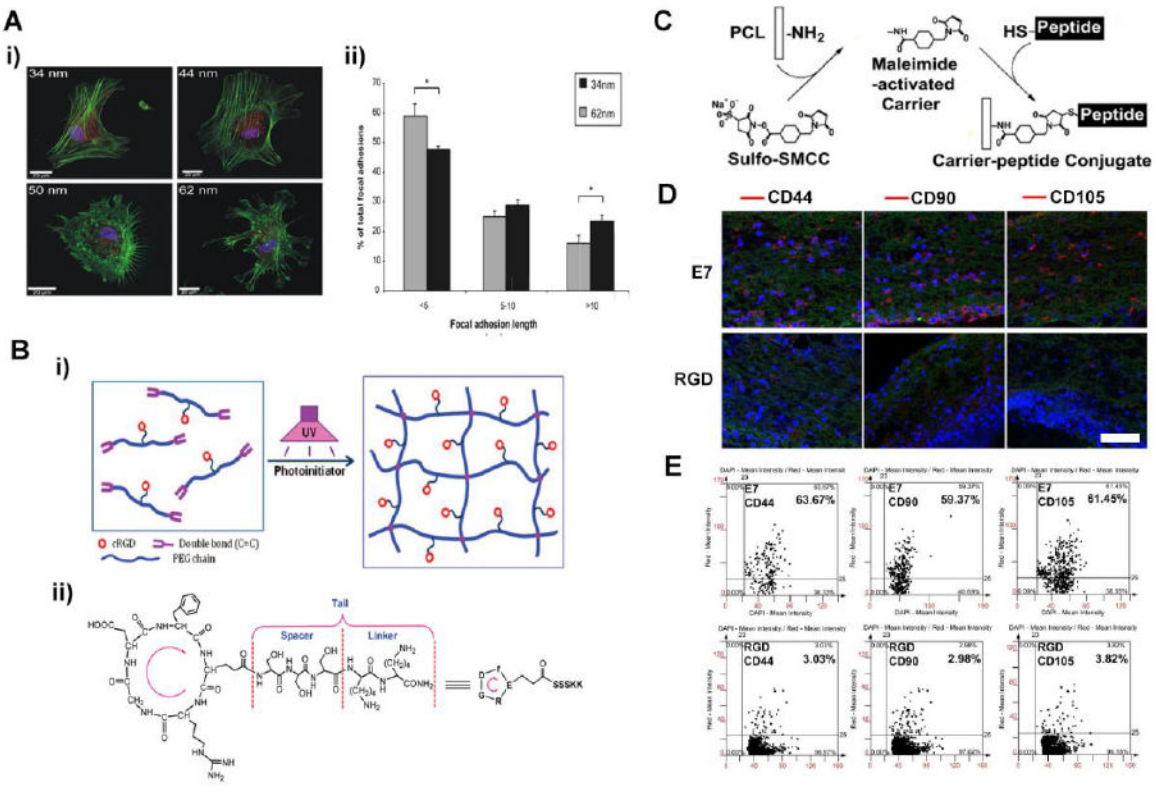

Figure 1.

Strategy for stem cell adhesion and recruitment using adhesive peptides. **A)** i) Human mesenchymal stem cells (hMSCs) staining with actin (green), focal adhesion kinase (FAK) (red) and Hoechst 33342 (blue) seeded on polystyrene-blockpoly(ethylene oxide) (PS-PEO) scaffolds. Scale bar =20 μm. The morphology of hMSCs changes according to the lateral spacing of RGD peptides in the scaffolds ii) Quantification of focal adhesions length in the different groups. **B)** Schematic representing polyethylene glycol diacrylate (PEGDA) functionalized with the cRGD peptide, which can form a bioactive tridimentional hydrogel upon UV irradiation ii) Chemical structure of the designed cRGD peptide which possess a tail of three serine as spacer and two lysine as a linker motif. **C)** Scheme of reaction for chemical conjugation of the E7 cell adhesive peptide to PCL scaffolds **D)** Comparison of the immunofluorescence staining of CD44, CD90 and CD105 positive cells recruited *in vivo* in the peptide conjugated scaffolds. Higher staining was present in the E7 group. (Scale bar = 50 μm) **E)** TissueFAXS image cytometry results indicating a larger presence of CD44, CD90 and CD105 positive cells in the E7-peptide PCL scaffolds.

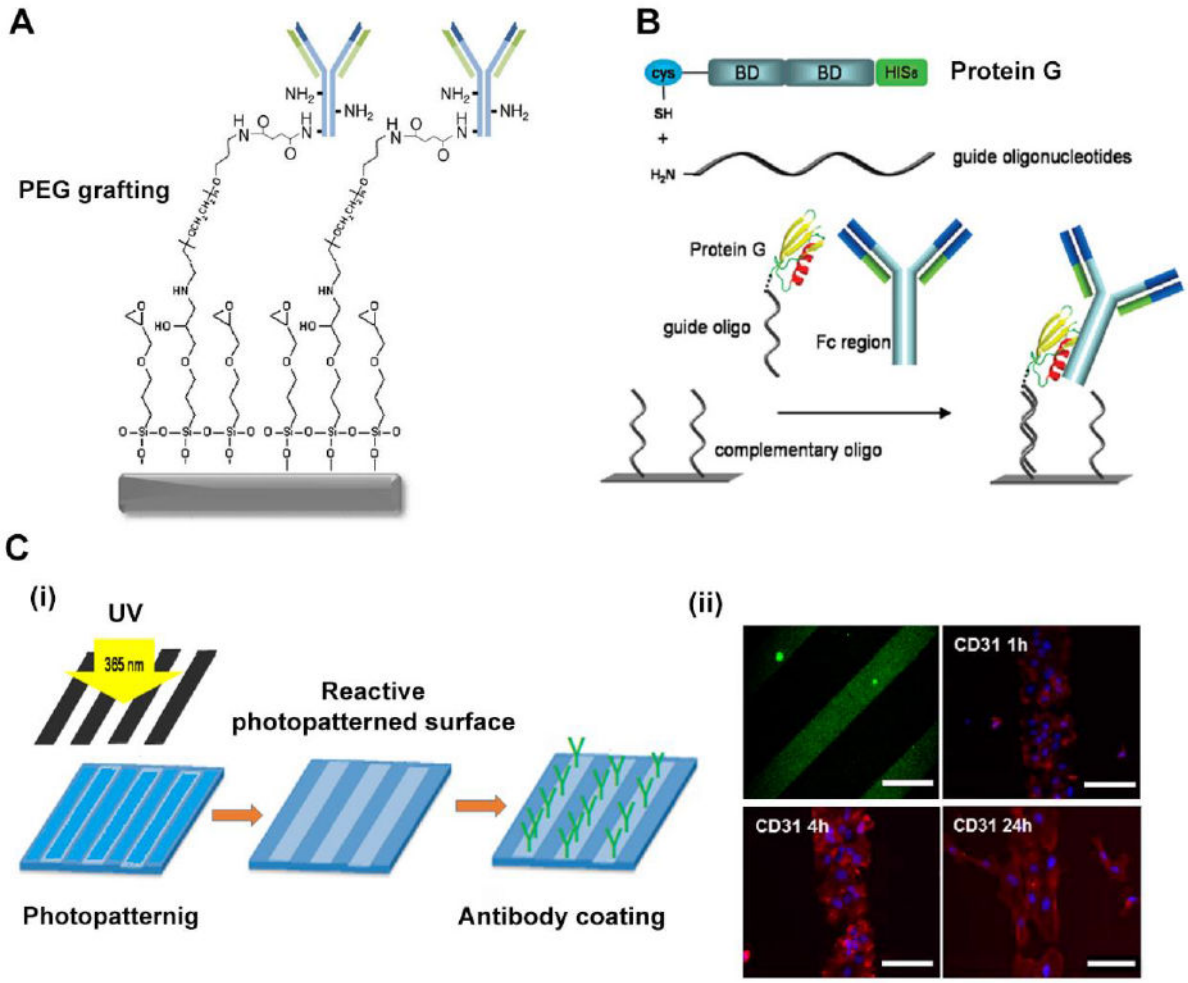

Figure 2.

Possible approaches for antibody immobilization. **A)** Schematic representing antibody immobilization on a reactive titanium surface using PEG grafting. **B)** Strategy for oriented antibody conjugation using the bacterial protein G. The presence of the protein G establishes a selective binding with the Fc region of the antibody. **C)** (i) Schematic indicating the process of photopatterning to create a reactive surface for antibody conjugation. (ii) Fluorescence images of conjugated antibody on the micropatterned surface. Actin Dapi staining of CD31 positive cells adhering on the micropatterned surfaces functionalized with antibody at different time points. (Scale bars =100 μm).

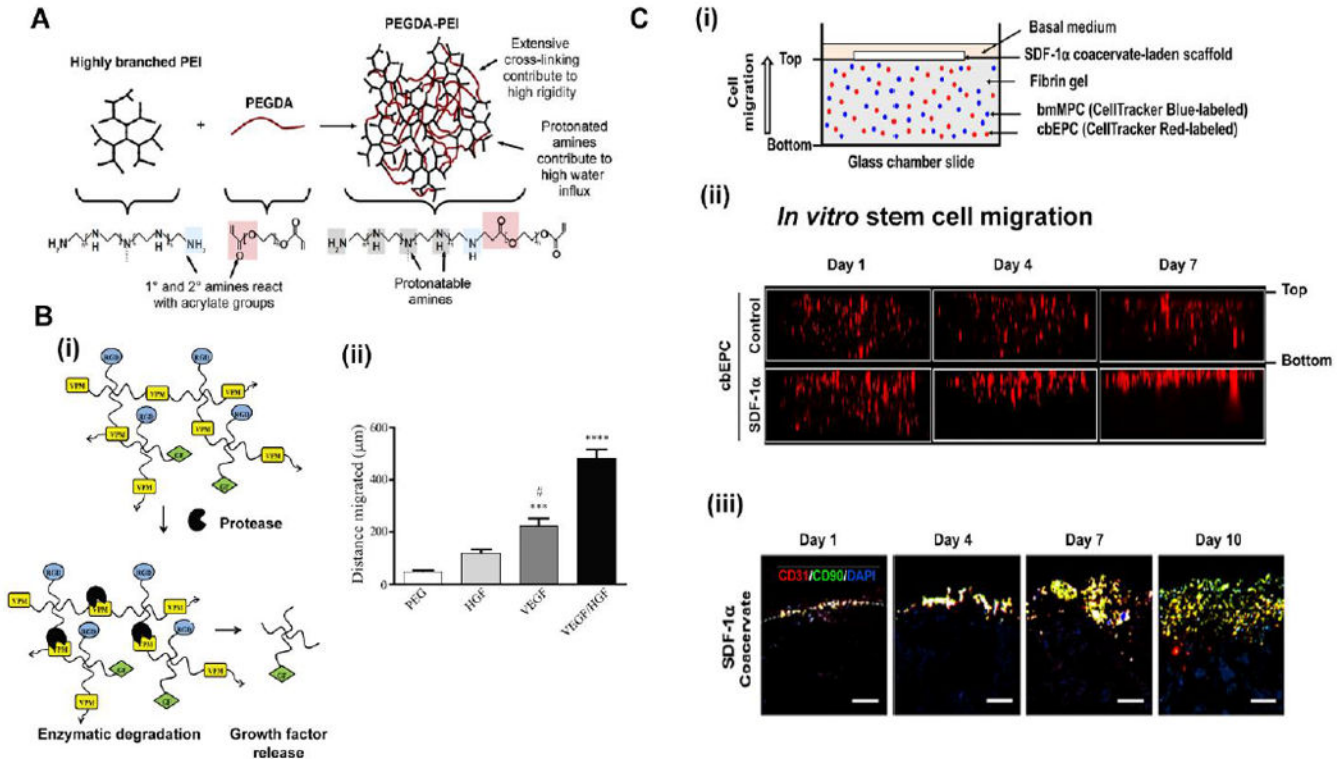

Figure 3.

Stem cell recruitment using growth factor release strategies. **A)** Design of a synthetic scaffold based on PEGDA-PEI with tunable swelling and degradability properties for growth factor release. **B)** (i) Schematic representing the enzymatic degradation of the degradable protease scaffolds and corresponding growth factor release. (ii) Quantification of stem cell migration *in vitro* in enzymatically degradable hydrogel carrying both VEGF and HGF. Higher migration was observed when both growth factors were released from the scaffold. **C)** (i) Schematic indicating the design of the *in vitro* migration assay for progenitor stem cells using SDF-1a coacervate-laden scaffolds. (ii) Cross-sectional images of fibrin gels indicating the migration of fluorescence-labeled EPCs in response to empty scaffold (Control) and SDF-1α coacervate-laden scaffold. (iii) Immunofluorescent staining for CD31 and CD90 positive progenitor stem cells recruited into SDF-1α coacervate scaffolds (Scale bar = 100 μm).

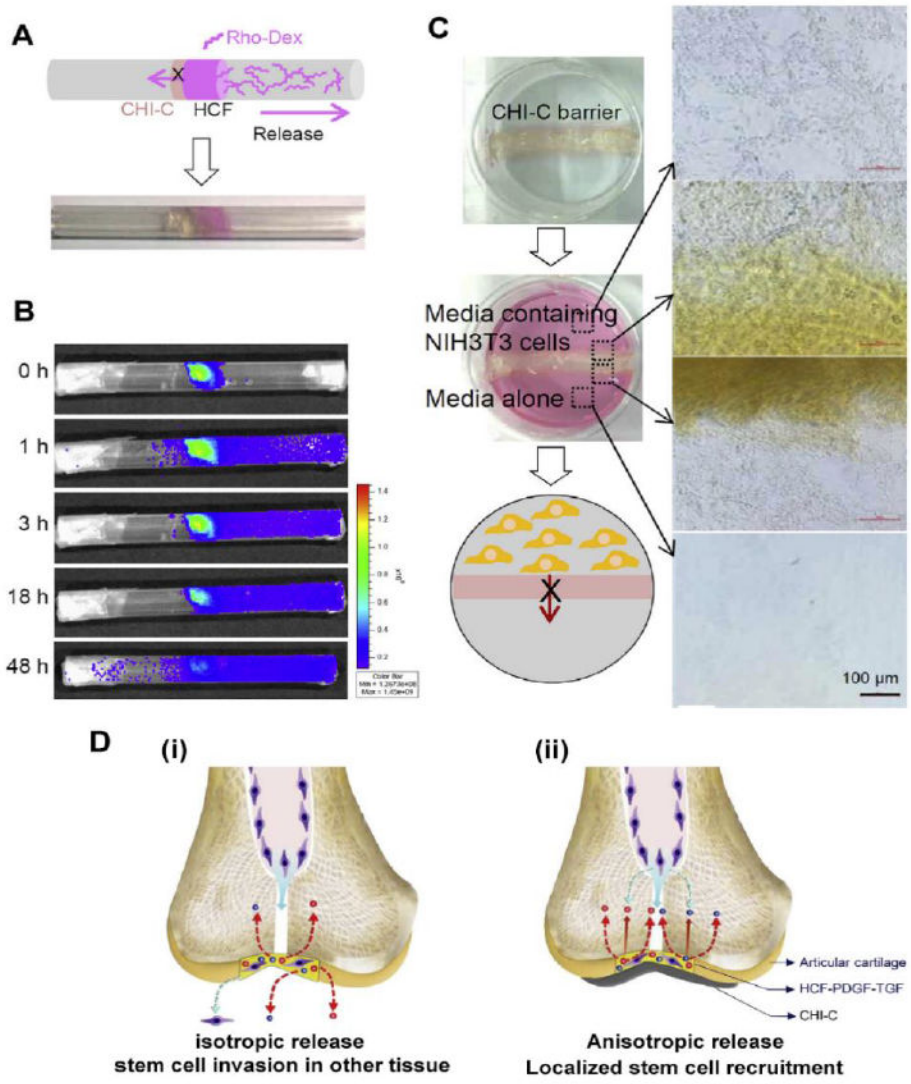

Figure 4.

Anisotropic release of growth factors for localized stem cell recruitment. **A**) Schematic illustrations of the directional release of the model polymer rhodamine dextran (Rho-Dex) from water-resistant catechol-conjugated chitosan (CHI-C) adhesive gel patch. **B)** Fluorescent images of the released Rho-Dex in heparin-conjugated fibrin (HCF) gels at pre-determined time intervals up to 48 hours. **C)** Illustration describing the inhibition of the cell migration using the CHI-C gel patch. The side pictures indicate the different layers of the designed system. **D)** *In vivo* functional mechanism of the CHI-C barrier for effective stem cell recruitment and articular cartilage repair.

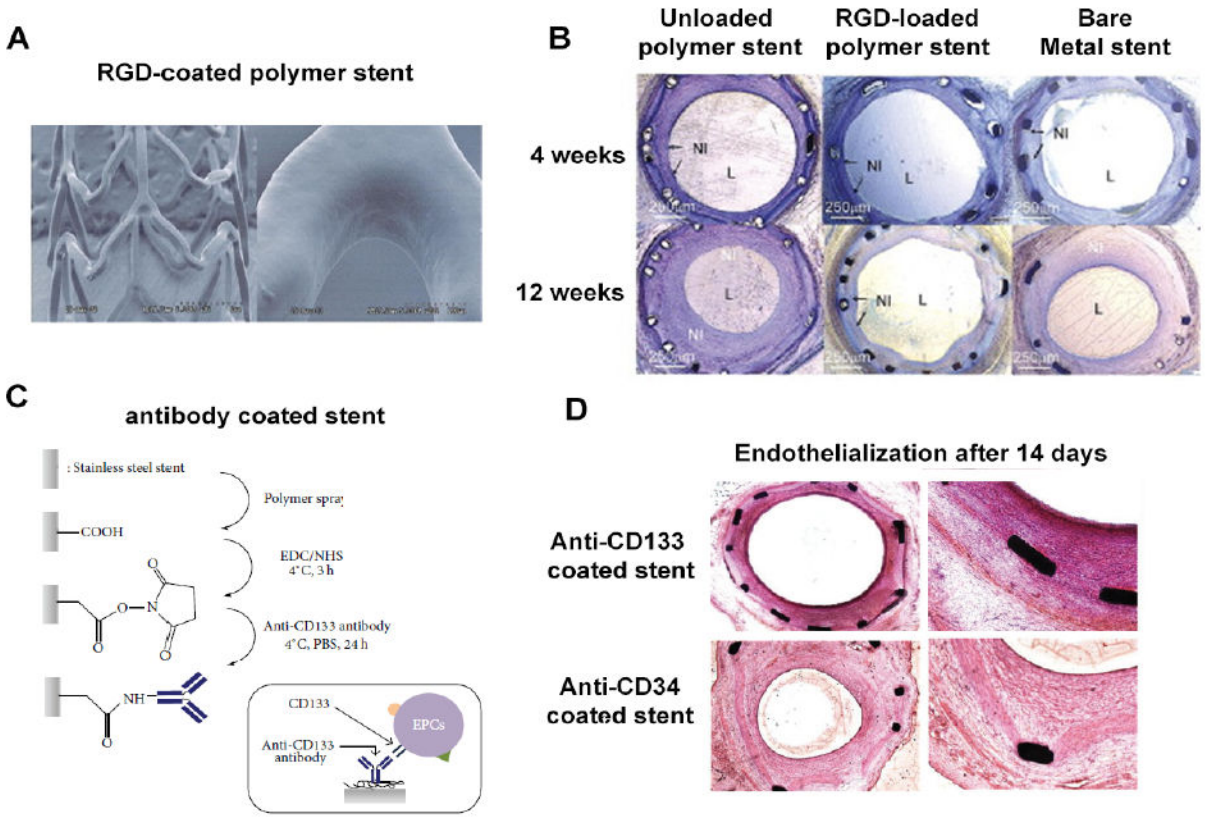

Figure 5.

Strategies for endothelialization of stents. **A)** SEM images of RGD-coated polymer stent displaying uniform coating distribution on the surface. **B)** Representative photomicrographs of coronary tissue sections used to determine stenosis rates after implantation of unloaded polymer, integrin-binding cyclic Arg-GlyAsp peptide (cRGD)-loaded, or bare-metal stents after 4 and 12 weeks Scale bars = 250 μm. The letter L stands for lumen, NI for neointima and the arrows indicate the neointimal area. **C)** Covalent conjugation of anti-CD133 antibody on stainless steel stent using ethyldicarbodiimide and N-hydroxysuccinimide EDC/NHS chemistry. The presence of the antibody is necessary for the capture of endothelial progenitor stem cells (EPCs) **D)** Hematoxylin and eosin staining of stents coated with anti-CD133 and anti-CD34 antibodies after 14 days of implantation in coronary arteries of small pigs. The coating with the anti-CD133 was more effective in promoting endothelialization of the stent.

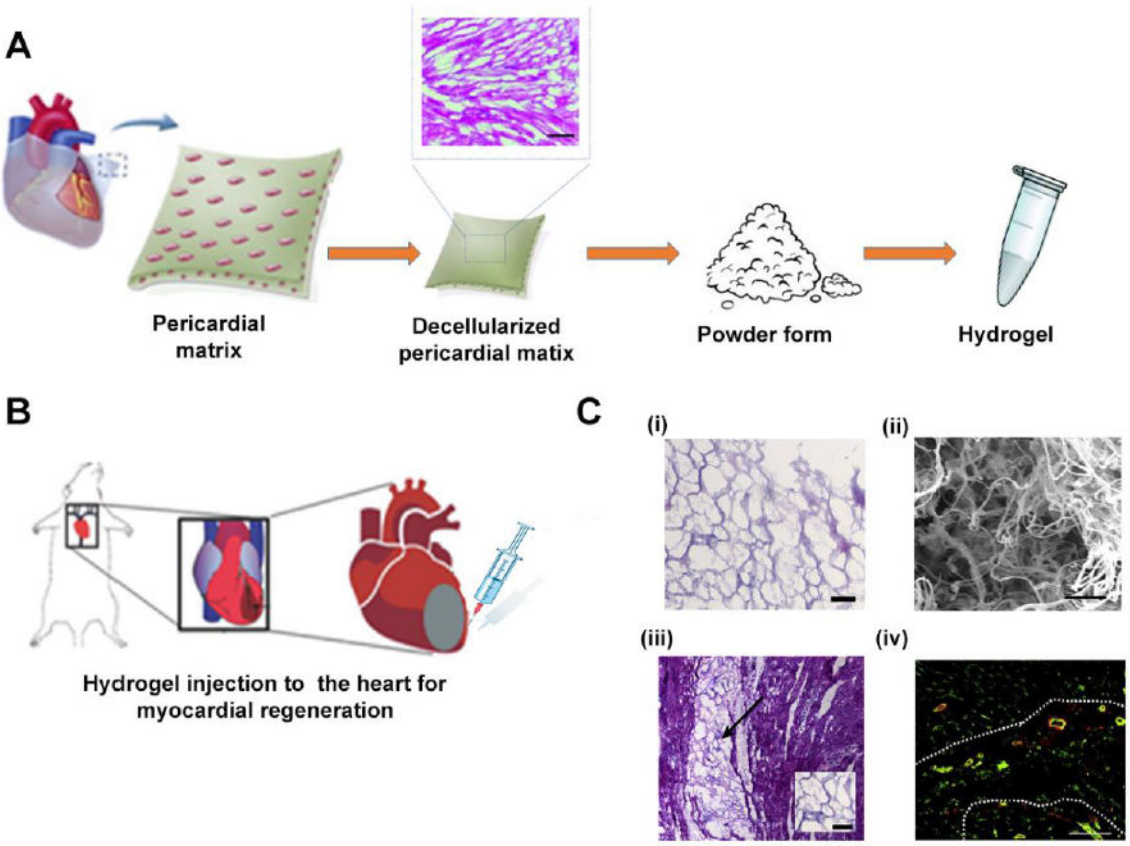

Figure 6.

ECM based scaffold for cardiovascular regeneration. **A)** Schematic representing the steps necessary for the fabrication of ECM based hydrogel derived from pericardial matrix. **B)** The ECM based hydrogels can be injected into the peri-infarct area to promote stem cell recruitment and myocardial regeneration. **C)** (i) Hematoxylin and eosin staining of decellularized myocardial matrix. Scale bar = 100 μm. (ii) SEM image of the myocardial matrix gel with nanofibers approximately 40–100 nm. Scale bar = 1 μm. (iii) Hematoxylin and eosin stained section of the myocardial matrix gel within the rat myocardium 30 min post-injection. Arrow indicates the area of the injected myocardial matrix. Insert image displays the decellularized intact myocardial ECM. Scale bar = 100 μm. (iv) Merged image showing endothelial cells (green) and smooth muscle cells (red) infiltrating into the myocardial matrix gel (white dots area), within the rat myocardium after 4 h post-injection. Scale bar = 100 μm.

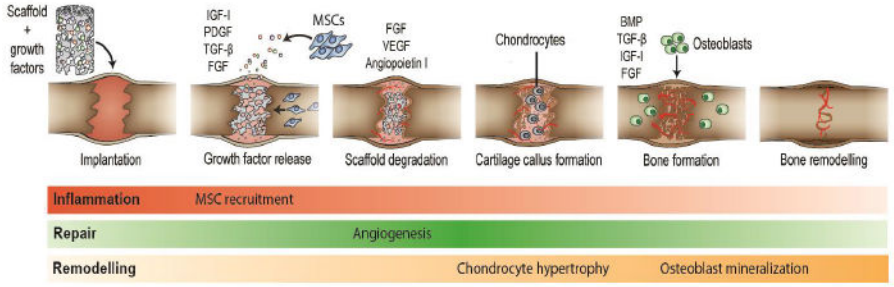

Figure 7.

Schematic representing the process of bone repair using a synthetic or ECM-derived scaffold loaded with growth factors to promote mesenchymal stem cells (MSCs) recruitment in the defect site. The different phases of bone regeneration and the main biological signals involved in each step are highlighted as they represent important targets for the design of novel therapeutic approaches to accelerate the process of bone repair. Main abbreviations: Insulin-like growth factor-I (IGF-I) platelet-derived growth factors (PDGF), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP).

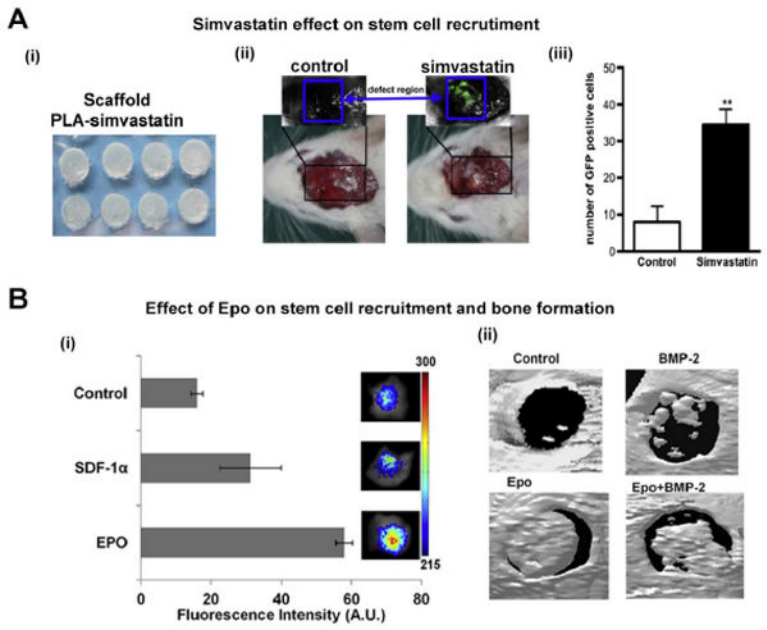

Figure 8.

Strategies for stem cell recruitment for bone regeneration. **A)** (i) Image displaying the PLA-simvastatin scaffolds prior implantation. (ii) Simvastatin recruitment of green fluorescent protein (GFP) labeled BMSCs to the bone defects *in vivo.* A higher GFP signal was detected in the simvastatin loaded scaffolds compared to the control group. (iii) Corresponding quantification of GFP positive cells recruited in the different scaffolds. (** = $p < 0.01$). **B**) (i) Quantification and fluorescent images of the recruited NIR dye labeled MSCs into microbubble scaffolds loaded with erythropoietin (Epo) and SDF-1α showing higher presence of MSCs in the Epo loaded system. (ii) Comparison of CT scan images of calvarial defects after 8 weeks, displaying a higher bridging effect in Epo loaded scaffolds compared to the other groups.

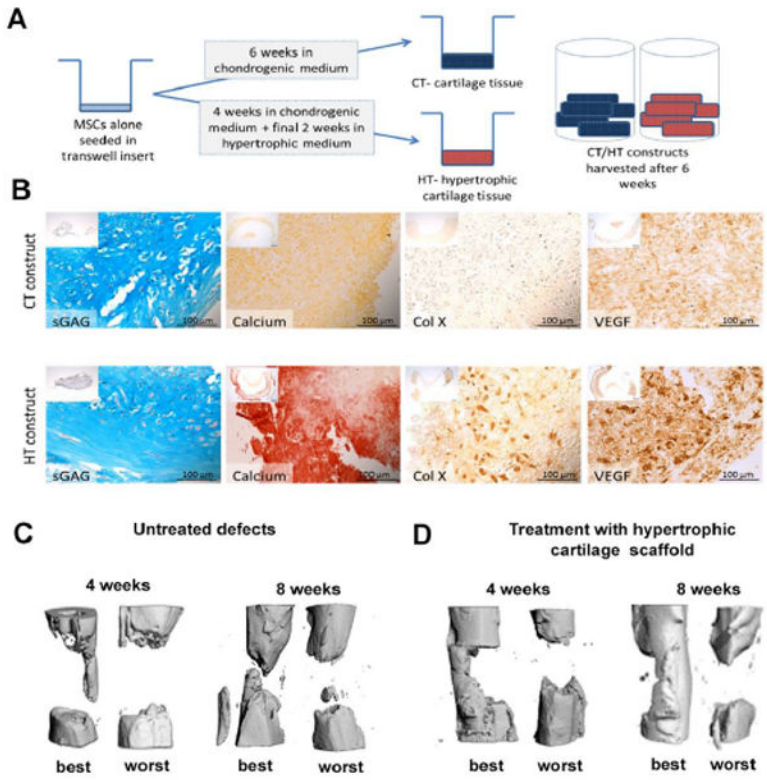

Figure 9.

In vitro hypertrophic cartilage ECM as strategy for bone regeneration. **A)** Schematic representing the difference between the preparation of cartilage tissue (CT) and hypertrophic cartilage ECM (HT). **B)** Histological analysis demonstrating positive deposition of GAG in both CT and HT ECM constructs. Higher calcium content and presence of both Col X and VEGF were found in the HT ECM group. Scale bar = 100 μm. **C-D)** Reconstructed μCT images of femoral defects left empty or treated with HT constructs harvested after 4 and 8 weeks *in vivo* showing the best and worst samples.

Table 1

Endothelialization of stents and vascular grafts by recruiting EPCs in animal models

Type of Implant	Coating	Linking strategy	Implantation site	Ref.
Stent	LLA/MBC Anti-CD33	Covalent conjugation	porcine coronary artery	[140]
Stent	Gelatin/Anti-CD33 Anti-CD34	Physical entrapment	rabbit abdominal aorta	[141]
Stent	HBC/Anti-CD33	Physical adsorption	porcine coronary artery	[142]
Stent	PEG/anti-human VE-cadherin	Covalent conjugation	rabbit iliac artery	[143]
Vascular graft Gelsoft™	FN/SDF-1α	Physical adsorption	ovine carotid artery	[144]
Vascular graft ePTFE	Anti-CD34	Covalent conjugation	porcine carotid artery and internal jugular vein	[145]
Vascular graft	PLCL/Substance P	Covalent conjugation	subcutaneous implantation in rats	[146]

Abbreviations: L-lactide (LLA), 5-methyl-5-benzyloxycarbonate-1,3-dioxan-2-one (MBC), Hydroxybutyl chitosan (HBC), polyethylene glycol (PEG), Fibronectin (FN), stromal derived factor-1α (SDF-1α), expanded polytetrafluoroethylene (ePTFE), poly(L-lactide-co-ε-caprolactone).