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Complete List of Authors:	Amirsadeghi, Armin; Shiraz University, Jafari, Arman; Shiraz University Eggermont, Loek; Northeastern University, Chemical Engineering Hashemi, Seyyedeh-Sara; Shiraz University of Medical Sciences Bencherif, Sidi; Northeastern University, Chemical Engineering; Harvard University, John A. Paulson School of Engineering and Applied Sciences Khorram, Mohammad; Shiraz University,

Vascularization Strategies for Skin Tissue Engineering

Armin Amirsadeghi[a], Arman Jafari[a], Loek J. Eggermont[b], Seyedeh-Sara Hashemi[c], Sidi A. Bencherif[b,d,e,*], Mohammad Khorram[a,*]

[a] Department of Chemical Engineering, School of Chemical and Petroleum Engineering, Shiraz University, Shiraz 71348-51154, Iran

[b]Department of Chemical Engineering, Northeastern University, Boston, MA 02115, United States

[c]Burn & Wound Healing Research Center, Shiraz University of Medical Science, Shiraz 71345-1978, Iran

[d]Department of Bioengineering, Northeastern University, Boston, MA 02115, United States

[e]John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02128, United States

* To whom correspondence should be addressed:

Sidi A. Bencherif. E-mail: s.bencherif@northeastern.edu

Mohammad Khorram. E-mail: mkhorram@shirazu.ac.ir

Abstract

A number of drawbacks in skin grafting for wound healing have drawn researchers to focus on skin tissue engineering as an alternative solution. The core idea of tissue engineering is to use scaffolds, cells, and/or bioactive molecules to help the skin to properly recover from injuries. Over the past decades, the field has significantly evolved, developing various strategies to accelerate and improve skin regeneration. However, there are still several limitations that should be addressed. Among these challenges, vascularization is known as a critical challenge that needs thorough consideration. Delayed wound healing of large defects results in an insufficient vascular network and ultimately ischemia. Recent advances in the field of tissue engineering paved the way to improve vascularization of skin substitutes. Generally, these solutions can be classified into two categories as (1) use of growth factors (GFs), reactive oxygen species (ROS)-inducing nanoparticles (NPs), and stem cells to promote angiogenesis, and (2) *in vitro* or *in vivo* prevascularization of skin grafts. This review summarizes the state-of-the-art approaches, their limitations, and highlights the latest advances on therapeutic vascularization strategies for skin tissue engineering.

Keywords

Skin, Tissue engineering, Angiogenesis, Growth factor delivery, Nanoparticles, Scaffolds

1. Introduction

Skin is the largest organ of the body, making up 15% of human body weight and comprising an area of 1.8 m^2.[1, 2] This gigantic organ is responsible for a number of critical functions. It plays important roles in sensation, temperature regulation, water evaporation, and most importantly, it acts as a physical barrier that protects internal organs against the external environment.[3, 4] The skin is composed of three layers: epidermis, dermis, and hypodermis.[5] The epidermis, the outermost layer of the skin, provides a barrier against exogenous physical, chemical, and biological hazards. This layer, which is mostly comprised of keratinocytes, has no intrinsic vascular network. Consequently, for their survival, these cells depend on oxygen and nutrient supply from the microvascular networks in the dermal layer.[2, 6] The second layer, the dermis, has a complex architecture consisting of various components with pivotal roles in skin function. Fibroblasts, being the most abundant cells in the dermal layer, are responsible for synthesizing the extracellular matrix (ECM), a scaffold providing physical strength and elasticity.[2, 6] This layer also contains a lymphatic system that has several fundamental functions, including the regulation of immune responses and maintenance of regular tissue pressure via removal of waste products and interstitial fluid.[7-11] The blood vascular network is another important component of the dermis. Blood vessels are responsible for transporting oxygen and nutrients to cells within the skin.[12, 13] The final and innermost layer of skin is the hypodermis, a vascularized adipose tissue that is important for the preservation of body temperature and fat storage.[2]

Upon injury, the integrity of these layers is disrupted and consequently, rapid wound healing is required to restore skin functions. This is a complex, well-orchestrated cascade of events that includes hemostasis, inflammation, proliferation, maturation and remodeling,[1, 14] which could be accelerated by dressings that keep the wound area moist.[15] Traditionally, wound dressings like gauzes and tulles have been applied to open wounds for this purpose. However, they are

not suitable for patients requiring long-term treatments or with substantial exudating wounds.[16, 17] Recently, more advanced polymeric dressings, including dressings composed of poly(vinyl alcohol),[18] chitosan,[19] polyurethane (PU),[20] polycaprolactone (PCL),[21, 22] and alginate[23] have been used that do not only keep the wound moisturized, but also exhibit antibacterial activity to prevent infection of the wound site. As the size and depth of a wound increases, the complex wound healing process may not occur properly, leading to delayed or improper wound contraction.[24] In this case, treatments are required to support wound healing. The use of skin grafts (i.e., autografts, allografts, and xenografts) is currently the gold standard to promote complex wound repair. However, this approach encompasses several major drawbacks, including the need to create a new wound at the donor site, lack of donor tissues, susceptibility to infections, and rejection of the grafts.[25-29]

Tissue engineering is a promising alternative that could address several of these drawbacks. Through the use of biomaterials, bioactive molecules, cells and their combination, tissue engineering aims to develop engineered scaffolds that can assist tissue reconstruction.[30] Over the past two decades, a large number of researchers has used natural/synthetic polymers,[31-33] proteins,[34] and lipids[35] to fabricate hydrogels.[36, 37] Additionally, nano/micro-fibers,[38-40] nano/micro-particles, foams,[41] and sponges[42-44] have been used as tissue scaffolds. These scaffolds are designed to cover and interact with the damaged wound site to accelerate and optimize the healing process. Although tissue engineering could circumvent many of the challenges associated with skin grafts, the success of these approaches is still limited due to their inability to supply blood and nutrients in the early stages of wound healing.[45] The largest distance for efficient oxygen and nutrient diffusion from a blood vessel is limited to just 200 μm.[46, 47] As a result, cells suffer from oxygen deprivation and nutrient shortage when they are situated further away from a capillary. This limits cell proliferation, which is essential for wound repair and contraction. Thus, a successful tissue engineering approach needs to provide

a construct that supports blood vessel formation, leading to a sustainable supply of oxygen and nutrients to the cells.[48]

Although vascularization could take place via growth and invasion of the patient's own blood vessels into the construct, this process is usually slow.[49] Several studies have reported that new blood vessels can form at a rate of approximately 5 μm/h,[50] which indicates that it will take several weeks to achieve complete vascularization of relatively large (few millimeters long) wounds.[45] Non-vascular cells are unable to survive extended nutrient and/or oxygen deficiency. Additionally, incomplete vascularization leads to non-uniform nutrient and oxygen gradients within the scaffolds. This causes increased survival and proliferation of cells at the scaffold borders, resulting in a non-uniform cell density.[51] Ultimately, complications induced by insufficient vascularization can result in infections, partial necrosis, delayed healing and immune reactions leading to scaffold rejection.[52, 53]

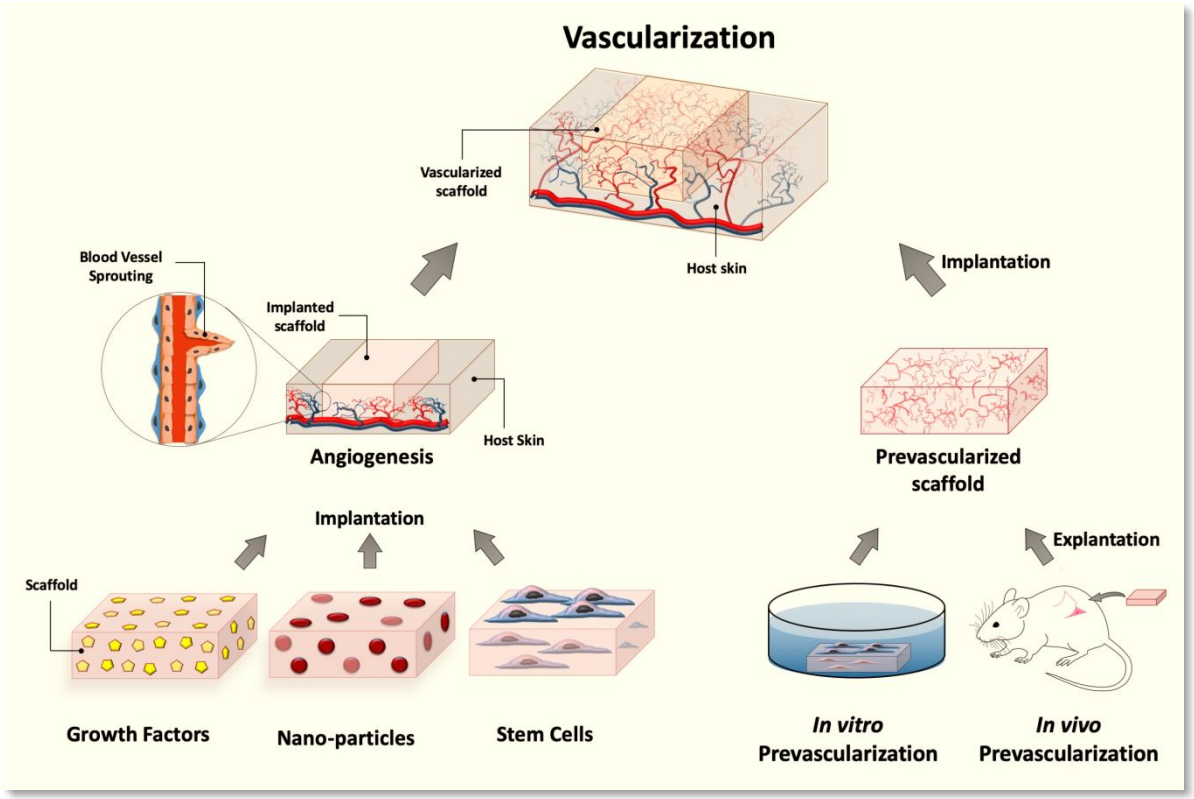

Figure 1. State-of-the-art strategies to promote the vascularization of skin substitutes.

To address these challenges, the development of new strategies to boost vascularization of skin scaffolds have been actively investigated (**Figure 1**). Broadly, these strategies can be classified into two approaches: (1) angiogenesis and (2) prevascularization. Angiogenesis comprises all the strategies that stimulate blood vessel formation inside the scaffold once implanted. This strategy itself can be subdivided based on the utilization of growth factors (GFs), reactive oxygen species (ROS)-inducing nanoparticles (NPs), or stem cells. Prevascularization, on the other hand, is known as a technique that introduces scaffold vascularization prior to its clinical application in patients.[54] A major benefit of this approach is that prevascularized scaffolds can be rapidly integrated within the patient's vascular network to prevent vasculature deficiency.[55] This approach can be subdivided into *in vitro* and *in vivo* approaches. In this review, we will cover the state-of-the-art strategies, their limitations, and highlights the latest advances on therapeutic vascularization strategies for skin tissue engineering.

2. Strategies for angiogenesis

2.1. Angiogenic growth factors

GFs comprise a class of proteins that can manipulate cell activity, including cellular metabolism, differentiation, proliferation, recruitment, and morphogenesis.[56] GFs play a substantial role in various phases of the wound healing process.[57] When the barrier function of skin is disrupted by an injury or a disease, cells in the damaged area begin to secrete signaling molecules that alert the surrounding tissues. Subsequently, the wound healing process starts when the release of growth factors like epidermal GF (EGF), fibroblast GF (FGF), and transforming GF (TGF) from different sources stimulates epithelial migration and proliferation. Additionally, release of vascular endothelial GF-A (VEGFA), angiopoietins and platelet-derived GF (PDGF) at the wound site stimulate angiogenesis.[58-60] **Table 1** summarizes

the most important angiogenic GFs and their functions during wound healing. These GFs work in a sequence that starts by vessel destabilization, followed by endothelial cell (EC) migration and/or proliferation to form new blood vessels, and finally vessel maturation to complete the angiogenic process.[61] A detailed overview of this process is depicted in **Figure 2.**

Table 1. GFs and their roles in angiogenesis.

Growth factors	Relevant activity	Ref
Vascular Endothelial Growth Factor-A (VEGFA)	• EC migration and sprouting • EC proliferation • EC survival	62-66
Angiopoietin-1 (Ang-1)	• Stabilizes vessels by reinforcing interactions of ECs, smooth muscle cell and pericytes • Promotes EC survival	62, 65, 67
Angiopoietin-2 (Ang-2)	• Destabilizes vessels by weakening EC-smooth muscle cell-pericyte interactions • Stimulates EC migration	62, 65, 67
Platelet-Derived Growth Factor (PDGF)	• Stabilizes and matures the nascent vessels by recruiting smooth muscle cells and pericytes	65, 68, 69
Basic Fibroblast Growth Factor (bFGF)	• EC migration • EC proliferation • EC survival • Capillary growth stimulation	63, 65
Placental Growth Factor (PlGF)	• Stimulates angiogenesis by enhancing VEGFA efficacy • Recruitment of monocytes/macrophages	70, 71
Transforming Growth Factor (TGF)	• Stabilizes nascent blood vessels by increasing ECM deposition	72

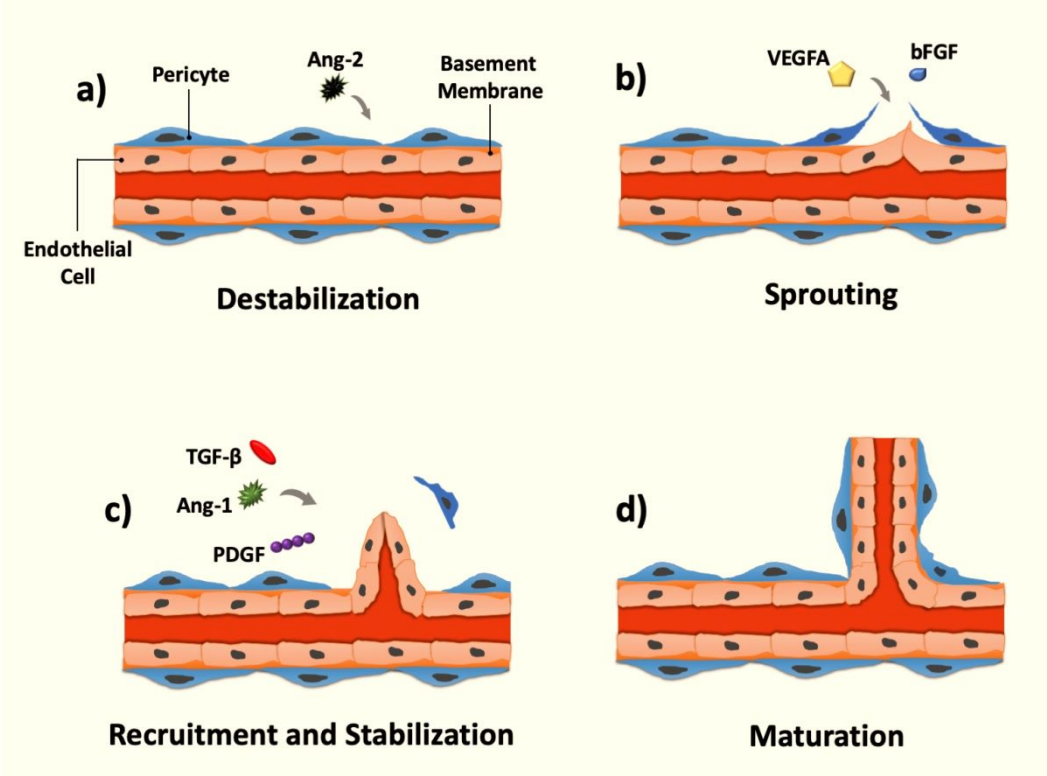

Figure 2. Sequential roles of growth factors during angiogenesis; (a) Ang-2 released as a result of hypoxia destabilizes pericyte/EC interactions, leading to pericyte detachment; (b) VEGFA and bFGF stimulate sprouting and proliferation of exposed ECs, creating a new branch; (c) PDGF release leads to the recruitment of pericytes, while Ang-1 stabilizes pericyte/EC interactions, and TGF-β increases basement membrane deposition, all together promoting maturation of nascent blood vessels; (d) mature blood vessel.[73-75]

Although these GFs are essential for wound healing, their low concentrations often lead to poor vascularization, tissue hypoxia, and as a result, an inability to prevent cell necrosis.[76] Consequently, researchers and clinicians have tried to supply additional GFs to enhance angiogenesis and ultimately wound healing.[77]

2.1.1. Vascular endothelial growth factor

VEGFA is a member of the platelet-derived growth factor family and the main GF that regulates angiogenesis inside the human body. VEGFA plays an essential role in both physiological and pathological angiogenesis via the stimulation of EC proliferation.[78, 79] Leung

et al. first identified and purified VEGFA, and showed that VEGFA could potentially act as a mitogen for vascular ECs.[80] Later studies demonstrated that VEGFA also has considerable effects on other types of cells,[81] making this protein one of the most investigated GFs in the fields of tissue engineering and wound healing.[82-88] For instance, Demeter et al. showed that keratinocyte-derived VEGFA is a major player in the regulation of skin vascularization.[89] Furthermore, Supp and Boyce demonstrated that overexpression of VEGFA in cultured skin substitutes promotes accelerated graft vascularization while significantly reducing graft contraction, resulting in enhanced full-thickness wound healing.[90]

2.1.2. Angiopoietins

Angiopoietins are another group of GFs that have critical roles in neovascularization. Ang-1 reinforces the interactions of ECs with smooth muscle cells and pericytes, leading to the maturation of newly formed capillaries and blood vessels.[91-93] Suri et al. demonstrated the angiogenic properties of Ang-1 by generating transgenic mice that overexpressed Ang-1 in skin.[94] These transgenic mice showed increased size, density and branching of blood vessels compared to wildtype mice. Furthermore, this study suggested that the overexpression of Ang-1 in combination with VEGF could further increase the number, size and branching patterns of blood vessels. This finding demonstrates that combination regimens may be suitable for therapeutic angiogenesis. On the other hand, Ang-2, which is generally secreted by ECs in tissues during inflammation and vascular renewal, loosens the interactions of ECs with smooth muscle cells and pericytes.[62] More specifically, Ang-2 is an antagonist to Ang-1 since they both compete for the same receptor (Tie-2). Although Ang-2 may initiate the angiogenesis cascade, elevation of Ang-2 expression results in vascular regression and decreased VEGFA levels.[95-97] Therefore, Ang-2 has mostly been investigated for its anti-angiogenetic effect in other medical conditions such as tumor therapy.[98]

2.1.3. Platelet-derived growth factors

PDGF, another key GF, promotes the maturation and stabilization of newly formed blood vessels and prevents vessel regression via the recruitment and activation of pericytes and smooth muscle cells.[62, 99, 100] Amaral et al. investigated the impact of PDGF on vascularization using a collagen–glycosaminoglycan scaffold infused with human umbilical vein endothelial cells (HUVECs) and multipotent mesenchymal stromal cells (MSCs). This study demonstrated that early addition of PDGF does not significantly alter vascular regression compared to PDGF-free control scaffolds.[101] On the other hand, late and continued exposure to PDGF prevented vessel regression in these scaffolds. In another study conducted by Wan et al., a dual layer gel scaffold was used to deliver PDGF for diabetic wound healing.[102] This scaffold contained a top layer of a silver-loaded gelatin gel and a bottom layer of a PDGF-loaded three-dimensional (3D) printed gelatin gel. *In vivo* tests showed that these PDGF-loaded scaffolds can improve vascular and granular tissue formation and induce accelerated re-epithelization.

2.1.4. Other growth factors

In addition to the GFs described above, recent studies have established that other GFs, such as basic fibroblast GF (bFGF), can also play an indirect role in the regulation of angiogenesis.[103] bFGF is mainly involved in tissue reconstruction as a factor to mediate survival, proliferation, migration, and differentiation of cells.[104] Additionally, this particular GF has an important role in angiogenesis by stimulating the expression of other GFs such as VEGFA, PDGF, and hepatocyte GF.[105] However, bFGF-mediated angiogenic stimulation is indirect, as it is still VEGFA dependent for the formation of new blood vessels.[103]

The combination of VEGFA and FGF2 has also been shown to synergistically promote vascularization. In a study by Nillesen et al., five different scaffolds (collagen,

collagen/heparin, collagen/heparin with VEGFA or FGF2, collagen/heparin with both VEGFA and FGF2) were subcutaneously implanted in 3-month-old Wistar rats to evaluate how these GFs would induce vascular network formation. Hydrogels that contained both VEGFA and FGF2 induced the highest number of blood vessels with more mature vessels.[106]

Placenta GF (PlGF) is another pro-angiogenic protein that promotes skin vascularization both independently and by enhancing VEGFA-driven angiogenesis.[107] Furthermore, it was demonstrated that PlGF-induced vessels are more mature and stable than VEGFA-induced vessels as a result of this dual functionality.[108] Applications of other GFs involved in angiogenesis are reported in **Table 3**.

2.2. Growth factor delivery systems

Although GFs are essential to promote vascularization, they often have a short effective half-life due to their poor stability or fast blood clearance, which can limit their use for regenerative applications.[92] As a result, various strategies have been used to achieve a therapeutic effect at the wound site (**Figure 3**).[109] In some cases, growth factors are used in higher concentrations than present in physiological conditions.[110] However, this may lead to adverse side effects, such as cancer development.[111] These drawbacks, in addition to a demand for an adequate therapeutic outcome, highlight the critical need for drug delivery systems (DDS).[112] In the field of skin tissue engineering, DDS for the delivery of GFs can be divided into (a) polymer-based micro/nanostructures,[113, 114] (b) lipid micro/nanostructures,[115, 116] (c) hydrogels,[117, 118] (d) smart scaffolds,[119] and (e) any combinations of these groups.[120]

With the exception of smart and responsive systems, the release kinetics of GFs from nanofibers, hydrogels, and polymeric micro and nanospheres are generally based on the matrix degradation and/or protein diffusion rate.[121, 122] Although diffusion-controlled systems for the

sustained release of GFs have been reported,[123] most delivery systems rely on degradation, which is easier to control.[124, 125]

To achieve a better control over GF release rates, dual DDS have been investigated in which GFs were incorporated into micro or nanospheres before being embedded into a scaffold.[126-129] Compared to free GF, pre-encapsulating GF guaranteed a prolonged GF release from the scaffolds. For instance, by loading insulin-like GF I (IGF-I) into poly(lactic-co-glycolic acid) (PLGA) microparticles embedded in a silk fibroin matrix, Wenk et al. achieved a more sustained release when compared to scaffold-free IGF-I-loaded microparticles.[130] In their study, dual-encapsulation (i.e., embedding IGF-I-loaded microparticles in a scaffold) could decrease the release rate of IGF-I by at least 50% in comparison to bare IGF-I-loaded PLGA microparticles. In another study, Jiang et al. used PCL nanofibers together with VEGFA-loaded gelatin particles as their DDS.[131] Their platform showed a fast release profile, releasing 50% of VEGFA in the first 2.5 days, and exhibited a more sustained delivery over the next 7.5 days, reaching up to 80% release of their payload. As this scaffold stimulated MSC differentiation, it could be implemented for promoting microvascular formation and vessel maturation.

Each of the GFs participating in vascularization and/or the healing process has its own temporal and spatial specificity, which emphasizes the need for the controlled sequential release of multiple factors from the same scaffold.[57, 132] As indicated previously, VEGFA induces angiogenesis, while PDGF promotes blood vessel maturation. Richardson et al. were the first to fabricate a system for controlled delivery of two different GFs (VEGFA and PDGF) with different release kinetics.[133] To that end, they blended VEGFA with a PLGA polymer matrix, while PDGF was encapsulated in microspheres of same polymer. The polymer degradation rate was fined tuned by varying both PLGA molecular weight and the lactic acid/glycolic acid ratio. When tested in animals, the synergistic delivery of VEGFA and PDGF from their DDS resulted

in the formation of a dense and mature vascular network.

To promote wound healing, Lai et al. fabricated collagen and hyaluronic acid (HA)-based nanofibers embedded with gelatin NPs to form skin substitutes that exhibit stepwise release of multiple angiogenic GFs.[134] PDGF-loaded gelatin NPs and EGF were incorporated into collagen nanofibers. Similarly, VEGFA-loaded gelatin NPs and bFGF were incorporated into HA-based nanofibers. *In vitro* GF release studies demonstrated that embedding GFs into NPs resulted in slower release rates in comparison to GFs that were directly blended with the nanofibers. Moreover, in contrast to HA-based nanofibers, the encapsulation of GFs in collagen nanofibers resulted in a more sustained release. This composite scaffold enhanced growth of HUVECs and formation of thread-like tubular structures *in vitro*, as well as enhanced wound healing on streptozotocin-induced diabetic rats.

2.2.1. Core-shell structures

Another technique for the controlled and sequential release of GFs is based on electrospun core–shell structures (**Figure 3**).[135] This method has been extensively utilized to encapsulate delicate bioagents such as GFs to preserve their bioactivity and control their release.[136, 137] In this method, a shell layer acts as a physical barrier to slow down the release of GFs entrapped in the core structure.[138, 139] Adjusting the composition and structure of these constructs allows for controlling and fine tuning GF release rates. During the fabrication process, the use of core-shell structures also prevents direct contact of water-soluble GFs with organic solvents, thereby preserving their bioactivity.[140-142] On the other hand, this technique has the potential for the controlled and sequential delivery of various GFs through the core layer, shell layer or both layers.[138, 143]

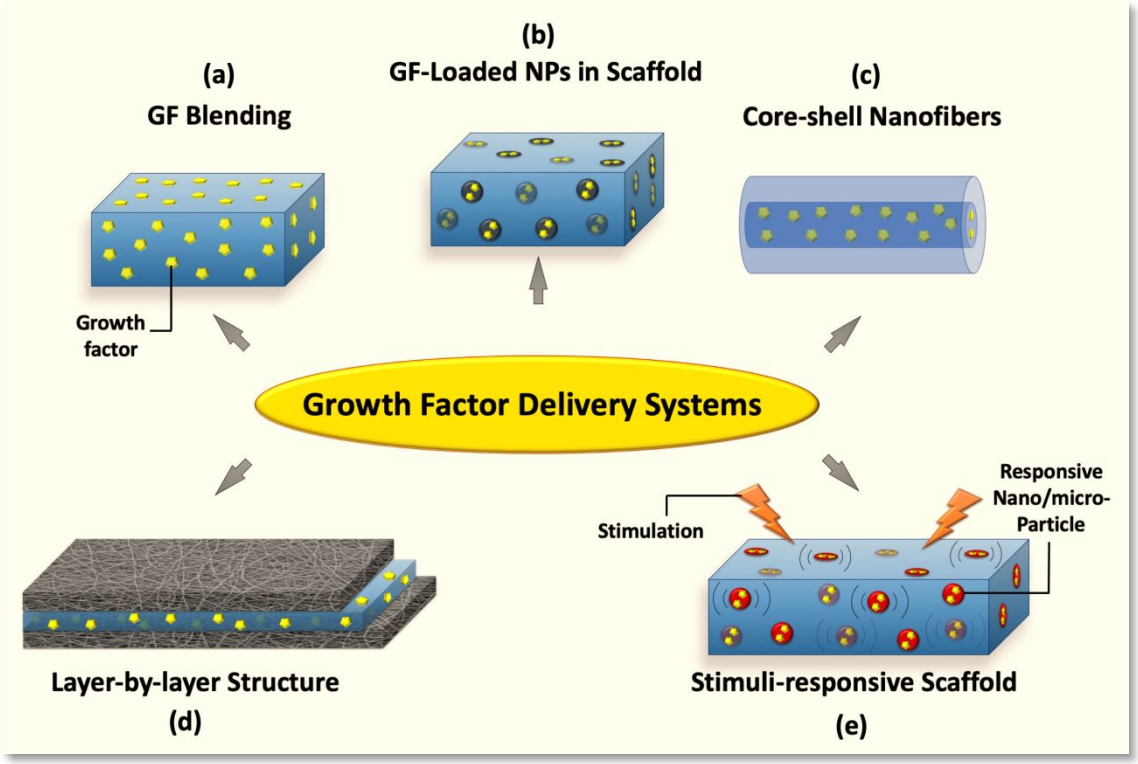

Figure 3. Schematic representation of the main GF delivery strategies used in skin tissue engineering. (a) GFs are blended with biopolymers to form the scaffolds, (b) GFs are first embedded within nano/micro structures and then blended with biopolymers to form composite scaffolds, (c) core-shell nanofibrous structure in which GFs are encapsulated in the core layer, (d) layer-by-layer structure in which GFs are embedded to offer prolonged release, and (e) stimuli-responsive scaffolds that offer on-demand GF release.

Core-shell nanofibers have been fabricated with two coaxial capillaries to electrospin different polymers and form simultaneously the core and shell structures.[135] In another approach, emulsion electrospinning has been used in which the dispersed phase formed the core layer while the continuous phase produced a shell layer.[144, 145] Zhang et al. performed a study comparing bFGF release from various core-shell nanofibers, containing either a chitosan hydrogel core or PLGA-based emulsion-core (PEG-b-PLGA/heparin, PLGA/heparin). All nanofibers contained a PEG-b-p(lactide-co-caprolactone)-based shell layer.[146] Nanofibers with a hydrogel core exhibited a burst release within the first 5 days, followed by a slow and sustained GF release, reaching up to 90% payload delivery after 35 days. However, emulsion-core nanofibers showed a faster GF release, reaching up to 90% of their payload within 35

days.

Emulsion electrospinning was also utilized for the controlled release of VEGFA. For example, negative-voltage emulsion electrospinning has been applied to make use of electrostatic interactions between positively charged VEGFA molecules and a negatively charged scaffold.[147] This method resulted in steady VEGFA release that was sustained for up to 18 days. Sandwich or layer-by-layer approaches can also be used for GF delivery. In this approach, sequential layering or electrospinning of different polymer solutions is used to provide sustained GF release and preserve their intrinsic bioactivity. The release mechanism of this system is similar to that of core-shell structures. The middle layer consists of water-soluble polymers loaded with GFs, which helps to protect these GFs from organic solvents used for the fabrication of the outer layer. Moreover, the outer layer acts as a physical barrier to allow sustained release of entrapped agents.[56] Zhao and Wang investigated this fabrication method and compared it with the other electrospinning techniques.[148] They fabricated PLGA/bFGF single layer (type 1), bilayer (type 2) and trilayer nanofibers with sequential electrospinning of cellulose acetate (CA) and bFGF/PLGA emulsion (type 3). Additionally, they fabricated core-shell structures using coaxial electrospinning of bFGF-containing PLGA as core and CA as shell (type 4). They showed that a burst release of 60% of bFGF occurred within the first three days when using type 1 nanofibers as a DDS. On the contrary, addition of CA to this system significantly hindered this fast burst release of bFGF via electrostatic interactions. Furthermore, their results indicated that type 2, type 3, and type 4 fibers exhibited a more desirable sustained release profile, showing a bFGF release of 80%, 40%, and 60% after 15 days, respectively. Among these samples, type 4 fibers displayed the longest release profile, reaching up to 75% GF release over 28 days. Taken together, co-axial and emulsion core-shell strategies could significantly improve GF release, providing a superior delivery

strategy. However, these methods often rely on the optimization of many process parameters, making this approach challenging.

2.2.2. Smart release systems

Although the aforementioned strategies can be used to achieve desirable sustained release profiles in some cases, in other situations there is a need for systems that can respond to local or external stimuli for controlled drug release.[149] These types of DDS are called smart or "release on-demand" systems. Based on the specific stimuli they respond to, smart delivery systems can be classified as (a) pH-responsive,[111, 150] (b) temperature-responsive,[150, 151] (c) light-responsive,[152, 153] (d) mechanical pressure-responsive,[154] (e) ultrasound-responsive,[155] (f) electric or magnetic field-responsive,[149] (g) enzyme-responsive,[156-160] and (h) ion-responsive.[161] These smart systems can offer an active release which can result in a more effective temporal release of therapeutic agents when needed during the process of wound healing. Furthermore, these systems can emulate aspects of wound healing mechanisms by releasing GFs in a sequential fashion.[162]

To promote vascularization, Lee et al. were among the first to develop stimuli-responsive GF-releasing scaffolds.[154] They fabricated an alginate hydrogel that released VEGFA upon mechanical stimulation. While alginate scaffolds exhibited a steady VEGFA release in a stimulation-free environment, VEGFA release was significantly increased following stimulation. When tested *in vivo*, non-obese diabetic mice had increased blood vessel formation upon stimulation. In another approach, Moncion et al. designed stimuli-responsive fibrin scaffolds with on-demand bFGF release capability.[163] In this strategy, focused ultrasound was used to trigger and controllably release their GF. Additionally, GF release was further controlled by varying several parameters, including the ultrasound intensity and exposure time.

Heparin-based DDS are inspired by the composition of the native extracellular matrix to effectively control the release of various GFs and proteins within the body.[164] Several polypeptides and GFs can bind to heparin or heparan sulfate, which helps the temporal, spatial, and sequential delivery of GFs, including FGF, hepatocyte GF, VEGFA and PDGF.[165, 166] Heparin binding can also slow down the degradation of GFs and in some cases improve their binding to cell surface receptors.[162, 167] Therefore, heparin-containing scaffolds have been widely investigated to control the release of GFs involved in angiogenesis.[168, 169] To date, heparin-containing scaffolds composed of fibrin,[162, 170] collagen,[171] alginate,[172-175] poly(ethylene glycol) diacrylate (PEGDA),[176] hyaluronate,[177] and pluronic,[178] were explored for this purpose. For instance, a study by Edelman et al. was one of the first to apply a system consisting of alginate microspheres loaded with bFGF-bound heparin-Sepharose beads. As shown in **Figure 4,** they showed that the binding between heparin and bFGF could be controlled by ionic interactions and enzymatic bond cleavage with heparinase to manipulate bFGF release as desired.[179] In a recent study by Rensburg et al., heparin and heparan sulfate were used to bind VEGFA and control its release rate from PEG hydrogels.[180] Once implanted in rats, these heparinized hydrogels were able to controllably release VEGFA and promote local tissue vascularization. In another study, Freeman et al. demonstrated that heparin-binding GFs can also bind to alginate sulfate with similar affinity.[173] In their later study, they fabricated alginate scaffolds and alginate/alginate sulfate scaffolds loaded with either VEGFA or a combination of VEGFA, PDGF-BB, and TGF-β1.[166] Alginate scaffolds displayed a significant burst release (~85%) within the first 2 days, while alginate/alginate sulfate scaffolds exhibited a sustained burst-free release, reaching up to 20% and 50% delivery of their payload after 2 and 8 days, respectively. Additionally, alginate/alginate sulfate scaffolds exhibited different release kinetics across the three GFs (VEGFA, PDGF-BB, and TGF-β1) investigated, most likely due to different binding affinities with alginate and/or alginate sulfate. Interestingly,

unlike alginate scaffolds, GF-loaded alginate/alginate sulfate scaffolds induced more mature and stable blood vessels when tested in animals. Additionally, several studies have focused on predicting more precisely the release profiles of these smart DDS by mathematical and computer modeling.[181-184] This could potentially minimize the need for trial-and-error experimentations, and ultimately reducing costs.[164] Although stimuli-responsive DDS offer many advantages, their widespread applications for skin vascularization remain limited. This is partially attributed to the challenges associated with targeted and controlled drug delivery as well as potential side effects.[149, 185, 186] The advantages and limitations for each release strategy are summarized in **Table 2**.

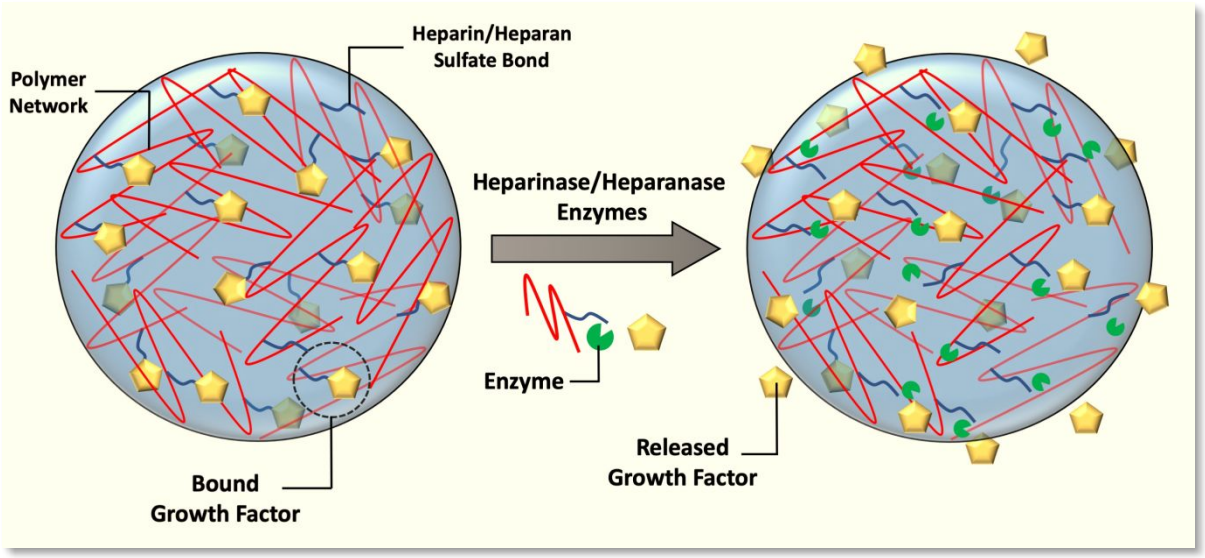

Figure 4. Active release mechanism of heparin-binding growth factors. Heparinase/heparanase enzymes cleave the heparin/heparin sulfate bonds, resulting in GF release.

Table 2. Advantages and limitations of different GF delivery systems.

Delivery system	Advantages	Limitations	Relative release time
Simple blending	• Simple fabrication	• Burst release • Lacks sequential release of multiple GFs • Lacks on-demand	• Short

		release	
NP-loaded scaffolds	• Control release • Simple fabrication • multiple GFs sequential release	• Lacks on-demand release	• Long
Core-shell NF	• Controlled release • multiple GFs sequential release	• Lacks on-demand release • Complicated fabrication process	• Long
Layer-by-layer structure	• Controlled release • Simple fabrication • Allows sequential release of multiple GFs	• Lacks on-demand release	• Moderate
Stimuli-responsive	• Controlled release • Allows sequential release of multiple GFs • On-demand release	• Complicated fabrication process • Requires external stimulation source • Potential side effects	• On-demand

2.3. ROS-inducing nanoparticles

Drawbacks associated with the use of GFs, such as their short half-lives and high cost, can be avoided by using materials that stimulate their production by cells *in situ*.[187, 188] During the inflammation phase in which neutrophils arrives at the wound site, cellular activity results in the production of ROS.[189] ROS (i.e., $O_2^{\bullet-}$) have unpaired electrons in their outer orbit, making them highly reactive.[190] Although ROS are cytotoxic and causing oxidative stress, EC dysfunction, and chronic inflammation at high concentrations,[191-193] they have shown to stimulate cell proliferation, migration, and differentiation at low concentrations. Furthermore, ROS can mediate angiogenic-related gene expression and GF secretion across various cell types.[192] The process by which ROS stimulate angiogenesis is schematically depicted in **Figure 5**.[190]

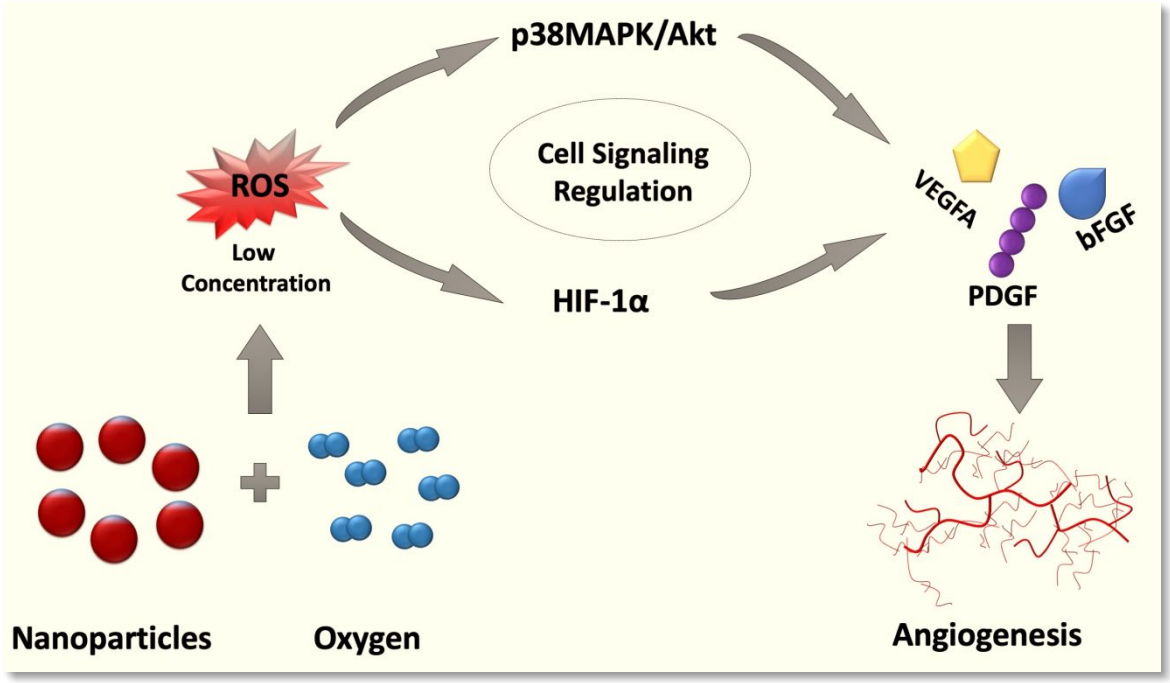

Figure 5. General mechanism of ROS-inducing NP-mediated GF release. While interacting with cells and tissues, NPs can induce ROS. At low concentrations, these ROS induce cell signaling through a number of pathways, including activation of HIF-1α and the p38MAPK/Akt pathway. These signaling pathways result in the release of angiogenic factors, such as VEGFA, PDGF and bFGF, that work together to promote angiogenesis.

Several studies have demonstrated that both metallic and non-metallic NPs can induce ROS formation and therefore contribute to angiogenesis.[194, 195] Examples of these materials include Europium(III) Hydroxide ($Eu(OH)_3$),[196-198] cerium oxide (CeO_2),[199] Zinc oxide (ZnO),[195, 200] Zinc peroxide (ZnO_2),[200] Titanium peroxide (TiO_2),[201, 202] Carbon nanotubes (CNT),[203] graphene oxide (GO),[204] silver (Ag),[205] and gold (Au).[206] Mukherjee et al. demonstrated that graphene oxide (GO) and reduced graphene oxide (rGO) exhibit pro-angiogenic properties at concentrations below 50 ng/mL.[204] For both GO and rGO, *in vitro* cell migration assays showed that GO and rGO (10-50 ng/mL) were more effective in stimulating HUVEC migration than VEGF (40 ng/mL). Moreover, in a Chick Chorioallantoic Membrane (CAM) assay, GO and rGO induced blood vessel formation comparable in size, junction and length to VEGF-induced vessels. However, at high GO and rGO concentrations, they observed disruption of preexisting

vasculatures and inhibition of new blood vessel formation, most likely due to excessive ROS-mediated oxidative stress.

Zinc has several important biological functions in the body and has been applied for skin treatment (e.g., acne, ulcers, infections).[207, 208] Therefore, zinc oxides (ZnO and ZnO_2) are intensively investigated in wound care.[208] A number of studies have also investigated the effect of zinc oxide NPs on angiogenesis. Augustine et al. studied the potential of ZnO to stimulate blood vessel formation and wound healing using ZnO-loaded PCL nanofibers.[187] Their findings suggested that scaffolds containing less than 2 wt.% ZnO NPs were cytocompatible and improved cell density *in vitro*. Furthermore, scaffolds containing 1 wt.% ZnO NPs boosted angiogenesis and promoted full-thickness wound healing 5 days following implantation in guinea pigs.[209] This effect was associated with a concentration-dependent increase of VEGFA and FGF secretion.

$Eu(OH)_3$ nanorods have also been studied *in vitro* and *in vivo* for their pro-angiogenic potential.[196, 197] For instance, Augustine et al. investigated this approach using PCL/$Eu(OH)_3$ nanofibrous scaffolds.[210] The presence of 0.5 wt.% $Eu(OH)_3$ nanorods in these scaffolds enhanced ECs adhesion, growth, and proliferation, and markedly upregulated expression of angiogenic proteins. The same team also investigated CeO_2 NPs for their capacity to stimulate angiogenesis.[211] In this study, they showed that PCL-based nanofibers containing 1 wt.% CeO_2 significantly improved HUVEC proliferation *in vitro*, as well as vessel diameter and branching in a CAM model. Additionally, they demonstrated that CeO_2 NPs promoted angiogenesis by upregulating angiogenic factors such as VEGFA and hypoxia-inducible factor (HIF)-1α. However, at higher CeO_2 NP concentrations, the nanofibers reduced cell viability *in vitro* and vessel density in the CAM model, and induced severe inflammatory responses *in vivo*. Collectively, these studies highlighted that the angiogenic properties of ROS-inducing NPs are concentration dependent. Therefore, the concentration of ROS-inducing NPs need to be tightly

controlled and optimized to achieve the desired results.[194]

2.4. Stem cell-laden scaffolds

Another approach to enhance angiogenesis at the wound site is to use cellularized scaffolds containing stem cells. This approach, like the use of DDS and NPs, is an attractive alternative to the direct application of GFs. Stem cells are involved directly or indirectly in the angiogenesis cascade.[212] They can secrete various GFs, including VEGFA, FGF and TGF-β, to induce migration and proliferation of ECs at the wound site. Alternatively, they can differentiate to ECs and form new blood vessels.[213-215]

Stem cells from different origins have been incorporated in scaffolds and investigated for their capacity to induce vascularization. These include sweat gland–derived MSCs,[216] placental MSCs,[217] adipose-derived MSCs (ADMSCs),[218-222] bone marrow-derived MSCs (BMSCs),[223, 224] Wharton's jelly-derived MSCs (WJ-MSCs),[225] glandular-derived stem cells,[226] pancreas-derived stem cells,[227] skin-derived stem cells,[228] endothelial progenitor cells (EPCs),[229] embryonic stem cells (PSCs),[230, 231] and induced pluripotent stem cells (iPSCs).[232, 233]

MSCs are most commonly derived from bone marrow, making BMSCs the main cells being investigated for their potential to promote blood vessel formation.[234] For instance, Chen et al. used an ion and light-based dual crosslinking technique to fabricate BMSC-laden collagen/gellan gum hydrogels that promoted BMSC differentiation into ECs.[224] Although BMSCs can be very effective in promoting tissue vascularization, their invasive harvesting and laborious isolation process make this approach challenging.[234, 235] Furthermore, the ability of BMSCs to differentiate and proliferate decreases with age. As a result, other sources (e.g., Wharton's jelly) to isolate MSCs have also been investigated.[234, 236]

The use of ADMSCs is an interesting alternative, as fat tissues are usually available in large

quantities.[237] Moreover, there is low morbidity at donor sites following fat tissue resection and up to 500-fold more stem cells can be harvested in comparison to bone marrow.[220] The potential of ADMSCs delivery for blood vessel formation during wound healing was examined by Eke et al., who incorporated ADMSCs into gelatin/HA-based hydrogels.[221] Their *in vivo* studies demonstrated that ADMSC-laden hydrogels induced a three-fold increase in vascular growth when compared to cell-free hydrogels. The process of vascularization was attributed to the release of paracrine signaling molecules by ADMSCs to surrounding cells.

Sweat gland–derived MSCs have also been investigated as they are easy to harvest and expand, making them a viable option for therapeutic applications.[237] A study by Danner et al. confirmed that combining these cells with collagen-based scaffolds resulted in a high proliferation rate and a capacity to form microvessels in a full-thickness wound model.[216] They also confirmed that these cells promoted angiogenesis via their differentiation into ECs and other blood vessel components, as well as the release of various pro-angiogenic biomolecules.

Wharton's jelly of the human umbilical cord is another alternative source for MSC isolation.[238] Compared with other sources, these cells are inexpensive, exhibit low immunogenicity, and require less invasive harvesting protocols than those used for BMSC isolation. Furthermore, these cells have an ability to proliferate rapidly, resulting in an improved regenerative capacity.[239-241] Moreover, a study comparing WJ-MSCs and ADMSCs showed that the key angiogenic growth factors, including VEGF, FGF, and Ang-1 are expressed and secreted to a greater extent by WJ-MSCs. Their results highlighted the angiogenic potential of WJ-MSCs through their ability to enhance paracrine release of angiogenic factors to surrounding cells.[225] Collectively, these advantages made WJ-MSCs a viable candidate for tissue engineering including skin tissue repair.[225, 242-245] For example, Millan-rivero et al. examined the effect of WJ-MSC-laden silk fibroin nanofibers in a murine excisional wound model.[245] Their findings

showed that combining these cells with silk-based biomaterials resulted in the formation of well-organized and vascularized granulation tissues, an accelerated wound contraction, and reduced scar formation, highlighting the potential of WJ-MSCs in wound healing.

EPCs exhibit features of both endothelial and progenitor cells, and can be isolated from various sources, including bone marrow, cord blood, adipose and vascular tissue.[246-248] Zhang et al. showed that vascular-resident EPCs enhanced angiogenesis through the secretion of pro-angiogenic GFs such as VEGF and PDGF. Moreover, they demonstrated that after 2 weeks, vascular-resident EPCs seeded on Integra® matrix, a porous collagen and glycosaminoglycan-based wound dressing, significantly increased *in vivo* skin vascularization in comparison to cell-free scaffolds.[249] In a more recent study, Wang et al. investigated the role of nanofibrous collagen-PCL-bioactive glass scaffolds seeded with bone marrow-derived EPCs on wound healing. They established that EPCs were able to form new capillaries through HIF-1α, VEGF, and SDF-1α pathways. In their *in vivo* studies, they also showed that wounds treated with cell-laden scaffolds significantly boosted angiogenesis when compared to cell-free scaffolds after 7 days, which resulted in enhanced wound healing.[250]

Endothelial colony forming cells (ECFCs), a rare subset of EPCs, are mostly found in umbilical cord blood. ECFCs are highly proliferative and have an intrinsic capacity to induce new capillary formation.[247, 251-253] Baltazar et al. engineered 3D printed scaffolds with designated dermis and epidermis layers.[254] The dermis layer was first printed using a collagen solution containing human fibroblasts, placental pericytes (PCs), and ECs derived from cord blood ECFCs. Next, the epidermis was printed on this layer using human keratinocytes. They showed that after 7 days, PCs in association with ECFCs formed a vascular network *in vitro* with no sign of regression for at least 50 days. Furthermore, when implanted in animals, the 3D printed structures promoted the formation of perfused vascular networks within 4 weeks.

ESCs, originating from the inner cell mass of mammalian blastocysts, have the ability to differentiate into all three germ layer cells during embryonic development. Therefore, they could potentially be stimulated to differentiate into vessel forming cells .[255, 256] For example, Kusuma et al. stimulated ESCs to differentiate them into ECs and pericytes. They showed that ECs and pericytes derived from ESCs can form a vascular network in HA-based hydrogels after 3 days.[256] Although ESCs have indicated to have a strong pro-angiogenic potential,[1,2] their application is hampered due to ethical concerns and allograft rejections.[248, 255, 257] To tackle these challenges, Takahashi and Yamanaka showed that somatic cells, as known as induced pluripotent stem cells (iPSCs), could be generated and reprogrammed to have the morphology and characteristics of ESCs.[255] Since then, many researchers have investigated the angiogenic capacity of various iPSC-derived cells.[232, 233, 258, 259] For instance, Tan et al. investigated the angiogenic potential of iPSC-derived ECs (iPSC-ECs) seeded on PCL-gelatin nanofibrous scaffolds.[260] *In vitro* gene expression showed an upregulation of pro-angiogenic GF secretion for these iPSC-EC-laden scaffolds after 7 days of culture. After implantation in a subcutaneous rat model, laser Doppler perfusion monitoring for measuring blood flow demonstrated that, unlike untreated animals, blood perfusion was significantly higher in rats treated with cellularized scaffolds. Immunohistochemistry also showed that only 2 days after implantation, GF concentrations and capillary densities were significantly higher in the treated group. In a comparative study, Gorecka et al. compared the wound healing and angiogenic properties of iPSC-derived smooth muscle cells (iPSC-SMCs) and ADMSCs encapsulated in collagen scaffolds. Both, the secretion of VEGFA and bFGF from iPSC-SMC-laden scaffolds were significantly increased in comparison to ADMSC-laden scaffolds. Furthermore, an *in vivo* study showed that wounds treated with iPSC-SMC-laden scaffolds contained higher numbers of VEGFA and bFGF positive cells and displayed increased capillary formation.[261] These investigations revealed that iPSC-derived cells can stimulate angiogenesis effectively.

However, iPSCs are limited by their reduced capacity of cellular reprogramming and a number of safety concerns, including the risk associated with tumor formation. As a result, this approach needs further investigation before its widespread clinical application.[262, 263]

Collectively, stem cells derived from different sources have many advantages, making them promising candidates for tissue vascularization. However, their limitations associated with invasive harvesting procedures, risks of disease transition, immunogenicity or tumor formation, age-dependent impairment of stemness, and donor dependence should be carefully considered.[264-266] Moreover, a comprehensive study that compares the angiogenic properties across various stem cells could help identify better candidates for skin tissue engineering.

3. Prevascularization

Although the strategies discussed in the previous sections can effectively promote vascularization, they are limited by a slow growth of blood vessels at a rate of approximately 5 μm/h. This means that even for small wounds, it may take several days or even weeks before a vascular network is fully formed.[50, 59, 267] In large critically-sized defects, the use of scaffolds with a pre-established vascular network can be beneficial and further facilitate the delivery of nutrients and oxygen to cells. This technique aims to create microvessels within the biomaterials prior to being introduced into the body.[268, 269] The prevascularized scaffolds should be permeated by a network of capillary-like tubes to prevent cell death in the first few days following implantation.[270, 271] Furthermore, the pre-established vascular network should allow for a hierarchical and functional vasculature, consisting of arteries, veins, and capillary beds.[272] Lastly, upon implantation, the vascular network from the prevascularized scaffolds should be rapidly anastomosed with the host vasculature, thus improving chances of promoting construct integration with the surrounding tissues.[273]

Prevascularization strategies can be classified into *in vitro* and *in vivo* approaches.[54, 274] For *in vitro* prevascularization, the focus is on the incorporation or seeding of vessel-forming cells into the scaffolds.[275] This strategy is different from the previously discussed applications of stem cell-laden scaffolds, as *in vitro* prevascularization allows sufficient time for the cell-laden scaffolds to form mature capillary and vessel-like structures prior to being implanted into the target sites.[54] This approach aims to guide therapeutic angiogenesis in a remarkably shorter time. In early studies, ECs have been used as they can rapidly induce immature microchannels that become perfused following implantation.[276-278] Despite their ability to induce rapid vessel formation, ECs lack high proliferative turnover *in vitro*. As a result, they cannot always be cultured in therapeutic quantities needed for clinical applications. Additionally, some donor tissues used for harvesting ECs may negatively impact their performance and angiogenic properties.[279, 280] Therefore, studies have focused on using ADMSCs, BMSCs, glandular-derived stem cells, amniotic fluid-derived stem cells, and iPSCs as alternatives for ECs in prevascularization approaches.[281-283] Hanjaya-putra et al. investigated the potential of synthetic HA-based hydrogels infused with human ECFCs and multiple factors (VEGFA, bFGF, Ang-1, tumor necrosis factor-α, and stromal cell-derived factor-1 (SDF-1).[253] They demonstrated in their *in vitro* studies that vacuoles are formed within 3 hours following encapsulation, which was followed by tube formation, sprouting, and branching on day 2, and a mature vascular network on day 3. When these prevascularized scaffolds were subcutaneously implanted in mice, the host vascular networks invaded the scaffolds, rapidly anastomosed with the pre-established vessels, and eventually promoted cell survival. In a recent study by Kong and coworkers, iPSC-derived ECs and human coronary artery smooth muscle cells were co-cultured in porous polyurethane scaffolds.[284] Capillaries that were formed within the scaffold survived up to 3 days. Upon implantation, the preformed vascular network survived and was integrated with the host ingrowing capillaries.

Prevascularization can also be achieved with cell sheet technology.[285] This method produces prevascularized constructs without using a pre-existing scaffold.[286] This is achieved by seeding cells on a smart cell culture substrate, such as temperature-responsive biomaterials. In response to temperature change, cells would detach from the surface spontaneously and form an intact cell sheet.[54, 287] For example, in an approach used by Lee et al., sheets of keratinocytes or keratinocytes, fibroblasts, fibrin and ECs were used as prevascularized fibrin-based sheets for deep oral wound healing.[288] They demonstrated that the prevascularized sheets had faster wound closure rates with a higher number of blood vessels a week post-implantation. Furthermore, they used these cell sheets for full-thickness excisional wound healing, which also confirmed their potential for skin wound healing.[289] In a different study, Radke et al. combined MSCs and ECs. They fabricated a prevascularized cell sheet to enhance survival of skin grafts during full thickness wound healing.[273, 290] When tested *in vivo*, their engineered cell sheet improved split-thickness skin graft outcome, as the construct prevented graft shrinkage, reduced inflammatory responses, and enhanced microvessel formation.

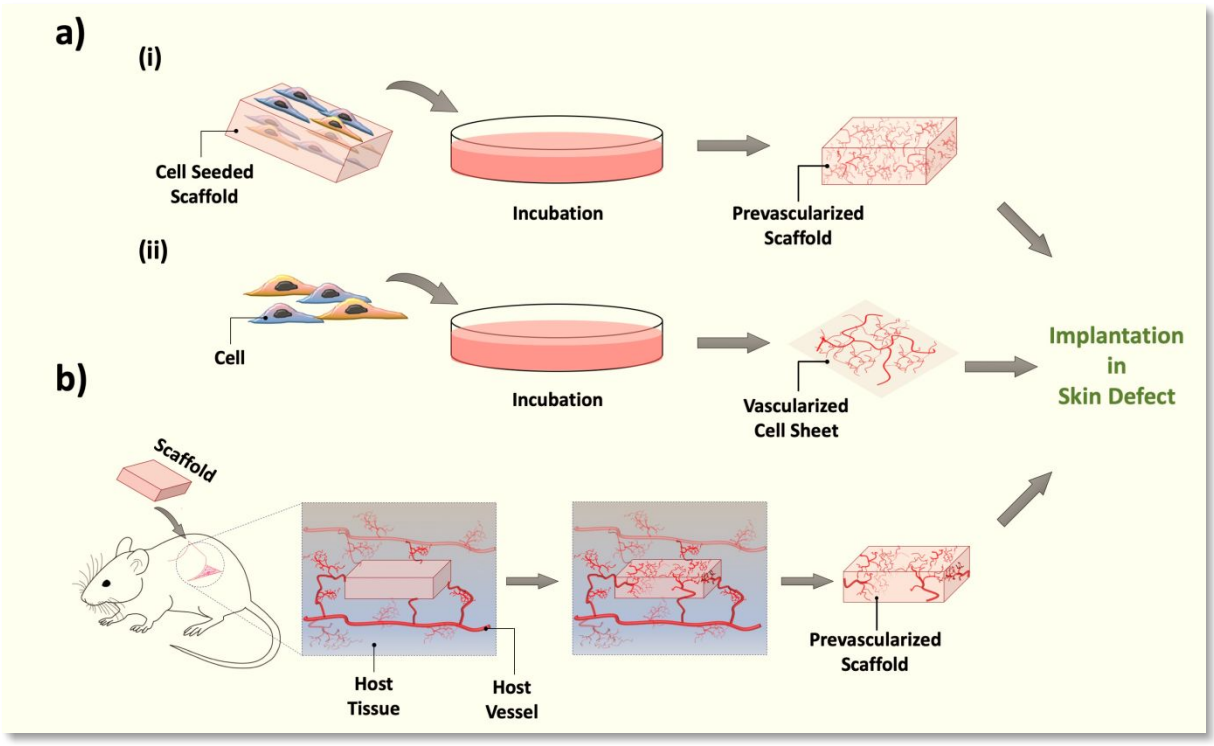

Fig. 6. Schematic displaying the main prevascularization strategies in skin tissue engineering. (a) *In vitro* strategies: (i) Cells are seeded on a scaffold and cultured to form a vascular network in 3D, and (ii) using cell sheet technology, cells are cultured in two-dimensions to produce a sheet of prevascularized tissue. (b) *In vivo* approach: Scaffolds are subcutaneously introduced into the body to promote neovascularization.

In vivo prevascularization strategies utilize the body itself as a bioreactor to form new blood vessels within the implanted scaffolds.[291] The simplest prevascularization approach is to temporarily implant the scaffold into an easily accessible and well-vascularized tissue, such as subcutaneous pockets or muscle pouches. Typically, random microvessels grow within the scaffolds. Next, the vascularized scaffolds are retrieved and transplanted to the desired tissues.[292-294] This approach was used by Laschke and coworkers where they implanted PLGA scaffolds in mice.[291] After 20 days, the vascularized scaffolds were transferred into dorsal skinfold chambers to evaluate blood perfusion, further vascularization, and cell survival. They demonstrated that blood perfusion for the prevascularized PLGA scaffolds was nearly 20 times higher than those non-vascularized, and the blood supply was sufficient to prevent cell apoptosis after 6 days.

The flap technique and the AV-loop are two important *in vivo* prevascularization strategies. In the flap technique, the scaffold is implanted into a muscle flap to allow development of microvessels from the surrounding tissue. At this point, the implant and surrounding tissue are removed and transplanted to the targeted defect site.[295] The AV-loop approach uses an arteriovenous fistula that is shaped in a loop to induce random growth of blood vessels.[296-298] Combining *in vitro* and *in vivo* prevascularization could be a synergistic strategy to promote scaffold vascularization. Zhang et al. investigated a hollow channel-modified porous silk-based hydrogel for its ability to form a prevascularized network.[270] When prevascularized, either *in*

vivo or *in vitro*, their hydrogel construct exhibited increased cell survival, proliferation, and vascular infiltration. However, when their *in vivo* and *in vitro* prevascularization approaches were combined together, they reported accelerated vascularization, leading to increased survival and engraftment of the transplanted stem cells.

Despite encouraging evidence, prevascularized constructs still face a number of challenges, preventing their broad implementation into clinical practice. For instance, many of the discussed scaffolds do not have the appropriate porous microstructure needed to efficiently accelerate blood vessel formation and perfusion.[285] Studies into the vascularization of engineered scaffolds with large and interconnected macropores, such as cryogels, could be a game changer and improve current strategies for prevascularization.[300, 301] Moreover, in order to avoid hypoxia, it is important that established vessels are not only mature but also perfusable. The use and availability of appropriate autologous cells for the formation of pre-established networks, such as ECs and MSCs, are other major considerations. Treatments utilizing allogeneic cells or incompatible cells may increase the risk of rejection and prevent anastomosis.[272] Furthermore, the invasive nature of surgical transplantations combined with a slow ingrowth of the host's vasculature into the implants are among other major drawbacks for the *in vivo* prevascularization method. At this point, there is a limited number of reported studies on prevascularization approaches in skin tissue engineering and additional pre-clinical and clinical trials are necessary to assess their full potential for clinical applications.[54, 298, 302]

Table 3. Summary of therapeutic studies aiming at enhancing angiogenesis.

Pro-angiogenic strategies	Bioactive components	Model system	Effect on vascularization	Ref.
GFs	• VEGFA • PDGF • EGF • IGF-1 • TGF- β	GF-loaded Matrigel embedded in porous PLGA scaffold	GF-loaded scaffolds significantly increased the density and sprouting area of the vascular network	[303]
GFs	• VEGFA • SDF-1 • IGF • Ang-1	GF-loaded Dextran hydrogel	Co-delivery of VEGFA/Ang-1, VEGFA/ IGF/SDF-1, or all GFs together remarkably increased the number and size of newly formed vessels compared to application of any individual GF	[304]
GFs	• bFGF	bFGF-loaded core-sheath Poly(ethylene glycol)-poly($_{DL}$-lactide) nanofibers	Mature vessels with high density in two weeks	[305]
GFs	• VEGFA • PDGF-BB	PDGF-BB-loaded PLGA NPs embedded in VEGFA-loaded chitosan/PEO nanofibers	Co-delivery of VEGFA and PDGF-BB induced higher number of vessels *in vivo* after 1 and 2 weeks	[306]
GFs	• VEGFA	GFs were loaded in coacervate of	Co-delivery of VEGFA	[307]

	• TGF-β3	PEAD polymer and then coated on PLGA nanofibers	and TGF- β3 with this system resulted in a higher number of vessels with higher blood perfusion compared to single delivery of each GF	
GFs	• VEGFA	PCL nanofibers containing VEGFA-loaded gelatin particles	Direct MSC differentiation to ECs along with stimulation and stabilization of EC angiogenesis	[131]
GFs	• VEGFA • PDGF-BB	PDGF-loaded PLGA microspheres embedded in VEGFA-loaded PLG scaffolds	Dual delivery of GFs significantly increased the density of vessels and their maturation	[133]
GFs	• VEGFA • PDGF • bFGF • EGF	VEGFA and PDGF were separately encapsulated in gelatin NPs and then embedded in bfGF-loaded HA-based nanofibers and EGF-loaded collagen nanofibers, respectively	Increase in EC growth rate, better formation of thread-like tubular structure *in vitro*, and improved vessel maturation *in vivo*	[134]
GFs	• VEGFA	VEGFA-loaded PLGA nanofibers were produced by negative-voltage emulsion electrospinning	Improved EC proliferation and cytoskeleton development	[147]
GFs	• VEGFA	VEGFA-loaded heparinized PEG hydrogels embedded in polyurethane	Sustained release of VEGFA in the presence	[180]

		disks	of heparin/heparin sulfate increased vascularization	
GFs	• bFGF	bFGF bound to alginate microsphere hydrogels with or without alginate sulfate	Alginate/alginate sulfate microspheres hydrogels containing bFGF showed significantly higher vascularization and vessel maturation compared to bFGF or alginate sulfate-free microspheres	[173]
GFs	• VEGFA • PDGF-BB • TGF-β1	GFs were bound to alginate/alginate sulfate hydrogels	GF release with different kinetics leads to formation of mature and stable vessels within the scaffolds when implanted in rats	[166]
GFs	• PDGF-BB	Bilayer scaffold with bottom layer of 3D-printed PDGF-BB-loaded gelatin and top layer of silver-loaded gelatin cryogel	*In vivo* improvement of angiogenesis and accelerated wound closure	[102]
GFs	• FGF2 • VEGFA	Collagen scaffolds with heparin, FGF2, and VEGFA	Scaffolds with heparin and both GFs showed increased vessel number and maturation compared to pure collagen scaffold or	[106]

			scaffolds with one GF	
GFs	• bFGF	bFGF-loaded ultrasound-responsive fibrin-based scaffolds	Megahertz-range ultrasound-controlled GF release, resulting in increased blood perfusion and vessel formation	163
GFs	• VEGFA • bFGF	GF-loaded PEG hydrogels	After 11 days, hydrogels loaded with GFs showed significantly higher blood vessel numbers in CAM assays	308
GFs	• NGF • BDNF • NT-3 • GDNF	Cells were cultured on collagen sponges and GFs were added to culture medium	Presence of neurotrophic factors dramatically increased the number of vessels formed	309
GFs	• PDGF	HUVECs and hMSCs cultured on collagen-glycosaminoglycan scaffolds	Addition of PDGF prevented vascular regression	101
ROS-inducing NPs	• ZnO NPs	ZnO-loaded P(VDF-TrFE) nanofibers	Low NP concentrations (<2%) induced higher number of vessels	310
ROS-inducing NPs	• Potassium doped ZnO NPs	Chitosan/cellulose porous hydrogel containing potassium doped ZnO NPs	Higher concentrations of potassium stimulated angiogenesis	311
ROS-inducing NPs	• ZnO nanoflowers	NA	Formation of mature blood cells *in vitro* and enhanced EC migration	195

			in wound healing	
ROS-inducing NPs	• ZnO NPs	ZnO NP-loaded PCL nanofibers	*In vivo* studies showed that scaffolds containing 1 wt.% ZnO improved angiogenesis after 5 days of implantation	[187, 209]
ROS-inducing NPs	• ZnO NPs • ZnO_2 NPs	NP-loaded chitosan cellulose hydrogels	ZnO_2-loaded hydrogels showed higher *in vitro* vascularization responses compared with ZnO-loaded and NP-free hydrogels	[200]
ROS-inducing NPs	• CeO_2 NPs	CeO_2 NP-loaded PCL nanofibers	Scaffolds loaded with 1% wt.% CeO_2 improved angiogenesis both *in vitro* and in CAM assays	[211]
ROS-inducing NPs	• CeO_2 NPs	NA	Use of 1 µg of CeO_2 NPs could stimulate higher vessel sprouting compared to 50 ng of VEGFA in CAM assays	[199]
ROS-inducing NPs	• Ag NPs	Matrigel containing polyvinylpyrrolidone (PVP)-coated Ag NPs	0.5-5 µg/ml Ag NPs increased angiogenesis both *in vitro* and *in vivo*	[205]
ROS-inducing NPs	• $Eu(OH)_3$ nanorods	PCL nanofiber-loaded $Eu(OH)_3$ nanorods	NP-loaded scaffolds increased proliferation rates and promoted	[210]

			blood vessel growth	
ROS-inducing NPs	• $Eu(OH)_3$ NPs	NA	NPs promoted EC proliferation *in vitro* and improved vascular sprouting *in vivo*	196, 197
ROS-inducing NPs	• Graphen oxide (GO) • Reduced graphen oxide (rGO)	Filter papers soaked in GO or rGO	Both GO and rGO treated groups showed higher numbers of matured cells compared with untreated groups in CAM assay	204
Stem cell-laden scaffolds	• BMSCs	Denatured acellular dermal matrix	Angiogenesis was accelerated in presence of BMSCs, leading to larger vessel diameters compared to cell-free scaffolds	312
Stem cell-laden scaffolds	• ADSCs	Thermosensitive Pluronic F-127 hydrogels	Higher of microvessel density for cell-laden hydrogels compared to pure hydrogels	313
Stem cell-laden scaffolds	• BMSCs	Collagen/gellan gum hydrogels	Improved differentiation of BMSCs to ECs	224
Stem cell-laden scaffolds	• ADMSCs	Photocrosslinkable methacrylated gelatin/methacrylated HA-based hydrogels	Implanted cell-laden scaffolds resulted in 3-fold increased vascularization in comparison with cell-	221

			free hydrogels	
Stem cell-laden scaffolds	• Sweat gland–derived MSCs	Integra® matrix	Cells secreted angiogenic factors *in vitro* and enhanced vascularization *in vivo*	216
Stem cell-laden scaffolds	• WJ-MSCs	Silk fibroin nanofibrous scaffold	Improved vascular surface area at wound site compared to empty scaffolds	245
Stem cell-laden scaffolds	• ADMSCs	Dextran-based hydrogels	Hydrogels promoted angiogenic GF secretion	314
Stem cell-laden scaffolds	• Vascular-resident EPCs	Integra® matrix	Two weeks after implantation, cell-laden scaffolds significantly increase vascularization compared to cell-free scaffolds	249
Stem cell-laden scaffolds	• Bone marrow-derived EPCs	Collagen-PCL-bioactive glass nanofibrous scaffold	Cell-seeded scaffolds significantly increased vascularization compared to cell-free scaffolds after 1 week	250
Stem cell-laden scaffolds	• Umbilical cord blood drived EPCs • VEGFA	Porous PCL scaffold immobilized with heparin	Cell-seeded scaffolds increased vascularization *in vivo* after 1 week VEGFA showed synergy with cell-seeded	315

			scaffold, significantly increasing vascularization *in vivo*	
Stem cell-laden scaffolds	• ECs and pericyte-derived PSCs	HA-based hydrogel	Vascular network was formed in HA-based hydrogels after 3 days of culture	256
Stem cell-laden scaffolds	• iPSC-ECs	Peptide-functionalized PEG hydrogel	Cells self-assembled into vascular structures after 3 days of *in vitro* incubation	258
Stem cell-laden scaffolds	• iPSC-ECs	PCL-gelatin nanofibrous scaffold	Cell-seeded scaffolds upregulate pro-angiogenic GF secretion, blood perfusion, and capillary density after two days	260
Stem cell-laden scaffolds	• iPSC-SMCs • ADSCs	Collagen scaffold	iPSC-SMC-laden scaffolds significantly increased pro-angiogenic GF secretion and capillary formation compared to ADSC-laden scaffolds	261
In vitro prevascularization	• HUVECs	Artificial dermis via endogenous matrix deposition by cells	Fabricated matrix featured complex vessel network and connected with host vessels 1 week	316

			post implantation	
In vitro prevascularization	• HUVECs • VEGF	PLGA microfibers covered with HUVECs entrapped in a collagen scaffold	Fibers guided formation of primary vessels and promoted branching by inosculating with capillaries formed in collagen scaffold	[317]
In vitro prevascularization	• human aortic ECs	Cell sheets of human aortic ECs sandwiched between two sheets of human fibroblast cells	Higher HGF and PlGF secretion *in vitro* and formation of microvessels 3 days after subcutaneous implantation	[275]
In vitro prevascularization	• hiPSC- ECs • Human vascular smooth muscle cell	NovoSorb™ scaffolds seeded with hiPSC-EC and human vascular smooth muscle cells	Capillary formation after 1 day *in vitro* and enhanced vascular density in vivo	[284]
In vitro prevascularization	• Human ECFCs • VEGFA • bFGF • Ang-1 • Tumor necrosis factor-α • SDF-1	Human ECFCs encapsulated within synthetic HA-based hydrogels	*In vitro* vessel formation after 3 days and perfusion of vessels after implantation *in vivo*	[253]
In vitro prevascularization	• Tissue-derived microvascular fragments (MVFs)	Integra scaffolds seeded with adipose MVFs	Improved microvessel density, MVFs perfused rapidly, and accelerated vascularization *in vivo*	[318]

In vitro prevascularization	• Stromal vascular fraction-derived ECs	Fibrin or collagen hydrogels co-cultured with stromal vascular fraction-derived ECs	Formation of branched and mature capillaries 3 weeks and rapid perfusion 4 days after implantation	281
In vitro prevascularization	• Amniotic fluid-derived stem cells (AFSC)	Fibrin/PEG hydrogels co-cultured with AFSC and AFSC-derived ECs	*In vitro* prevascularization showed comparable vascular formation of this approach with co-culture of HUVECs and MSCs	282
In vitro prevascularization	• HUVECs • Human MSCs	Cell sheets made from co-culture of HUVECs and human MSCs	Higher secretion of angiogenesis related factors *in vitro* and improved blood perfusion *in vivo*	287
In vitro prevascularization	• ECs	Fibrin-based sheets cultured with keratinocytes, fibroblasts, and ECs	Prevascularized structures stimulated neovascularization of wound in early stages	288, 289
In vitro prevascularization	• HUVECs • Human MSCs	Cell sheets of HUVECs cultured on top of human MSC sheets	Enhanced neovascularization and blood microcirculation	273, 290
In vitro and *in vivo* prevascularization	• HUVECs	Hollow channel-modified porous silk-based hydrogel	Increased capillary formation *in vitro* and enhanced host vessel infiltration *in vivo*	270

In vivo prevascularization	• NA	Prevascularized PLGA scaffolds were generated by 20 days of implantation in mice	Significantly higher blood perfusion compared to unvascularized scaffold	[291]
In vitro prevascularization	• ECFCs	ECFC encapsulated in HA-based hydrogels	Host vessel infiltration into the scaffold	[319]
In vitro prevascularization	• ECFCs	ECFC sheets sandwiched between fibroblast cell sheets	Network formation after 3 days of culture and functional microvessel formation after seven days of *in vivo* implantation	[320]

4. Conclusions and future perspectives

Over the last decades, various strategies have been explored to improve skin vascularization, which is essential for adequate healing of large wounds. To improve angiogenesis, VEGFA and other supporting GFs have been extensively applied for this purpose. In particular, their controlled release from wound healing scaffolds in a fashion that mimics natural wound healing mechanisms can lead to highly vascularized scaffolds. Although it is challenging to accurately mimic spatiotemporal and sequential release of several growth factors, the development of advanced and smart delivery systems has shown considerable promise. Furthermore, delivery of GF-releasing stem cells within these scaffolds allowed for a more natural GF release profile. Nonetheless, the speed of angiogenesis as a result of GF delivery is limited by the natural growth rate of blood vessels, which may be insufficient for vascularization of large defects. *In vitro* or *in vivo* prevascularization of scaffolds can address some of these challenges, leading to a functional vascular network throughout the constructs within a few days.

Looking forward, combinations of prevascularization and angiogenesis-promoting strategies should be further investigated, as these approaches are most likely to be successful in accelerating wound healing. It is expected that such a combinational approach will help engraft the pre-vascularized scaffolds to the host vasculature more quickly. Furthermore, the application of scaffolds containing large, interconnected macropores could further help guide the growth of blood vessels into implanted constructs. Additionally, advanced strategies that compensate for the lack of blood supply during the first few days following scaffold implantation, such as oxygen-releasing biomaterials, should be explored more extensively. Importantly, the lymphatic system, known to play a key role in immune protection and tissue regeneration, should also be integrated within the vascularized constructs, thereby replicating the intricate compositional and architectural organization of skin tissues.[321, 322] In summary,

the successful translation of these vascularization strategies may pave the way for improved clinical trial design and implementation of skin tissue engineering.

Conflicts of interest

There are no conflicts to declare.

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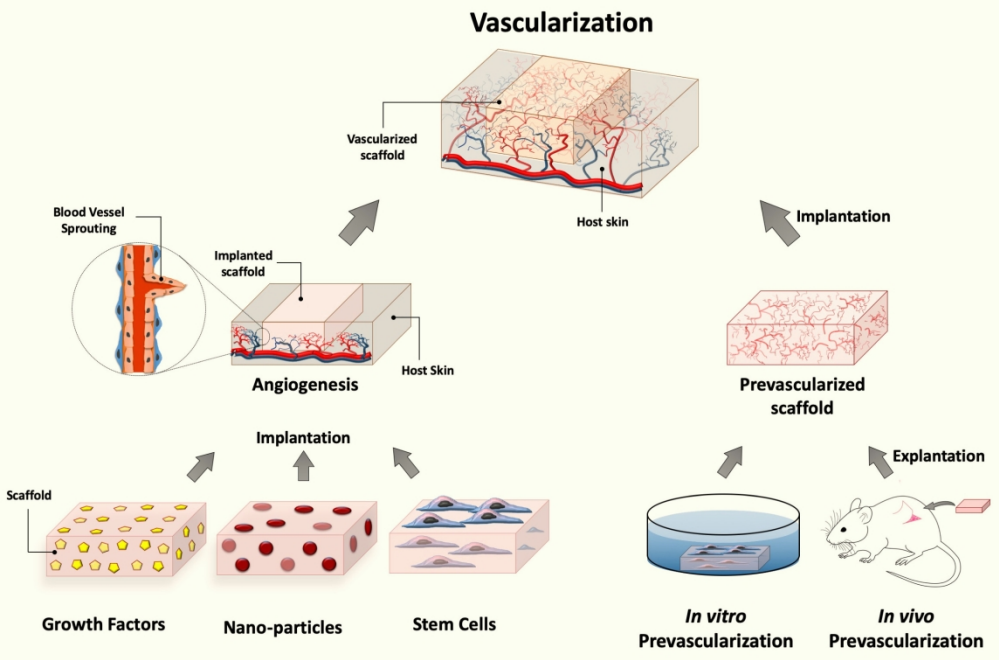

State-of-the-art strategies to promote the vascularization of skin substitutes.

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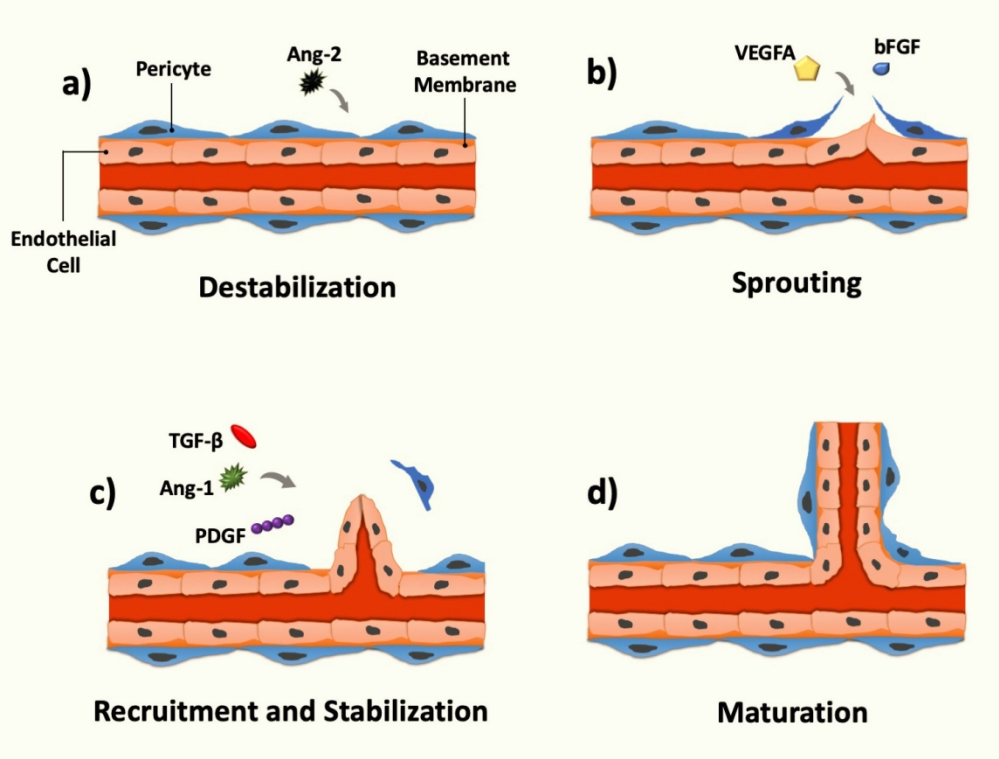

Figure 2. Sequential roles of growth factors during angiogenesis; (a) Ang-2 released as a result of hypoxia destabilizes pericyte/EC interactions, leading to pericyte detachment; (b) VEGFA and bFGF stimulate sprouting and proliferation of exposed ECs, creating a new branch; (c) PDGF release leads to the recruitment of pericytes, while Ang-1 stabilizes pericyte/EC interactions, and TGF-β increases basement membrane deposition, all together promoting maturation of nascent blood vessels; (d) mature blood vessel.73-75

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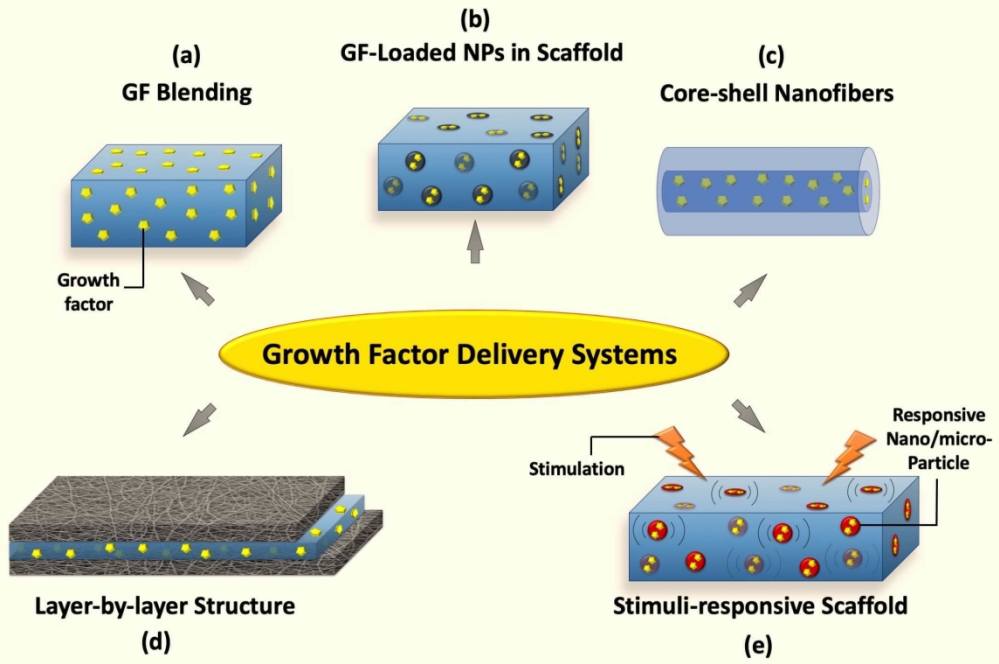

Figure 3. Schematic representation of the main GF delivery strategies used in skin tissue engineering. (a) GFs are blended with biopolymers to form the scaffolds, (b) GFs are first embedded within nano/micro structures and then blended with biopolymers to form composite scaffolds, (c) core-shell nanofibrous structure in which GFs are encapsulated in the core layer, (d) layer-by-layer structure in which GFs are embedded to offer prolonged release, and (e) stimuli-responsive scaffolds that offer on-demand GF release.

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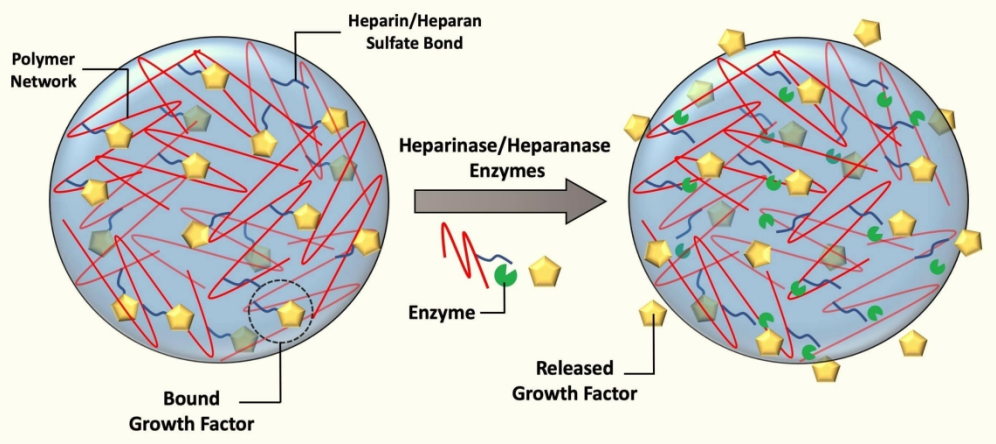

Figure 4. Active release mechanism of heparin-binding growth factors. Heparinase/heparanase enzymes cleave the heparin/heparin sulfate bonds, resulting in GF release.

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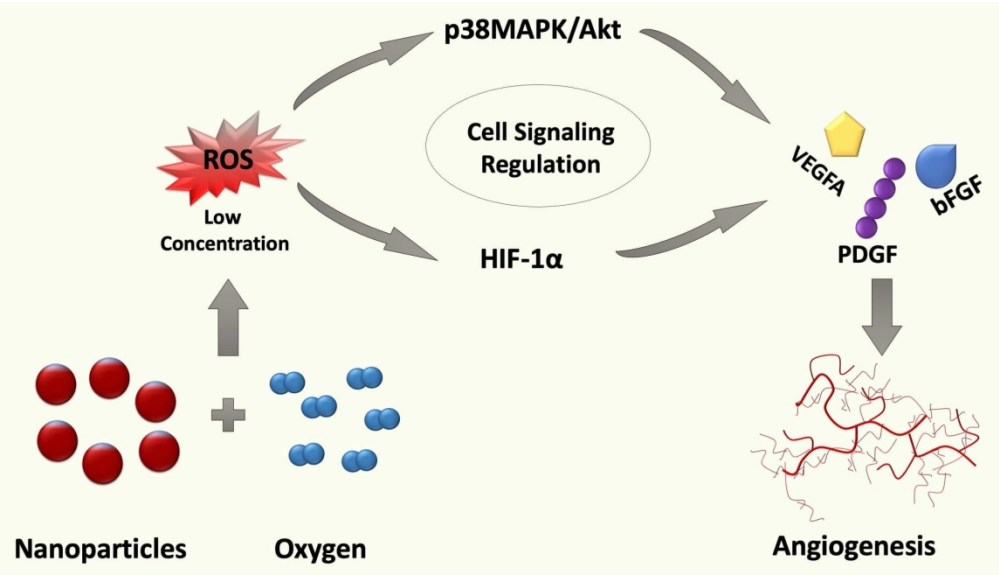
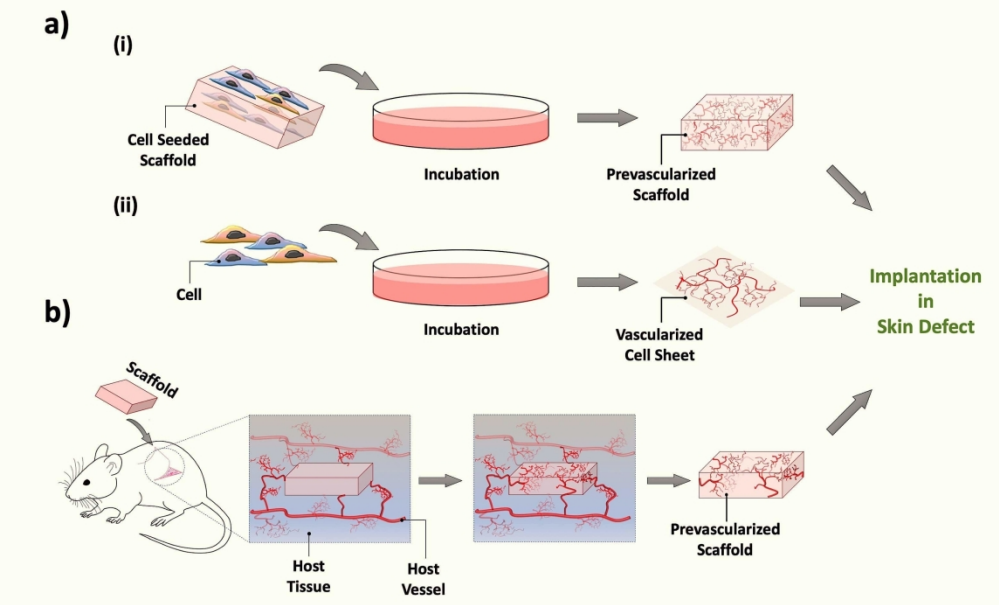


Figure 5. General mechanism of ROS-inducing NP-mediated GF release. While interacting with cells and tissues, NPs can induce ROS. At low concentrations, these ROS induce cell signaling through a number of pathways, including activation of HIF-1α and the p38MAPK/Akt pathway. These signaling pathways result in the release of angiogenic factors, such as VEGFA, PDGF and bFGF, that work together to promote angiogenesis.

854x490mm (72 x 72 DPI)



Schematic displaying the main prevascularization strategies in skin tissue engineering. (a) In vitro strategies: (i) Cells are seeded on a scaffold and cultured to form a vascular network in 3D, and (ii) using cell sheet technology, cells are cultured in two-dimensions to produce a sheet of prevascularized tissue. (b) In vivo approach: Scaffolds are subcutaneously introduced into the body to promote neovascularization.

1100x667mm (72 x 72 DPI)