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Engineered delivery strategies for enhanced control of growth factor activities in wound healing

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ABSTRACT

Growth factors (GFs) are versatile signalling molecules that orchestrate the dynamic, multi-stage process of wound healing. Delivery of exogenous GFs to the wound milieu to mediate healing in an active, physiologically-relevant manner has shown great promise in laboratories; however, the inherent instability of GFs, accompanied with numerous safety, efficacy and cost concerns, has hindered the clinical success of GF delivery. In this article, we highlight that the key to overcoming these challenges is to enhance the control of the activities of GFs throughout the delivering process. We summarise the recent strategies based on biomaterials matrices and molecular engineering, which aim to improve the conditions of GFs for delivery (at the 'supply' end of the delivery), increase the stability and functions of GFs in extracellular matrix (in transportation to target cells), as well as enhance the GFs/receptor interaction on the cell membrane (at the 'destination' end of the delivery). Many of these investigations have led to encouraging outcomes in various *in vitro* and *in vivo* regenerative models with considerable translational potential.

Contents

Abbreviations: GF, growth factor; ECM, extracellular matrix; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; Ang-1, angiopoietin-1; PDGF-BB, platelet-derived growth factor-BB; HGF, hepatocyte growth factor; FDA, the United States Food and Drug Administration; PEG, polyethylene glycol; NK1, first kringle domain; EGF, epidermal growth factor; PRP, platelet-rich plasma; PRF, platelet-rich fibrin; TGF-β, transforming growth factor beta; IGF-1, insulin-like growth factor 1; PEF, pulse electric field; TRAP, thrombin receptor activating peptide; HA, hyaluronic acid; HS, heparan sulphate; ASCs, adipose-derived stem cells; MHC, major histocompatibility complex; HUVECs, human umbilical vein endothelial cells; PNIPAM-PAA, poly(amidoamine)-based poly(*n*-isopropyl acrylamide); PAM/BIS, polyacrylamide crosslinked with bisacrylamide; KGM, konjac glucomannan; Hep, heparin; PCL, poly(γ-caprolactone); MFG-E8, milk fat globule-epidermal growth factor 8; GAGs, glycosaminoglycans; DEDMs, decellularised ECM-derived matrices; FN, fibronectin; VEGFR2, VEGF receptor 2; EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; NGF, nerve growth factor; NTR, neurotrophin receptor.

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1. Introduction: control of GF activities to meet the key challenges in application

Wounds caused by trauma, burn, surgery and chronic diseases pose a massive challenge to the global healthcare system, leaving multi-millions of patients suffering from disability, discomfort and distress [1, 2]. The World Health Organisation estimated that 11 million (as of 2004) suffered and 180,000 (as of 2018) people died of burn injuries, respectively [3]. In the United States alone, chronic and non-healing wounds affect over 6.5 million people [4]. The annual expenditure on wound care will reach $ 22.4 billion by 2024 from $14 billion in 2015 [5]. These numbers are unlikely to shrink as the global society is rapidly ageing with a soaring incidence of chronical diseases such as diabetes [6].

Wound healing is a well-orchestrated and highly regulated process which could roughly be divided into four overlapping phases: haemostasis, inflammation, proliferation and remodelling [7]. These events are driven and regulated by the complex interaction among multiple types of cells, extracellular matrix (ECM) and biological signalling molecules – such as numerous families of growth factors (GFs; defined to include also cytokines). GFs, which are soluble (glycol)proteins secreted by various cells, are known to play profound, essential and versatile functions in regulating wound healing in different aspects. For instance, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families stimulate the migration and proliferation of endothelial cells for angiogenesis [8, 9]; angiopoietin-1 (Ang-1) enhances blood vessel stability [10, 11]; platelet-derived growth factor-BB (PDGF-BB) facilitates pericyte adhesion and blood vessel maturation, as well as stem cell recruitment and granulation tissue formation [12, 13]; and hepatocyte growth factor (HGF) regulates the proliferation, migration and differentiation of mesenchymal stem cells [14, 15]. In the past two decades, researchers continue to uncover more GFs functioning in wound healing (or previously unknown regenerative functions of known GFs), and wound management technologies have evolved to become more physiologically active. In this context, delivery of GFs to the wound milieu using biomaterials vehicles, aimed at actively mediating the healing process (contrary to only providing a passive covering on the wound bed) has emerged as a promising strategy.

However, compared with small bioactive molecules, GFs have inherent unfavourable properties that require extraordinarily delicate control of their delivery. Usually, in the form of recombinant proteins, GFs have poor stability in a physiological environment, non-specific bioactivities and clear adverse effects. Specifically, due to their short half-life, rapid diffusion/degradation and low effective dose *in vivo*, GFs are often repeatedly injected at a supraphysiological dose, in order to maintain their concentration at the wound sites. This does not only raise the cost but more worryingly brings about concerns as severe as increasing the risk of cancer [16]. A notable example is the Regranex® gel; its active ingredient is 0.01% becaplermin (a recombinant human PDGF-BB) and is the first and only GF-based product approved by the United States Food and Drug Administration (FDA) for diabetic wound management. Nevertheless, FDA also warns that this product has increased rate of malignancy when three or more tubes have been applied. To date, the multiple strategies to deliver GFs for wound healing have achieved little clinical success.

The key to overcoming the challenges with the GF delivery lies in better control of their activities throughout the entire delivery process. Recently, many attempts have emerged to increase the stability and maintain the activity of the GFs, which show interesting and positive outcomes to varying extent. In this review, we collectively and critically summarise these approaches to aim for *one* major goal, fall into *two* technical means and target the *three* phases of the entire delivery procedure for improvement. The one major goal is clear: to achieve precise control of the activities of the delivered GFs for wound healing. The two technical means are: i) molecular engineering of the GFs and ii) design of GF-interactive biomaterials scaffolds. And we will discuss all the content in three sections organised corresponding to the three phases of delivery:

Phase I (Section 2): improving the conditions of GFs for delivery (at the 'supply' end), through molecular engineering of the GFs or employing new sources of GF supply;

Phase II (Section 3): improving the stability and efficacy of GFs around cells (during the 'trucking' process; GFs are shipped to target cells), through devising GF-affinitive scaffolds or molecular tools to retain and stabilise GFs in the ECM;

Phase III (Section 4): improving the interaction between GFs and their cellular receptors (at the 'destination' end), through molecular engineering of GFs to increase their binding affinity for receptors or inducing synergies with other signalling.

This review will focus on the molecular engineering and biomaterials-based approaches to improve the delivery of GFs for wound healing, with the emphasis on the control of the GF activities in the above three phases (Fig. 1). Nevertheless, two types of technologies that focus on – i) optimising the controlled release kinetics of classical vehicles and ii) replacing GF delivery with gene delivery approaches –

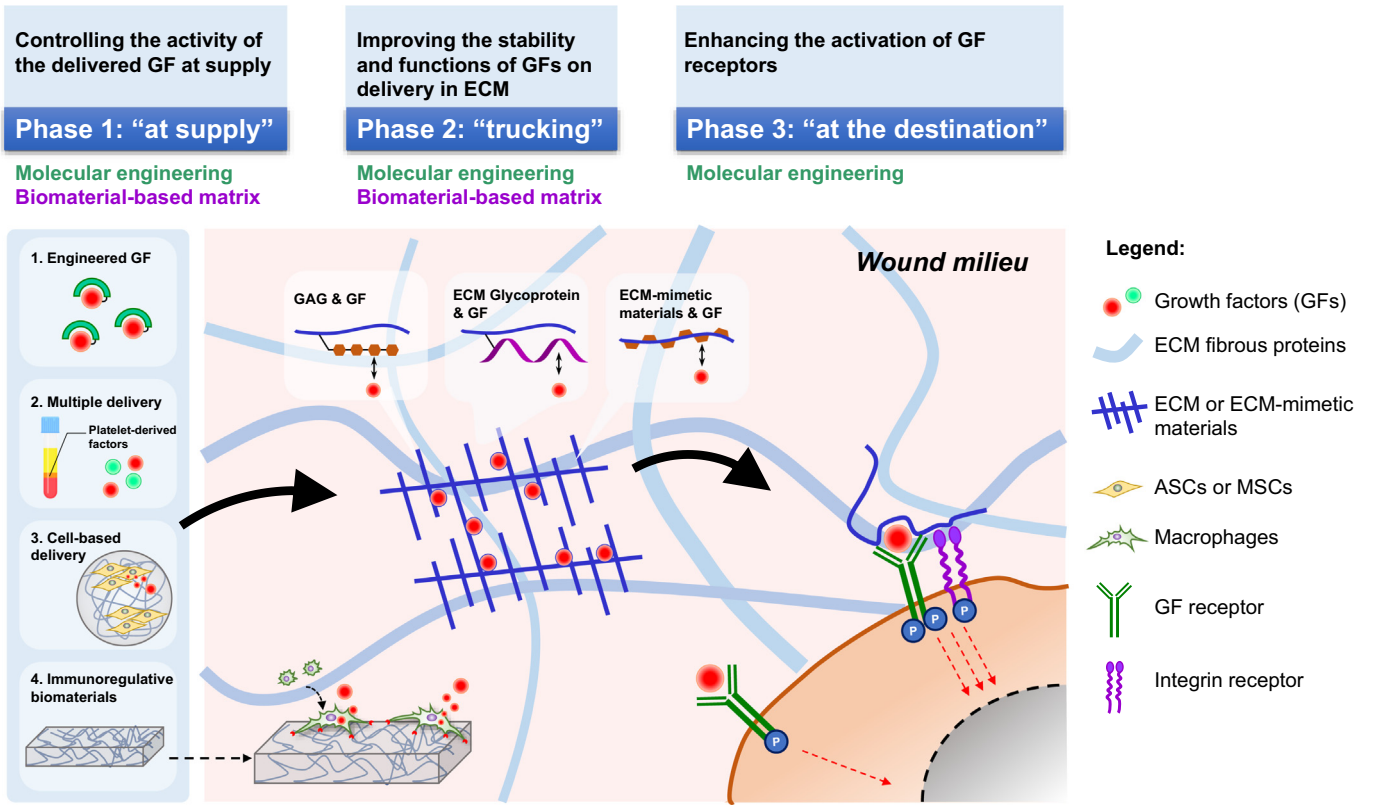

Fig. 1. Controlling the GF activities for wound healing. Strategies based on biomaterials matrices and molecular engineering have been devised to control the activity of pro-healing GFs throughout the three phases of delivery – improving the conditions of the GFs at the 'supply' end, enhancing the stability and function of GFs in the extracellular matrix, and finally strengthening the interactions between GFs and their cell receptors 'at the destination'.

fall out of the scope of this article and have been excellently reviewed elsewhere ([17, 18] and [19]; respectively).

2. Improving the conditions of GFs: controlling activity at the 'supply' end

Numerous strategies have been devised to improve the conditions of GF before delivering them to the wound milieu. These efforts have two focuses: i) increase the stability of exogenous GFs and ii) increase the types of GFs – either exogenous or endogenous for delivery. For the first aim, major approaches include chemical or genetic modifications of the recombinant proteins (Fig. 2a). For the second one, researchers have attempted to perform combinatory delivery of multiple GFs, employ platelet-derived products, as well as stimulate the production of endogenous GFs *in situ* by stimulating stem cells or macrophages with physical, chemical or materials tools (Fig. 2b–d). By improving GFs at the supply end, these studies aimed to realise the spatiotemporal control over a co-operative and sustained delivery of GFs to promote healing. However, as stated in the Introduction, approaches to – i) further stabilise the delivered GFs in the tissue matrix and ii) enhance their activities on the cells will be discussed later in Section 3 and 4.

2.1. Engineering exogenous GFs to increase their stability and half-life

As mentioned above, one of the critical challenges for the application of GFs is their instability *in vivo*, stemmed from their inherent limitation as recombinant proteins. Because they easily degrade or denature in the tissue milieu, multiple administrations of GFs in supraphysiological doses are clinically used to maintain their effective concentration at wound sites, causing unnecessary costs and various safety concerns as severe as inducing cancer [16]. In addition, the shelf life of GFs may be affected by many environmental factors, like temperature, pH, ionic strength, hydrolysis or oxidation [20]. Thus, attempts to deliver exogenous GFs for tissue regeneration have achieved little clinical success.

Protein engineering provides established tools for the invention of several approaches to tackle this fundamental obstacle, integrating the techniques of genetic mutation, polymer conjugation and variant fabrication. One such attempt is to mutate the amino acid sequences to enhance both thermostability and proteolytic resistance of the GF. Take FGF family as an example – they are potent mitogenic biomolecules involved in multiple physiological processes such as tissue development, angiogenesis and wound healing [21, 22]; but they also have low stability and a relatively short half-life *in vivo* [23]. The homology approach was applied to construct 16 mutants of FGF-1 and improve their thermodynamic stabilities [24]. The introduced mutations brought more stability (a 7.8 °C increase) compared with the wild-type protein, while having no impact on FGF-1 bioactivities. In another study, a triple mutant of FGF-1 in Q40P/S47I/H93G exhibited enhanced proteolytic resistance and stability, whose denaturation temperature raised by 21.5 K. The considerable improvement in FGF-1 stability resulted from the triple mutant triggered localised conformational changes, increasing van der Waals forces and releasing steric strains [25]. To enhance GF proteolytic resistance, the C-terminal region of FGF-1, which is the initial proteolytic cleavage site, was rigidified by introducing two single mutations (C117P, K118V). This variant demonstrated an up-to-100-fold increase in resistance to trypsin and chymotrypsin degradation, in comparison with wild-type protein [26].

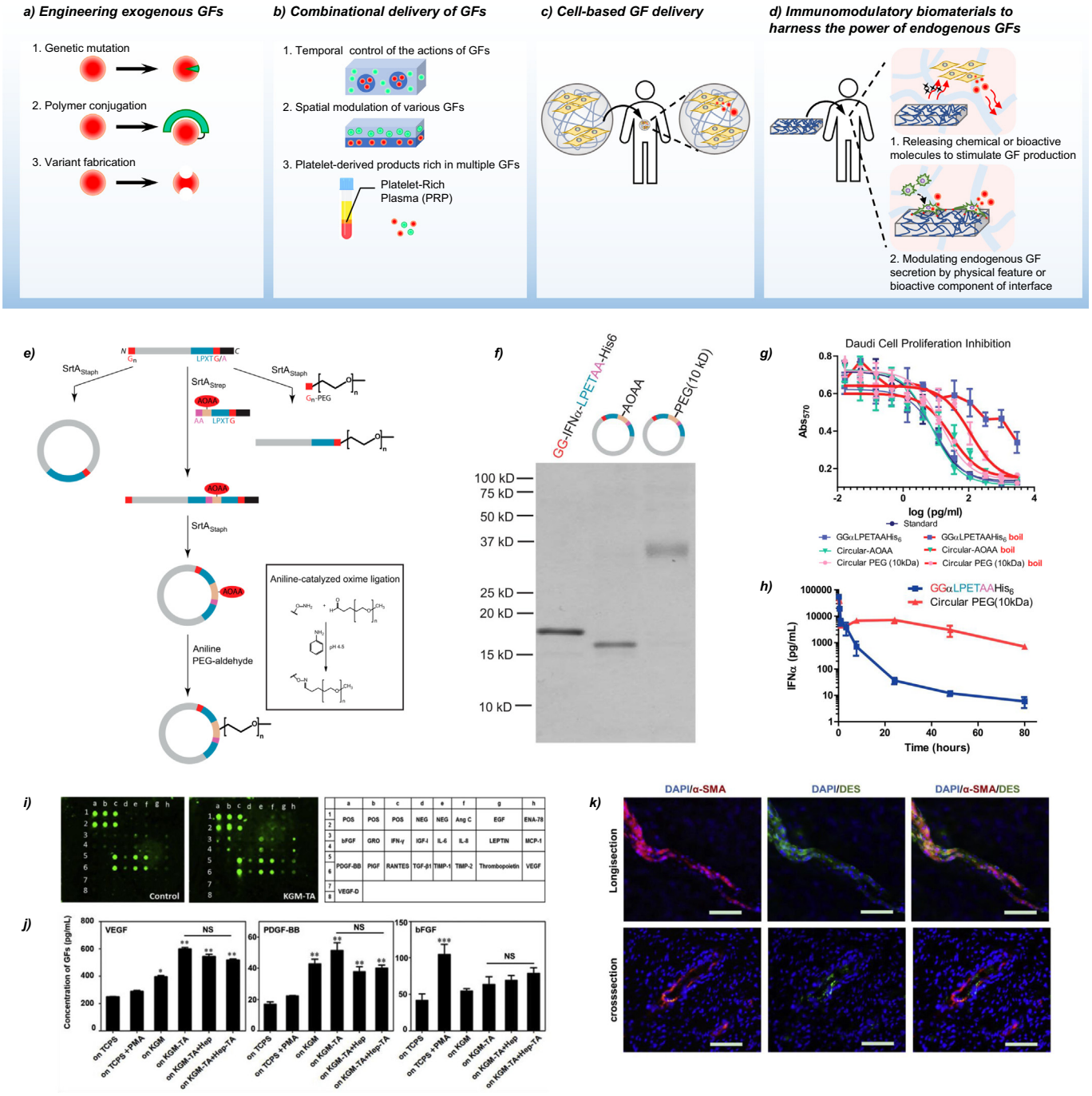

Fig. 2. Improving the conditions of GFs: controlling activity at the 'supply' end. a) Engineering exogenous GFs to increase their stability and half-life; b) combinational delivery of GFs for spatiotemporal release control and co-operative supply; c) Cell-based GF delivery strategy; d) utilising immunomodulatory biomaterials to stimulate the secretion of endogenous GFs *in situ*; e–h) Sortase-catalysed transformations facilitating GFs for both circularization and site-specific PEGylation, so as to improve the stability and half-life; e) the sortase reaction scheme; f) the backbone-cyclised IFN-α2 (cyclic GGIFN-α-LPETAAHis6) was PEGylated. And the product was resolved by 12.5% SDS-PAGE and stained with Coomassie; g) the Daudi cell proliferation inhibition assay revealed that circular cytokines remain biologically active after boiling; h) the changing trends of IFN-α levels in serum following tail vein injection of linear or circular, site-specifically PEGylated IFN-α; i–k) a biomaterials-based approach using bioactive KGM to exploit the power of endogenous GFs to encourage angiogenesis; i) Analysis of the proteins expressed in culture medium by angiogenic cytokine array; j) Determining pro-angiogenic GF concentrations of the cell culture supernatants using ELISA; k) Co-staining the harvested KGM-TA/Hep-TA gel with α-SMA (red), nuclei (DAPI, blue) and DES (green), as well as an overlap (yellow) (scale bar: 100 μm). Reprinted with permission from ref. [30, 87]. Copyright 2011 National Academy of Sciences and 2017 Elsevier, respectively.

Besides mutation of amino acid sequences, molecular modification with polyethene glycol (PEGylated) is another effective method. This strategy has been prevalently adopted in the development of GF-based biopharmaceutical products (*e.g.*, Omontys® and Lonquex®) [27]. It not only reinforces GF stability but also avoids their fast degradation in serum *via* masking the proteinase cleavage site. Following this approach, FGF-1 was conjugated with a 20-kDa mPEG-butyraldehyde, which retained approximately 60% mitogenic bioactivity of wild-type GF yet gained a remarkably enhanced thermal stability and prolonged half-life, leading to an improved performance in wound healing of diabetic rats [28]. However, non-site-specific PEGylation may cover up and interfere with the receptor binding sites of GFs, thus potentially

attenuating the therapeutic effect. To avoid this concern, a solid-phase N-terminus PEGylation strategy was developed to prepare mono-PEGylated FGF-2, which preserved the GF's secondary structure and receptor binding activity [29]. Also, a general platform using sortase-mediated transpeptidation to promote site-specific modification with PEG was designed to prolong the half-life of GFs with no impairment in their biological efficacy [30]. In this research, interferon alpha was annularly jointed through its N and C termini to generate circular polypeptides and site-specifically PEGylated (Fig. 2e and f). The product preserved the anti-proliferative effect of the cytokine while elevating its melting temperature of 4 °C, and may be applied at certain stages of healing to modulate excessive inflammation or fibrosis (Fig. 2g and h).

Another valuable approach is to develop a shorter substitute of GF to recapitulate its biological functions. A typical example HGF, a potent angiogenic mediator which interacts with Met receptor and signals through the downstream MAPK and PI3K pathways [31]. However, HGF has a complicated multi-domain structure that often results in low manufacturing yields and self-aggregation in physiological buffers [32]. The N-terminal and first kringle domain (NK1) of HGF is a natural HGF splice variant that behaves as a partial and weak agonist of the Met receptor [33]. Utilising protein engineering techniques, a small non-glycosylated active fragment (called 1K1), derived from NK1 and disrupted with the heparin-binding affinity, was engineered to improve the GF stability and amplify Met signalling [34]. Similarly, an engineered NK1 variant containing 8 mutations (termed M2.2) was created and exhibited a 15 °C increase in melting temperature compared to the native NK1, though its Met agonising potency was still weak [35]. Inspired by the heparin-induced dimerisation for receptor activation, NK1 homodimers were created by covalently coupling monomers through an introduced N-terminal cysteine residue with a disulphide bond; this homodimer demonstrated enhanced agonistic potency *in vitro* comparable to the activity of a wild-type HGF [36].

2.2. Combinational delivery of GFs to facilitate wound healing

Wound healing is a dynamic, complicated, multi-phase process driven by a spectrum of GFs and cytokines [37]. These GFs not only have versatile roles but also often act synergistically throughout the different stages of healing [38]. Compared to delivering a single GF, combinatory delivery of multiple GFs may allow for spatiotemporal control and cooperative actions of them, which better mimic the physiological scenario and may lead to more effective regeneration.

2.2.1. Temporal and spatial modulation of GFs

To implement temporal and spatial control of the actions of GFs may be desirable to their actions in all stages of healing. Nevertheless, most attempts in this direction that have gained encouraging outcomes are focused on modulating vascularisation. As a critical challenge in wound repair, vascularisation starting with endothelial sprouting required stabilisation to prevent regression and facilitate the maturation of newly formed tubes into functional vasculature [39]. Pro-angiogenic molecules Ang2 and VEGF cooperatively promote angiogenesis by facilitating pericyte detachment, existing vessels destabilisation and enhancing vascular sprouting in the early stage, while pro-mature factors Ang1 and PDGF mediate vessel maturation by antagonising with Ang2 and stimulating pericyte adhesion [40]. Hence, ideally, these two groups of biomolecules should be sequentially released. Otherwise, a well-initiated angiogenesis process at the beginning may fail to form functional vasculature in the end. Based on this concept, VEGF and PDGF-BB were directly incorporated in nanofibers or encapsulated into nanoparticles in advance, to achieve release in a relayed manner [41]. A similar method to incorporate nanoparticle in electrospinning fibres was adopted for temporal and combinatory delivery of four GFs of FGF-2 (also known as basic FGF, bFGF), epidermal growth factor (EGF), VEGF and PDGF, aimed at improving angiogenesis in full-thickness cutaneous regeneration [42]. In another strategy, individual GFs were separately wrapped within an *in situ* formed, thin polymer shells containing different ratio of the L- or D-chiral protease-specific peptide crosslinkers [43]. These enantiomers exhibited similar chemical specificity but distinct proteolytic kinetics: The D-chiral form was about 10-fold slower than the L-one. Through regulating the ratio of L and D enantiomers, researchers could tune the degradation speed of the polymer shells for different wrapped GFs. This system was capable of temporally delivering multiple GFs in response to the change in the level of protease expression in pathophysiological states. Last, macrophages, with their plastic phenotypes, are known to play central (but changing) regulatory roles in blood vessel formation and maturation: M1 macrophages initiate neovascularisation while M2 cells promote vascular maturation [44]. In order to optimise vascularisation *via* modulating the M1-to-M2 transition of macrophages, researchers devised a platform in which M1-phenotype mediator IFN-γ was physically adsorbed in scaffolds, while M2-phenotype regulator IL-4 was conjugated *via* biotin-streptavidin binding. This platform demonstrated an effective sequential delivery of GFs [45].

The delicate process of tissue reconstruction requires spatial modulation of various bioactive signals locally presented in regenerative graft. One representative case is the regeneration of small-diameter blood vessels, which may suffer from the thrombosis-induced restenosis and the risky bursting pressure. Grafts are increasingly commonly used; while a key expectation is a rapid and complete endothelialisation along the lumen of grafts to resist platelet adhesion and thrombosis, followed by proliferation of vascular smooth muscle cells around the outer layer to support the neovascular tissues [46]. For this purpose, a double-layered coaxial electrospun vascular scaffold was designed to load VEGF and PDGF in its inner and outer layer, respectively [47]. This double layered graft realised the dual delivery of GFs and their spatial regulation, therefore promoting the adhesion, proliferation of endothelial cells and smooth muscle cells, as well as vascular revascularisation.

2.2.2. Employing platelet-derived products rich in multiple GFs

Platelets play a critical role in haemostasis and wound healing based on the coagulation factors and abundant bioactive mediators principally stored in their α-granules [48]. The luxuriant growth factors and biomolecules can be released, triggered by either natural (*e.g.* thrombin) or artificial stimulations (*e.g.* freeze/thaw cycles triggered-platelet lysis, sonication or chemical treatment); their release is of significance for clotting and initiating wound healing [49]. Platelet-derived fractions, commonly termed platelet-rich plasma (PRP) or platelet-rich fibrin (PRF), have been clinically used for wound healing as an autologous source of multiple growth factors, such as PDGF, transforming growth factor beta (TGF-β), insulin-like growth factor 1 (IGF-1), VEGF, EGF, FGF-2 and HGF, among others [49, 50].

Several elements have been reported to influence the performance of platelet-derived factors, including platelet activation methods, centrifugation speeds, PRP concentration and formulations. For instance, as a physical method to activate platelets, pulse electric field (PEF) stimulation avoids the complications associated with the addition of exogenous thrombin, such as allergic responses and severe hemorrhagic or thrombotic complications [51]. Besides, PEF-treated PRP may be a better choice and leads to distinguished GF release profiles compared to bovine thrombin or thrombin receptor activating peptide (TRAP), which release more abundant EGF and similar levels of PDGF, VEGF and FGF-2.

In the preparation process of PRP or PRF, the low-speed strategy significantly promoted GF release (PDGF, TGF-β1, EGF and IGF) and enhanced fibroblast migration and proliferation [52]. This low-speed concept in the modification of centrifugation speed and time could

contribute to an elevated GF release from PRF clots and a fortified wound regeneration.

In another study, the wound healing potentials of different concentrations of PRP (10% and 20%, v/v) were compared in a non-contacted co-cultured keratinocytes and fibroblasts system [53]. It suggested that 20% PRP could remarkably enhance the expression of GM-CSF and collagen type I and III, therefore increasing inflammation and collagen deposition. Whereas, 10% PRP boosted the proliferation of keratinocytes and the release of HGF, MCP-1, CXCL5, VEGF-A, thus favouring tissue remodelling. Thus, the applied PRP concentration should correspond with the dynamic healing phases or diverse physiological wound conditions.

Concerning the counteractive effect of anti-coagulants in wound healing, a liquid injectable formulation of PRF (i-PRF) was developed without incorporating anti-coagulants [54]. This new liquid platelet concentrate was able to release higher concentrations of GFs, induce a greater extent of fibroblast migration and production of PDGF, TGF-β and collagen I.

In general, platelet-derived products prove a promising tool for inducing angiogenesis and wound regeneration, as a natural source of rich endogenous GFs. However, their preparation methods, applied formulations and the standardisation of protocols still demand a more detailed optimisation.

2.3. Cell-based GF delivery: exploiting the paracrine secretion

In addition to directly supplementing concentrated GFs, cell-based GF delivery strategies have attracted increasing attention. Such practices principally contain two parts: the differentiation of engrafted stem cells and their secretion of paracrine factors to communicate with resident cells (endogenous epidermal stem cells, endothelial progenitor cells and recruited immunocytes, *etc.*) [55]. The latter governs multiple healing effects, including recruitment of endogenous progenitor cells for wound regeneration, modulation of macrophage phenotypes, enhancement of angiogenesis and ECM reconstruction [56, 57].

Despite the importance of differentiation potential of graft stem cells, increasing evidence suggests that the engrafted stem cells possibly serve as initiators and regulators *via* releasing GFs, rather than effectors. For instance, a significant wound regeneration initiated by transplanted MSCs can continue following the clearance of the delivered stem cells, indicating their temporary effects and the prolonged contribution of released signals to recruiting endogenous progenitor cells and creating a suitable niche supporting injury recovery [58, 59]. In another study to treat an excisional wound in mice, a conditioned medium comprising the paracrine factors from BM-MSC, consisting of VEGF-A, IGF-1, EGF, SDF-1, MIP-1 and so on, could mobilise macrophages and endothelial lineage cells to initiate healing cascades [60]. Thus, based on the versatile paracrine effects, the secreted mediators from cell-based scaffolds may play a critical role in angiogenesis and wound healing process.

Specifically, a novel thermoresponsive PEG-hyaluronic acid (HA) hydrogel was developed for encapsulating human adipose-derived stem cells (ASCs) to improve chronic wounds [61]. The ASCs embedded in the PEGMEMA–MEO_2MA–PEGDA and HA hybrid hydrogel system could sustainably release VEGF and PDGF through 7 days, though the cellular proliferation was attenuated. In order to promote the cell retention and behaviours, an injectable gelatin micro-cryogel was fabricated with optimal elasticity and mechanical strength, which showed to maintain cell intactness during the injection process [62]. Moreover, the ASCs in this micro-cryogel exhibited elevated expression of pluripotency-related genes (OCT4, Nanog and SOX2) and pro-healing GFs (VEGF, HGF), compared to those in conventional 2D culture. This GF-delivering scaffold, which is facile, injectable and microscale, provides a minimally-invasive therapeutic strategy for intractable wounds.

An attractive feature of MSCs is that they not only secrete pro-healing (*e.g.* pro-angiogenic and mitogenic) GFs, but also uniquely possess immunosuppressive properties, because they lack a member of major histocompatibility complex (MHC) class II molecules (termed as human leukocyte antigen-DR, HLA-DR) and the T-cell costimulatory molecule B7 [63]. This feature makes them suitable for allogeneic engraftment transplant application [64]. In addition, MSCs also release various regulatory factors that inhibit the immune response of T and B lymphocyte and natural killer cells, suppress the activation of antigen presenting cells, as well as modulate macrophage polarisations [65]. To utilise the benefits of trophic paracrine factors, MSCs were encapsulated in a gelatin/PEG biomatrix as a living dressing for healing full-thickness wounds in rats. Following a 7-day treatment, this MSC-based therapy led to reduced immunocyte infiltration and foreign giant cell formation, as well as enhanced angiogenesis, granulation tissue formation [66]. In another MSC-laden hydrogel study, the delivered stem cells were used to renovate the inflammatory milieu of the chronic wound of diabetic ulcers *via* elevated paracrine production of TGF-β1 and FGF-2 to inhibit pro-inflammatory M1 macrophage polarisation [67]. Similarly, the co-culture of mesenchymal cells from human amniotic tissue or treatment with their conditioned medium could instruct the phenotype shift of macrophages from classically activated M1 to M2-like anti-inflammatory condition. This conversion was conducted according to the high content of IL-10, TGF-β1 and downregulated secretion of inflammatory IL-12, TNF-α, RANTES and nitric oxide in the cellular environment of mesenchymal cells [68].

Although the cell-based GFs strategies exhibit a high potential for accelerating wound repair and modulating the chronic wound microenvironment, there are still areas requiring attention and improvement, including the mode of cell delivery, the lack of specific markers for stem cell tracking, as well as relatively low survival and engraftment. Fortunately, a few attempts have been carried out by formulating multicellular spheroids or aggregates ahead of their engraftment, which facilitates the yields of trophic paracrine factors compared to the direct application of cell suspensions [69–74].

2.4. Harnessing the power of biomaterials-evoked endogenous GFs in situ

Besides transplanting exogenous cells to produce pro-regenerative GFs, a more recent strategy is to harness the functions of the GFs produced by endogenous cells *in situ*, through manipulating the biomaterial–host interactions. The endogenous tissue progenitor cells and immunocytes, especially monocytes and macrophages, exquisitely tuned by distinct physical/chemical cues and bioactive properties of materials (Table 1), could exert a great potential in therapeutic applications.

2.4.1. Stimulating GF production by physical cues

The physical features of biomaterials such as stiffness, roughness and topography are important for cell attachment, viability and phenotypic variation, which determine the cell fate and wound healing performance. In particular, it was revealed that the uniaxial cyclic stain (14%) could elevate the expression of VEGF and IL-6 expression in human dermal fibroblasts seeded on collagen-coated chitosan sponges [75]. And the upgrade of pro-healing GFs was maintained for at least 3 days under the stimulation of 14% cyclic strain. Besides, a stiffness-tunable hydrogel was fabricated with poly(amidoamine)-based poly(*n*-isopropyl acrylamide) to investigate the influence of the mechanical property of biomaterial on tissue response [76]. The results indicated that the hydrogel with medium stiffness expressed significantly higher amount of FGF-2 and accelerated the proliferation of keratinocytes. Similarly, compared to matrices with the rigidity of the human vitreous (1 kPa) and corneal epithelium (8 kPa), MSCs cultured on a

Table 1
Examples of biomaterial-mediated utilisation of endogenous GFs.

Category	Basic materials	Contributing factors	Modulated growth factors	References
Physical cues	Collagen-coated chitosan sponge	Uniaxial cyclic stain	VEGF, IL-6 ↑	[75]
	PNIPAM-PAA hydrogel	Stiffness	FGF-2 ↑	[76]
	PAM/BIS hydrogel (Matrigen Softwell®)	Rigidity	TGF-β1 ↑	[77]
Chemical signals	Cu-doped borate bioactive glass microfiber	Copper ions release	VEGF, TGF-β ↑	[78]
	Cu containing bioactive glass nanocoating on eggshell membrane		VEGF, HIF-1α ↑	[79]
	Chitosan-silica hybrid sponge	Silicon ions release	TGF-β1 ↑	[80]
	N/A	CKD712	VEGF ↑	[81]
	N/A	20 (*S*)-protopanaxadiol	VEGF ↑	[82]
Bioactive molecules	N/A	Platelet lysate	GM-CSF ↑	[83]
	Chitosan hydrogel	SIKVAV peptide	VEGF, FGF-2 ↑	[84]
Bioactive interface to activate macrophages	KGM-TA/Hep-TA hybrid hydrogel	KGM	VEGF, PDGF-BB ↑	[87]
	PCL fibre meshes	DMOG	IGF-1, HB-EGF, NGF, TGF-β1, IL-4 ↑ IL-1β, IL-6 ↓	[89]

matrix with corneal stromal rigidity (25 kPa) exerted better corneal re-epithelializing functions by activating integrin β1 in human corneal epithelial cells *via* TGF-signalling pathway [77].

2.4.2. Stimulating GF production by chemical signals

Chemical signals, including inorganic materials and small organic molecules, could invoke GF expression at the wounded site to stimulate angiogenesis and wound healing. For example, the ionic dissolution of Cu-doped borate glass microfibers was verified to be biocompatible and induced the expression of pro-angiogenic GFs such as VEGF and TGF-β in wounded diabetic mice [78]. Similarly, bioactive glass nanocoating on eggshell membranes (Cu-BG/ESM) containing different Cu doses was prepared *via* pulsed laser deposition technique [79]. Among them, Cu-BG/ESM containing 5 mol% copper elicited angiogenic activity by stimulating human umbilical vein endothelial cells (HUVECs) to produce VEGF, HIF-1α and nitric oxide. In another research, chitosan-silica hybrid sponge was devised to release silicon ions, which functioned to recruit inflammatory cells to wound sites and enhance GF secretion (particularly TGF-β at the early stage), giving rise to fibroblast proliferation and wound healing [80].

As for small molecules, a synthetic tetrahydroisoquinoline alkaloid (CKD712) was employed to accelerate cutaneous wound healing by activating VEGF production *via* haem oxygenase 1 induction in a full-thickness skin wound in mice [81]. Additionally, 20(*S*)-protopanaxadiol, an aglycone of ginsenosides in *Panax notoginseng*, increased nuclear trans-localisation of HIF-1α to promote VEGF secretion and capillary formation in HUVECs, and showed the capability to drive angiogenesis and wound healing in db/db diabetic mice [82].

2.4.3. Stimulating GF production by bioactive molecules

The delivery of bioactive molecules could also be utilised to modulate endogenous GF expression and initiate native healing cascades. For instance, platelet lysate was reported to regulate the paracrine activity in BM-MSC through NF-κB activation, triggering an inflammatory response in MSCs and maintaining pro-inflammatory polarisation of macrophage. These cellular activities uphold the inflammatory response towards injury that is crucial for initiating wound healing process [83]. Also, a laminin-derived synthetic peptide Ser-Ile-Lys-Val-Ala-Val (SIKVAV) was conjugated to chitosan hydrogels for cutaneous wound healing [84]. This peptide exhibited its function in promoting the proliferation and migration of fibroblasts and enhancing the synthesis of EGF, FGF-2, VEGF, PDGF, TGF-β1, and collagen I and III.

2.4.4. Stimulating GF production by activating macrophages with bioactive interface

Perhaps one the most attractive cellular targets for modulating the expression of pro-regenerative GFs is the macrophage. Macrophages play crucial roles during multiple wound healing phases, serving as both initiators and regulators to construct a pro-regenerative environment through secreting a diverse range of pro-inflammatory, anti-inflammatory, pro-healing and pro-angiogenic GFs [85, 86]. Therefore, therapeutic strategies for activating endogenous monocytes/macrophages to regulate GF functions have attracted increasing attention. For instance, konjac glucomannan (KGM) was demonstrated to be able to activate monocytes/macrophages to secrete pro-angiogenic and mitogenic GFs (VEGF and PDGF-BB) (Fig. 2i and j), which could be bound and stabilised by heparin (Hep) molecules. A KGM-TA/Hep-TA hybrid hydrogel was fabricated, with its KGM bulk acting to stimulate macrophages to produce pro-angiogenic GFs and its heparin moieties sequestering the fresh, endogenous GFs within the gel matrices. A series of *in vitro* and *in vivo* tests suggest the potential of this composite gel in activating GF production and further utilising them to promote vascularisation for wound regeneration (Fig. 2k) [87].

Of note, an efficient wound repair requires the involvement of macrophages in an anti-inflammatory phenotype – especially in the later stage of tissue remodelling. Hence, numerous strategies have focused on facilitating the transition of macrophage towards an M2 phenotype. For example, the ionic products from 45S5 bioglass were demonstrated to trigger M2-towards differentiation of macrophages and improve the secretion of anti-inflammatory and angiogenic GFs. Its application promoted capillary formation by endothelial cells and ECM deposition by fibroblasts [88]. Moreover, poly(γ-caprolactone) (PCL) fibre meshes embedded with the hydroxylase inhibitor dimethyloxalylglycine (DMOG) were fabricated by electrospinning [89]. RAW264.7 macrophages grown on this meshes showed to express higher levels of GFs (IGF-1, HB-EGF, and nerve growth factor, NGF) and anti-inflammatory factors (TGF-β1 and IL-4) and lower levels of pro-inflammatory factors (IL-1β and IL-6). Experimental outcomes indicated that PCL/DMOG meshes promoted wound healing by modulating macrophage responses. In another study, milk fat globule-epidermal growth factor 8 (MFG-E8), a novel anti-inflammatory factor, was demonstrated to reprogram macrophages from a pro-inflammatory M1 to pro-healing M2 phenotype [90]. The addition of MFG-E8 benefited wound closure in mice model, associated with an increased number of CD206-positive cells (M2 macrophages) and their elevated FGF-2 secretion. These trials demonstrated that it could be feasbile to increase the supply of endogenous, pro-healing GFs by fine-tuning the phenotypes of macrophages at the wound

site, using either soluble/diffusive bioactive agents or specifically designed surface of biomaterials implants.

3. Improving the stability and functions of GFs in ECM: controlling activity in transportation

3.1. Introduction to the role of ECM as the reservoir of GF

Cells reside in the three-dimensional ECM; and ECM is crucial for the cells. Its gel-like fibrous network provides both mechanical support and biological signalling, which dynamically and subtly dictate the behaviour of the cells [91–93]. ECM is also the reservoir of GFs; it binds, sequesters and enriches multiple GFs, enhancing their interaction with corresponding receptors. During an injury, not only the cells but also the ECM – including its components and their formed network structures – is lost. Reconstructing ECM, or functional mimics of ECM, is a rational and biomimetic strategy to stabilise the delivered GFs and improve their functions for wound healing.

Among the multiple types of ECM components, two groups of macromolecules are crucial building blocks of cellular microenvironment and play fundamental roles in regulating cell adhesion, survival, proliferation, migration and differentiation, in concert with a plethora of GFs and other small molecules [94]. The first group includes proteins presenting cell-adhesive domains, notably including the collagen family, elastin and laminin. It can also include fibrinogen, fibronectin and vitronectin, though their molecular weights are relatively smaller. These proteins form a scaffold to anchor the cells, confer tensile strength and accommodate other signalling molecules [95–99]. The cell-affinitive sequences on ECM proteins could be recognised by transmembrane receptors, such as integrins, and mediate the cell-ECM interaction [100, 101]. Besides mechanical anchorage, ECM proteins also transmit extracellular signals into cells (*e.g.*, adhesive state and surrounding environment) through integrin signalling to dictate cellular fates, such as migration, proliferation, differentiation and apoptosis [102, 103].

The second group comprises proteoglycans with their characteristic glycosaminoglycans (GAGs) chains. GAGs are sulphated (hence negatively charged), linear heteropolysaccharides, typically composed of repeating disaccharide units. Their highly hydrophilic carbohydrate chains provide the gel-like compressive characteristics of the tissue. More importantly, they bind and stabilise GFs to promote the latter's actions during tissue development and wound healing [104]. Through the different stages of the wound healing process, immune and tissue-resident cells secrete a multitude of GFs, which potently and rapidly regulate cell behaviours. GAGs sequester, stabilise and concentrate these soluble GFs, regulating their distribution and presentation to cells, before they interact with cell-surface receptors. The most representative type of GAG performing these functions is heparin sulphate, which binds to and modulates the activity of multiple GFs [105–110]. In the development of *Drosophila*, mutants in HS sequences result in morphogenic defects associated with inactivation of FGF-mediated signalling [111].

Apart from GAGs, some ECM proteins may also possess GF-binding capabilities, but the binding affinities may be less specific and weaker, as well as less elucidated, than those between GF and HS [112, 113]. For instance, vitronectin and fibronectin can bind manifold GFs from different families through their heparin-binding domains [114–117]. Once sequestered by the ECM, these signalling molecules could be released according to their binding kinetics and the hydrolysis of proteases. As such, the ECM could sequester and store GFs, establishing concentration gradients and modulating their bioavailability in a spatiotemporally controlled manner.

During the dynamic wound healing process, the ECM remodelling, as the extended final stage, determines the quality of wound repair. Chronic wounds arise due to prolonged pathogen infection or the impact of other diseases. In these cases, the wounds become trapped in a prolonged inflammatory phase, leading to the deterioration of the extracellular microenvironment. Take diabetic milieu as an example, turbulent redox signalling and increased oxidative stress level resulted from diabetes cause various complications, including impaired wound healing [118–121]. Normal redox signalling is disrupted, and the balance between the generation of reactive oxygen species and scavenging is broken, which directly damage both the cells and ECM at the wound site [122]. Meanwhile, persistently high levels of proteolytic enzymes result in the degradation of both matrix and the ECM-bound signalling molecules, which delays the wound healing. Therefore, it is vital to reconstruct a functional ECM and re-create a favourable niche for wound healing.

Hence, targeting the interaction between components of the ECM and GFs during wound repair may provide numerous attractive approaches. Among them, utilising the GF-binding mechanism of ECM constitutions and engineering a dynamic ECM-mimic material platform may be the key to enhance the delivery of GFs for wound healing.

3.2. Preparing naturally derived ECM macromolecules as delivering vehicles to stabilise GF

3.2.1. Decellularised ECM-derived matrices

Decellularised ECM-derived matrices (DEDMs) are prepared by removing cells from native tissues, such as the skin dermis [123], the submucosa of the small intestine [124], pericardium [125], urinary bladder [126], amniotic membrane [127] and placenta [128–131], among others (Fig. 3a). These DEDMs contain not only a variety of components retaining a close-to-native ECM structure, but also a composition of diverse endogenous growth factors facilitating wound healing. The similarity of the microstructure, composition, biomechanical and biological properties of decellularised matrices to those of native human ECM has spurred interest in the direct use of these materials for tissue engineering applications [132, 133].

The first advantage of DEDMs for wound healing is their content of endogenous GFs. In delicately processed DEDMs, the GFs can preserve their due bioactivities even after sterilisation and storage [134, 135]. For instance, a decellularised sheet was fabricated from human placentas [130] and a porous scaffold from human Wharton's jelly [136], both *via* freeze drying, for full-thickness wounds. These two ECM-derived dermal substitutes retained a cocktail of GFs involved in healing (*e.g.*, TGF-β1, FGF-2, EGF, PDGF, IGF-1, and VEGF-A) and demonstrated the capability to promote cell proliferation, accelerate wound closure and restore skin structure.

The second advantage of DEDMs is that they keep to the maximum extent the structure of ECM with cell-adhesive proteins and GF-binding GAGs. Compared with synthetic scaffolds, the decellularised matrices provide a platform for incorporation, delivery and sustained release of GFs. Specifically, an acellular injectable hydrogel produced from pericardial ECM confirmed the preservation of the GAG components and their FGF-2-binding ability relying on ionic association with the sugar chains [125]. Thus, this decellularised hydrogel could achieve sustained delivery of entrapped heparin-binding growth factors to the wound site.

Although DEDMs can retain the native components and structure of the living tissue, ways of manipulation may introduce changes to the physicochemical properties of the matrices [137–140]. Particularly, the freeze-thawing operation can alter the original structure of the ECM and thereby its mechanical integrity [141]. Similarly, acid or alkaline treatments may degrade vital components of the ECM, while ionic detergents (like sodium deoxycholate and SDS) can damage the ECM ultrastructure [142] and disrupt the electrostatic interactions among proteins [143]. Moreover, like many other natural materials-based

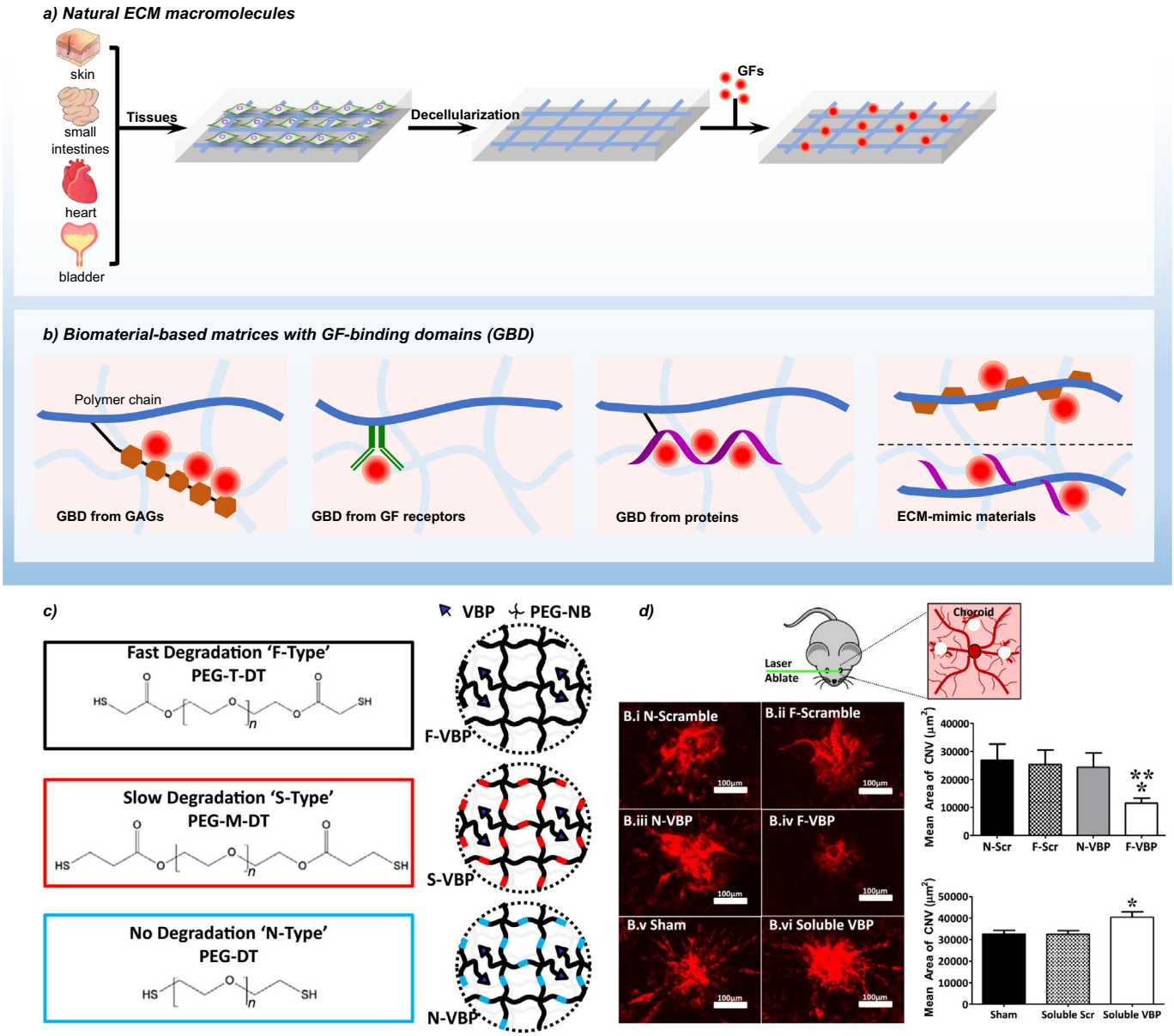

Fig. 3. Controlling GF activity in transportation: ECM-inspired matrices for improving the stability and functions of GFs. a) The procedure of preparing decellularised matrices for GF delivery; b) engineering biomaterial matrices by utilising GF-binding domains (GBD); c) schematic of VEGF binding domain peptide (VBP) microspheres crosslinked with fast-degrading PEG-T-DT, slow-degrading PEG-M-DT, and non-degrading PEG-DT; d) Degradable VBP microspheres reduced neoagiogenesis in a mouse choroidal neovascularization model. The results were illustrated by the representative fluorescent micrographs of ICAM2 + vessels in mouse CNV model after treatment with N-Scramble, F-Scramble, N-VBP, or F-VBP microspheres, or sham (saline) or Soluble VBP. Additionally, the mean CNV areas in mm^2 after treatment were also calculated. Reprinted with permission from ref. [176].

scaffolds, batch variation related to processing is another common issue and can influence the efficacy of the final DEDMs scaffolds.

3.2.2. GAG-based matrices

GAGs are the primary ECM components that bind, stabilise and promote the actions of various GFs in the wound site [144]. Because of this prominent property, major types of GAGs, including heparan sulphate, chondroitin sulphate, dermatan sulphate, keratin sulphate and hyaluronic acid, have been isolated and processed into tissue scaffolds, in an attempt to sequester the GFs *in situ* for healing.

Compared with using an entire piece of decellularised ECM, using purified GAGs as would healing materials has both technical and regulatory advantages – their composition, GF-binding activity (and specificity) and possible side-effects can be more clearly defined. Also, GAGs can be blended or crosslinked with another more inert polymer to fabricate a scaffold; by tuning the proportion of the two polymers, one can potentially achieve finer control of GF-binding strengths of the scaffold. Biomatrices based on GAG derivatives could promote the adhesion, proliferation and ECM-producing function of dermal fibroblasts [145]. It may be possible to modulate fibroblastic phenotypes by tailoring the matrix composition, and therefore optimise the wound healing process.

This GAG-GF interaction is largely based on the electrostatic interaction between the abundant negative charge from the sulphate and carboxylate groups of GAGs and the basic amino acids provided by the GFs [146]. But beyond the electrostatic interaction, the structures of both the oligosaccharides and the protein are also important. Take FGF-2 as an example, which is a well-known heparin-binding GF. Biomaterials in diverse forms have been designed with sulphated GAG molecules to control the release of this heparin-binding GF, such as *in situ* crosslinked

hydrogels [147], heparinised decellularised adipose matrix [148], GAG-containing nanoparticles [149] and a plasma polymerised allylamine surface decorated with heparin [150]. These strategies not only prolong the GF's half-life but also reduce the use of exogenous FGF-2 – usually in the form of recombinant protein that is highly unstable *in vivo* and is carcinogenic if overdosed. The GAG-based matrices have proven to protect the GFs from proteolytic degradation, in particular in the wound niche where the proteases are abundantly expressed. Besides, for theoretical research, a short-range cytokine gradient system has been set up using cell-sized agarose microparticles modified with different GAGs, to mimic and investigate paracrine cell interactions *in vitro* [151].

GAGs bind not only mitogenic growth factors but also pro-/anti-inflammatory cytokines. Because persistent and excessive inflammation is a major reason for impaired wound healing (especially in chronic wounds), scaffolds to bind immunological cytokines have been developed. For instance, a series of star-shaped PEG hydrogels decorated with heparin were fabricated to scavenge the inflammatory chemokines in the wound bed [152] or sustainably release IL-4 to regulate macrophages to a pro-healing phenotype (M2) [153]. These heparin-hybrid hydrogels offer valuable options to switch the local immunity of a wound microenvironment from a pro-inflammatory to pro-regenerative state, which is extremely needed in chronic, non-healing wounds such as diabetic ulcers.

3.2.3. ECM-derived proteins

Besides GAGs, some ECM-derived proteins, such as fibronectin (FN) and fibrin(ogen), can also bind various GFs and enhance their bioactivity *in situ* [112, 154, 155]. Like a bioactive glue, FN mediates the interaction between tissue cells, growth factors and other ECM proteins and reinforces the provisional matrix in wound healing [156, 157]. The heparin-binding domains of FN and fibrin(ogen) exert high affinities for growth factors of the PDGF/VEGF, FGF, TGF-β and neurotrophin families [112, 117]. Such interactions prepare FN and fibrin(ogen) to be potential carriers for the retention and delivery of GFs during wound healing [158]. For instance, a co-delivery system of fibrin functionalised with fibronectin to deliver CXCL11 was applied to facilitate wound healing in diabetic mice [159], leading to a remarkably enhanced granulation and accelerated re-epithelialization and wound closure.

Other ECM proteins, such as collagens and elastins, have long been developed into regenerative scaffolds. They may also possess the affinity for certain GFs to varying extent. Nevertheless, in comparison with FN or GAGs, their binding with GFs is weaker and less specific; even they bind a GF, it is unclear whether they promote the activity of this GF as a GAG does. But their major advantages – as is FN – are their abundant cell-adhesive and protease-sensitive domains that facilitate cell adhesion and further design of environmentally-responsive matrices. They may be used in combination with FN, GAGs or other GF-affinitive molecules, providing versatile tools for wound healing.

3.3. Engineering biomaterial matrices for GF delivery

Although decellularised ECM and isolated ECM components provide GF-binding features, their application as matrices for wound healing and tissue regeneration faces three major challenges. First, like many other naturally-derived substances, ECM or its components extracted from living sources can potentially lead to immunogenicity, pathogen transmission, batch-to-batch variability, and cost and ethical concerns. Second, they may be able to bind multiple GFs but lack specific affinity. For instance, a batch of heparan sulphate derived from the skin may simultaneously bind various pro- and anti-angiogenic GFs, which will make their overall efficacy uncontrollable when used as delivering matrices for VEGF or FGF-2. Third, these natural polymers may have useful bioactivities, but their physicochemical characteristics require further major modifications to satisfy the engineering demand – such as hyaluronic acid, which often requires crosslinking to gain an acceptable mechanical property. Hence, researchers have focused on designing non-ECM scaffolds with a range of materials for controlled delivery of GFs in the wound milieu (Fig. 3b).

3.3.1. Engineering biomaterial matrices by utilising GF-binding domains

Much information has been unveiled regarding the structural biology of GAG-GF binding [160]. For GFs, certain amino acid sequences, which present an electrostatic charge and a tertiary structure, are essential for the binding; for GAGs, their disaccharide profile and the number of repeating disaccharides are crucial [161–163]. For example, on the one side, the specific units of NS (highly sulphated sequences rich in GlcNS saccharides) on HS are required for FGF-1 and FGF-2 signalling through FGFR1c [164]. Similarly, *N*- and 6-*O*-sulfation have proven crucial for the binding of HS to TGF-β1 [165]. On the other side, the heparin-binding domain of VEGF-A has been elucidated to be the amino acid sequences from 142 to 165. Among these, the arginines presenting positive charges are required for the binding of sulphated GAGs [166].

Understanding such information offers insights for engineering HS with higher binding selectivity for one type of GF. In a series of studies, various peptides representing the heparin-binding domain on BMP-2, VEGF-A and TGF-β1 have been synthesised and modified onto the chromatographic column. This technology allows for the acquisition of the HS fraction with increased affinity for one type of GF. For instance, the crude HS was applied to the column modified with the heparin-binding domain of VEGF-A; its VEGF-affinitive fraction (wt., 9%) could be isolated from the other fractions. Compared with the crude HS with a low effect, this fraction remarkably enhanced the bioactivity of VEGF-A in inducing angiogenesis both *in vitro* and *ex ovo* [167]. This technology was creative, practical and readily translatable. Although it remains unknown how specific the fraction can be for one GF – in other words, whether one fraction affinitive for one GF loses its affinity for other GFs, further improvement in this technology may lead to mass production of GF-affinitive GAGs for the delivery of specific GFs for regenerative purposes.

Like the saccharide repeats on HS/GAG polysaccharides, the GF-binding domains of proteins derived from ECM, such as vitronectin [168], fibrin(ogen) [169], tenascin C [170], and fibronectin [171], are also investigated. For instance, a specific domain from fibronectin (FN III12–14) was confirmed to bind multiple GFs including VEGF family, FGF family and TGF-β family, with K_D values in the nanomolar range [171]. Similarly, a peptide named P12, derived from the first type III repeat of fibronectin, can bind with PDGF-BB (K_D value: ~200 nM). It facilitates PDGF-BB in promoting the survival of adult human dermal fibroblast under serum starvation and oxidative stress. Further *in vivo* evaluation indicated that P12 could significantly prevent the deterioration of burn injury in a rat hot comb model, compared with the control group [172]. These GF-binding peptides have been fabricated into bioactive materials for modulating the retention and delivery of GFs. Like fibrin(ogen), the specific binding domain of fibrin(ogen) named Fg β15–66 was incorporated into a synthetic fibrin-mimetic matrix, which presented high affinity for FGF-2 and PlGF-2. This synthetic matrix mimicked the effect of fibrin and might have the potential to substitute tissue grafts in the treatment of severe wounds [169].

Apart from ECM-derived peptide domains, researchers discovered a sequence (HSNGLPL) by phage display with a high binding affinity for TGF-β1 [173]. TGF-β1 could be immobilised on polycaprolactone surface functionalised with this peptide. The *in vivo* evaluation demonstrated that more TGF-β1 was retained and enriched on this peptide-grafted membrane, compared with the unmodified film. The sequestration of TGF-β1 increased the number of inflammatory cells at day 3 and promoted more fibrogenic response and neovascularisation *in situ* at day 7 [174].

Since GFs bind their corresponding receptors to trigger cellular signalling, the specific GF-binding moieties on these receptors may also be utilised to modify biomaterial scaffolds. For example, a peptide ligand was obtained from the VEGF receptor 2 (VEGFR2) and covalently incorporated into the synthetic hydrogel microspheres consisting of PEG

dithiol and 4-arm PEG norbornene, which mimicked the activity of natural ECM to not only sequester VEGF-A but, more intriguingly, release this GF in a controlled manner [175]. Moreover, PEG microspheres containing VEGF-binding peptides (VBPs) were prepared to bind, and modulate the activity of, VEGF-A, demonstrating the potential to reduce pathological angiogenesis (Fig. 3c and d) [176].

The above approaches suggest that the natural bindings between GFs and ECM components or receptors provide rich inspiration to the design of GF-affinitive matrices. Their advantages are obvious. First, compared with using naturally derived ECM which has a narrow range of physicochemical characteristics, one can employ various materials with a broad spectrum of mechanical strengths, inner-structural properties and surface chemistries. Modification of GF-binding moieties onto these materials in different densities allows for better control of the amount of GF to be sequestered. Second, wound healing is a dynamic process involving the actions of multiple GFs; such approaches help to achieve the combinatory delivery of multiple GFs with temporal control. Nevertheless, a critical pre-requisite of all these strategies is that the GF-affinitive sequence – either carbohydrates or peptides – are well elucidated and can be obtained in the laboratory.

3.3.2. Developing GAGs-mimetic materials

As discussed above, GAGs are the main macromolecules that bind and stabilise GFs in ECM, and their structural features responsible for mediating such bindings are increasingly being uncovered. However, despite the advancement in chromatographic technologies, it remains a challenge to batch prepare HS in high purity and specified sulphation patterns. Therefore, developing GAG mimetics based on other polymers has attracted considerable attention.

The first method to prepare GAG mimetics is to use natural, non-GAG polysaccharides as a framework and modify them with sulphates; because GAGs are highly sulphated polysaccharides and the type, position and degree of sulphation directly affect their GF-binding capability. For instance, alginate was modified with different degrees of sulphation using SO_3/pyridine and gained the ability to bind FGF-2. The degree of modification mattered, as samples with higher sulphation degrees showed a stronger affinity for this GF [177]. Alginate has widely been used as materials for wound dressing and has versatile physical advantages, while FGF-2 plays a crucial role in wound healing and tissue regeneration; thus, this invention may prove to be an elegant combination of the two substances. Similarly, cellulose was sulphated and fabricated together with gelatin into a scaffold affinitive for TGF-β3 *in vitro* [178]. Although this study continued to apply this scaffold in cartilage tissue regeneration and achieved positive outcomes, it is possible to translate the same system for wound healing, given the beneficial role of TGF-β3 in matrix deposition and remodelling.

Next, due to the complexity of the polysaccharide structures and the challenges in modifying polysaccharides at specific sites, researchers started to employ non-polysaccharide, synthetic polymers to prepare GAG-mimetic matrices with desirable GF-binding features. Again, sulphation is a main strategy of modification. For example, poly(sodium 4-styrenesulfonate-*co*-poly(ethylene glycol) methacrylate) was synthesised and immobilised on a gold substrate. The binding of VEGF-A and FGF-2 to this polymer was identified using surface plasmon resonance technology [179]. Another GAG-mimic polymer, poly(vinyl sulfonate) was prepared and its ability to bind FGF-2 was also confirmed, which could further strengthen the FGF-2/FGFR interaction [180].

Likewise, polypeptides were modified with sulphated groups for the same purpose. One representative example was a peptide nanofibre, named HM-PA/K-PA composed of lauryl-VVAGEGD(K-pbs)S-Am and Lauryl-VVAGK-Am. It could mimic the function of HS, showing stronger binding affinities for VEGF, HGF and FGF-2 than the control peptide fibre (lauryl-VVAGE, E-PA/K-PA). The heparin-binding domain of VEGF is required for the binding with these nanofibers [181]. Another nanofiber gel based on the HM-PA/K-PA self-assembled heparin-mimetic peptide was invented as an effective wound dressing in the rat model of full-thickness excisions. The gel induced a greater extent of angiogenesis, skin appendage formation and granulation tissue than the sucrose-treated group [182]. Similarly, a GAG-mimetic peptide nanofibre SO_3-PA (lauryl-VVAGEGD-K (*p*-sulfobenzoyl)-*S*-Am)/K-PA was also reported. Although it bound with BMP-2 and enhanced osteogenic activity [183], the strategy to design this gel may have implications for the development of other similar systems for wound healing.

These interesting explorations, which suggest that a variety of polymers can serve as the building block of GAG mimetics, undoubtedly open up new avenues for preparing GF-affinitive matrices for tissue regeneration. Nevertheless, two fundamental questions remain unanswered. First, apart from sulphation, little is known about what chemical features a polymer should possess to gain the GF affinity. Ideally, a general principle or code should exist to guide the modification of polymers into a GAG-mimetic scaffold. Second, most of these approaches could successfully prepare a GF-affinitive polymer; however, in the complex microenvironment of wounds, the efficacy of these tools in retention and release of GFs (and when to retain/release) awaits comprehensive evaluations in proper models.

An intriguing addition to the above methods based on chemical modification is a discovery of a natural polysaccharide from plants. Unlike GAGs of animal origin, a galacturonic acid-presenting – but non-sulphated – heteropolysaccharide was derived from a medicinal herb called *Eucommia ulmoides*. The polysaccharide, namely EUP3 (because it was the third polysaccharide fraction from chromatography based on acidity), showed a selective binding affinity for PDGF-BB and promoted PDGF-mediated vessel maturation [184]. EUP3 was further fabricated into a scaffold together with gelatin by electrospinning. It exhibited a distinctive feature of sequestering endogenous PDGF-BB at the wound site and subsequently release the GF in a sustained manner, leading to accelerated wound healing with improved tissue remodelling in mice [185].

3.4. Engineering growth factors themselves for sustained and specific release

As shown above, considerable progress has been made in developing GF-affinitive vehicles for wound healing. The vehicles may be GF-affinitive macromolecules derived from natural resources (such as GAGs) or polymeric scaffolds modified with GF-binding moieties. They bind and stabilise the GFs *via* non-covalent interactions, amplifying the latter's actions and achieving a controlled release in the healing microenvironment.

Alternatively, changes can be performed on the side of GFs, which are incorporated with enzyme-responsive domains or linked to biomaterials matrices – but covalently (Fig. 4a). These approaches aim to stabilise the delivered GFs while preserving their biological activities, as well as prolong their actions before degradation or internalisation.

3.4.1. Coupling growth factors with ECM-derived materials

GFs can be covalently anchored to a biopolymer *via* various types of chemical conjugations [186]. First, epidermal growth factor (EGF) was conjugated to hyaluronate (HA) by a coupling reaction between the generated aldehydes on the sugar chains of HA and amine groups of EGF (Fig. 4b). The HA-EGF preserved the activity of native EGF as confirmed by ELISA and fibroblast proliferation assay. The HA-EGF was incorporated into a patch-type film of HA and applied for wound healing in rats, exhibiting a desirable performance of prolonged retention of EGF in the wound niche, resulting in improved wound healing *in vivo* (Fig. 4c and d). It validated that HA conjugation inhibited the proteolytic degradation and increased the uptake of EGF into skin cells *via* HA receptors [187].

The second representative system was based on GFs and collagen. VEGF and Ang1 were covalently coupled to the porous collagen scaffolds using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) chemistry. More endothelial cells grew and formed

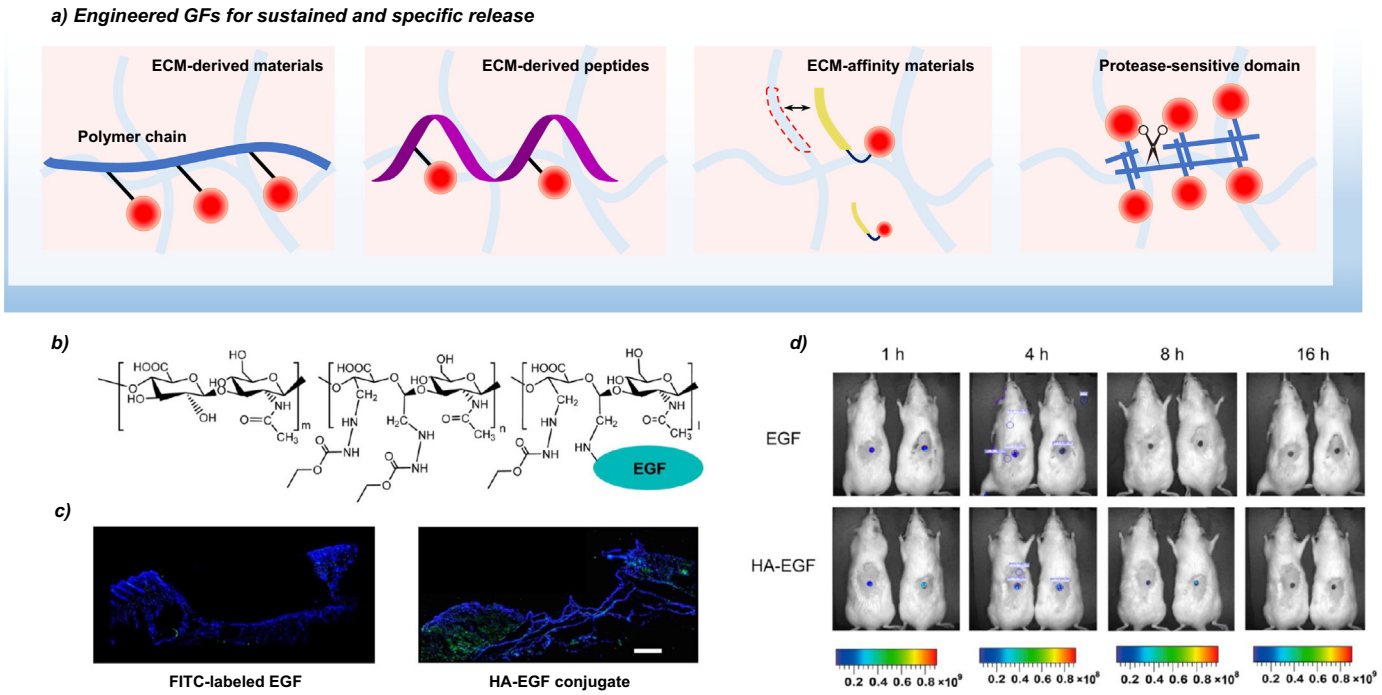

Fig. 4. Controlling GF activity in transportation: engineering GFs themselves for enhanced retention and controlled release. a) Engineering GFs with ECM-derived materials, peptides, ECM-affinity materials, and protease-sensitive fragments; b) the chemical structure of hyaluronate-epidermal growth factor (HA-EGF) conjugates; c) fluorescence images of the degradation behaviour of EGF and HA-EGF conjugate in patch-type HA films after topical application of FITC-labelled samples on wounded tissues; d) the immunofluorescent images of the skin tissues excised 1 day post-treatment with FITC-labelled EGF (left) and HA-EGF conjugate (right) (scale bar = 200 μm). Reprinted with permission from ref. [187]. Copyright 2016 American Chemical Society.

tubular structures in the fabricated scaffolds than in the EDC-activated scaffolds supplemented with native GFs. Moreover, coupling GFs with collagen also generate better effects in the chorioallantoic membrane assay. Such evidence suggested that protecting GFs from cellular inactivation and internalisation might be useful for improving the healing outcomes of the delivery [188].

Additionally, except for the ECM-derived biopolymers, one type of GF (FGF-2) was also coupled with a copolymer composed of styrene sulfonate units and methyl methacrylate units with PEG as side chains. This FGF-2–p(SS-*co*-PEGMA) conjugate could maintain the native bioactivity of FGF-2 and keep stable under harsh conditions. This copolymer showed the advantages of binding FGF-2 and inhibiting the denaturation of this GF [189].

3.4.2. Improve ECM-binding affinity of GFs through specific domains

Covalent conjugation provides firmer interactions between the carrier and the GFs than non-covalent binding and can thereby more potently enable sustained release; nevertheless, if designed inappropriately, covalent modification may affect the activity of the GFs. To avoid such limitation, researchers coupled GFs with ECM-derived fragments or specific domains. For example, 10th type III domain of fibronectin (FNIII10), VEGF-A mutant ($Mut_{Lys110–Pro111}$) and the $\alpha2PI_{1–8}$ substrate sequence were cloned into an eukaryotic expression vector pCEP-V97. The fusion product was expressed in human embryonic kidney cells and purified from cell supernatant. This engineered complex comprising FNIII10 and VEGF-A mutant exhibited affinity for both VEGFR-2 and integrin αVβ3, resulting in enhanced angiogenesis and cell adhesion compared with either protein used individually [190].

In another case, a collagen-binding domain which is a decapeptide with amino acid residues of WREPSFCALS derived from von Willeband's factor was coupled to the N-terminal of FGF-2. This conjugation could effectively improve neovascularisation and the migration of myofibers, showing considerable potential for treating full-thickness wounds [191]. Further, collagen membrane integrated with collagen-binding FGF-2 exhibited its ability to enhance the proliferation of human fibroblast cells *in vitro*. The collagen membrane fabricated by collagen-binding FGF-2 accelerated the healing Traumatic tympanic membrane and retrieved better structure and function of tympanic membrane, compared with native collagen films or non-treatment. The outcomes indicated that this platform has a promising prospect for treatment of Traumatic tympanic membrane perforation in the clinic [192]. Similarly, researchers engineered human BMP-2 combined with a collagen binding domain (CBD-BMP-2) and loaded into collagen scaffolds. The scaffolds were implanted into SD rats to mediate Lumbar 4–5 (L4–L5) posterolateral spine fusion. The results showed that CBD-BMP-2 group exhibited the highest fusion efficiency and bone mineral density among the tested groups, as well as prolonged retention of BMP-2 *in vivo* [193].

The discovery of specific GF domains responsible for binding other proteins such as those from ECM is providing new inspirations. For instance, a segment of placenta growth factor-2 ($PlGF\text{-}2_{123–144}$), found to mediate the binding with ECM-derived proteins, was coupled with GFs including VEGF-A, PDGF-BB and BMP-2. These engineered GF variants, potent in binding ECM, remarkably improved the healing of chronic wounds and regeneration of bone defects, compared with the native GFs [194]. Besides PlGF-2, native IGF-1 was conjugated with a heparin-binding domain of the heparin-binding epidermal growth factor-like growth factor (Xp-HB-IGF-1). This conjugation could bind heparin as well as various types of cell surfaces, which in turn improved the retention of the GF variant in the proteoglycan-rich environments [195]. Further, a substrate sequence tag derived from α_2-plasmin inhibitor ($\alpha_2PI_{1–8}$) was coupled with IGF-1, which can be covalently cross-linked into fibrin matrices. This conjugation showed higher bioactivity than normal IGF-1 and potential in promoting smooth muscle regeneration [196].

3.4.3. Incorporating protease-sensitive domains into GFs

As discussed above, increasing the binding affinity between GFs and matrices helps to avoid burst release of the GFs and offers more room for modulating its release rate. Meanwhile, creating responsiveness in the

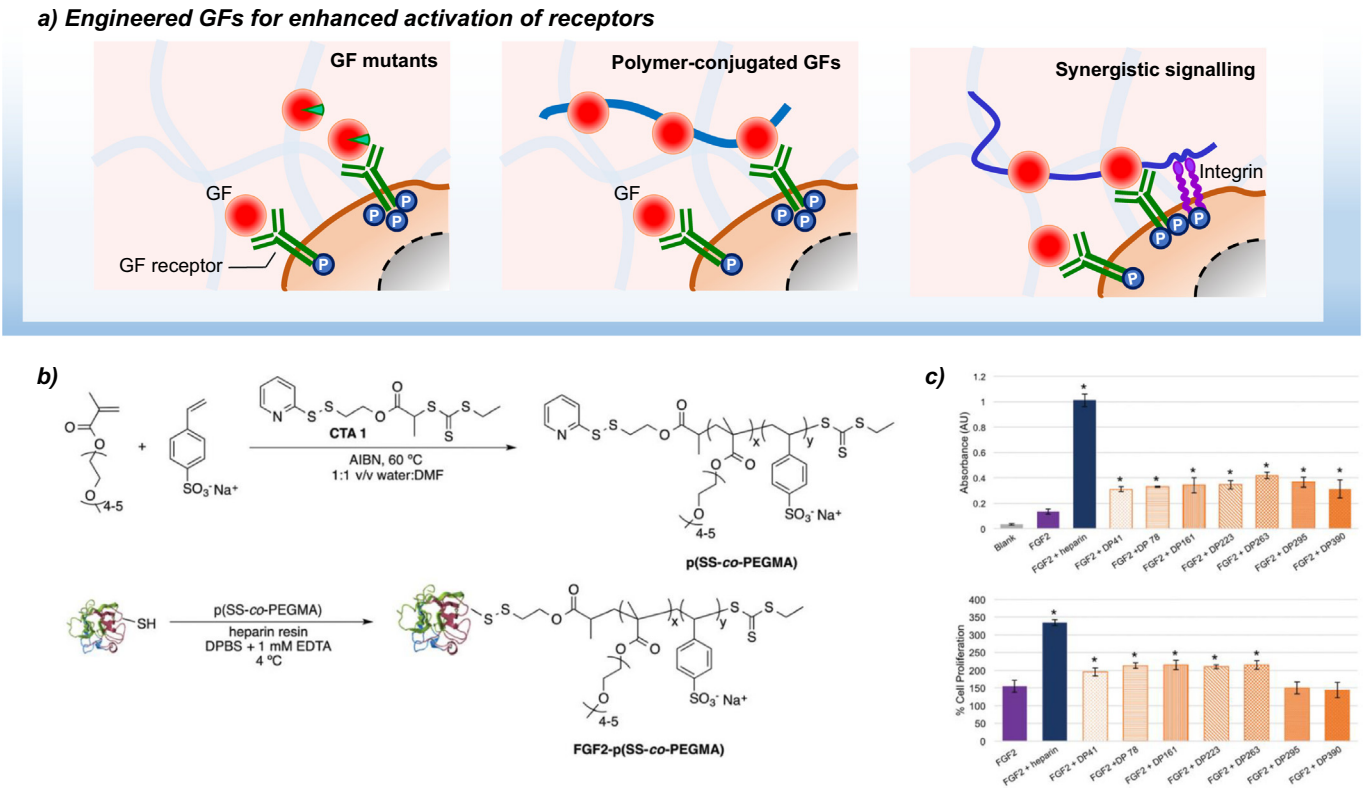

Fig. 5. Improving the activation of GF receptors – controlling the activity of GFs at the 'destination' end. a) Molecular engineering-based strategies for improving GF receptor activation (GF mutants, polymer-conjugated GFs, engineered GF material facilitating the synergistic signalling between integrins and GF receptors); b) synthesis of polymer-protein conjugate, FGF2-p(SS-*co*-PEGMA); c) assessment of the FGF receptor binding abilities by FGF2-p(SS-*co*-PEGMA) with diverse polymer sizes. Reprinted with permission from ref. [205]. Copyright 2017 The Royal Society of Chemistry.

GF-delivery system has emerged as another strategy. The injured tissues express elevated levels of proteases; synthesising protease-sensitive peptides and coupling them to the GFs can lead to the selective release of the latter on demand. One study reported the design and evaluation of polyethylene glycol-based synthetic hydrogel scaffold incorporating i) MMPs-sensitive domains, ii) cell-adhesion motifs and iii) GFs. This system demonstrated a proteolysis-dependent release of VEGF *in vivo* over 2 weeks, compared with the injection of soluble VEGF. As a result, an increased number of blood vessels migrated into the implants in early days, with a higher vessel density found in 1 month [197]. In another interesting study, an optimised fibrin-based platform was engineered for the delivery of VEGF. Murine $VEGF_{164}$ was coupled with a sequence derived from α_2-plasmin inhibitor (α_2PI_{1-8}), which is a substrate of the coagulation factor fXIIIa. The complex was further covalently cross-linked into a fibrin hydrogel, such that it was only released in response to enzymatic cleavage. The efficacy of this fibrin-α2-PI_{1-8}-$VEGF_{164}$ system was evaluated in ischemic hind limb and wound models, showing convincing outcomes in promoting angiogenesis and tissue perfusion [198]. Additionally, another hydrogel was engineered by polyethylene glycol maleimide (PEG-MAL) for delivering pancreatic islets to the small bowel mesentery and promoting graft vascularisation. A cysteine-flanked proteolytically degradable peptide (GCRDVPMSMRGGDRCG) was selected to cross-link the hydrogel. Human VEGF-A was physically incorporated into PEG-MAL hydrogel to be released in a controlled manner in response to collagenase degradation. The islets were loaded into PEG–MAL matrix and then were transplanted to the small bowel mesentery of rats. The results showed that islets grafted to the host tissue and revascularised by 4 weeks. Thus, this PEG-MAL engineered matrix with enzymatic release of VEGF-A can augment neovascularisation and may serve as a pro-angiogenic delivery system for various kinds of wound healing [199].

4. Improving the activation of GF receptors – controlling activity at the 'destination' end

GFs are delivered to the wound milieu, to the microenvironment, to the cells; but the 'real' recipients are cell receptors. GFs are delivered to bind and activate their corresponding receptors on the cell membrane to initiate intracellular signalling. We have discussed the approaches to improve the supply of GFs in Section 2 and stabilise/enhance the GFs during delivery in Section 3; here, we introduce the emerging attempts to improve the activation of the GF receptors for wound healing. Developing strategies to regulate the GF-receptor interactions may help to maximise the efficiency and bioactivity of the delivered GFs while minimising the dosage of administration (Fig. 5a).

4.1. Molecular engineering of GFs to strengthen their binding with the receptors

A major goal of improvement is to increase the binding affinity between a pro-healing GF and its receptor, and a series of approaches have been developed for this purpose [200]. GFs can be engineered into active mutants as therapeutic molecules for tissue regeneration, which often exhibit higher capability of binding receptors and enhancing signalling. For example, a chimeric protein, VA1, composed of receptor-binding fragments of VEGF and Ang-1 proved potent in enhancing angiogenesis, by binding to and activating VEGFR-2 and Tie2 receptor simultaneously. VA1 demonstrated both higher efficacy and safety than VEGF. It activated VEGFR-2 differently, decreased vessel leakiness and tissue inflammation, as well as increased perfusion in ischemic muscles. Meanwhile, it did not cause angioma formation, vascular permeability or inflammatory cell recruitment to the same level of VEGF, thereby reducing the potential risks [201]. Another protein fused with α_2-PI_{1-8} and $VEGF_{121}$, named TG-$VEGF_{121}$, was developed, which

bound to fibrin and was released in a controlled manner in response to proteolysis. *In vivo* evaluation demonstrated that TG-$VEGF_{121}$ induced more established and stable vascularisation than $VEGF_{121}$. In sum, the release of fibrin-conjugated mutant TG-$VEGF_{121}$ could induce lower activation of VEGFR2-luc, but more significantly enhance angiogenesis, compared with native $VEGF_{121}$ [202]. These fusion proteins directly aimed at manipulating the type and strengths of GF-direction interaction may have immediate applications in wound healing, especially in the early stages.

In addition to VEGF-related products, researchers engineered two EGF mutants, m28 and m123, which possessed different sequences at the specific sites. The two mutants showed faster kinetic on-rates and more potently activated EGFR than the wild EGF did. Similarly, eight NGF variants were generated and their binding affinity for neurotrophin receptor (NTR) was evaluated. A variant called I31R NGF showed more specific binding to NTR than other variants [203, 204]. Given the profound and direct role of EGF (and the possible effect of NGF) in wound healing, these two lines of GF variants may have broad applications in wound healing and skin regeneration.

Polymers are also useful tools to modify GFs for achieving greater bind affinity for receptors. For instance, FGF-2 was conjugated with a catalogue of copolymers of poly(styrene sulfonate-*co*-poly(ethylene glycol) methyl ether methacrylate) [p(SS-*co*-PEGMA)] with various degrees of styrene sulfonate but similar sizes (Fig. 5b). The binding affinity between the FGF-2 conjugates and FGFR was measured using ELISA and a cell-based assay. The results indicated that $p(SS\text{-}co\text{-}PEGMA)_{81}$, with a degree of 81% styrene sulfonate incorporation, most significantly enhanced FGFR binding, compared with both unmodified FGF-2 and all other modified products (Fig. 5c). Further, $p(SS\text{-}co\text{-}PEGMA)_{81}$ in varying sizes (containing from 41 to 390 monomer repeating units) were synthesised, but they showed a similar affinity for the receptor. It could be concluded that changing the degree of sulfonated monomer, instead of altering the size of the polymer, would be an effective strategy to modulate FGFR binding [205].

4.2. Synergistic signalling effects

Besides their functions to support cell adhesion and retaining GFs, the ECM components also participate in mediating the GF signalling through modulating the GF-receptor interaction. Certain fragments from ECM could be used to enhance the signalling in a synergistic mechanism and consequently lower the usage of GFs.

One of the most interesting examples is a fibronectin fragment containing both the α5β1 integrin-binding domain (III9 to 10) and VEGF-binding domain (III13 to 14), which significantly augmented VEGF-mediated endothelial migration and proliferation, as well as the activation of VEGFR/Erk signalling. However, the cell-binding or VEGF-binding fragment of fibronectin alone had no such effects. There is clearly a VEGF/Fn synergism, which is mediated extracellularly by the formation of a novel VEGF/Fn complex; and the formation of this complex requires both the cell-binding and VEGF-binding domains to be linked in a molecule [162, 206].

Such synergy also exists between integrin and GF receptors, which can be triggered by an engineered molecule combining two or more functional ECM fragments [207]. A multi-functional, short recombinant FN fragment was engineered to link the integrin-binding domain (FN III9–10) to the GF-binding domain (FN III12–14), meanwhile containing the substrate sequence $\alpha 2PI_{1-8}$ for factor XIIIa, which allowed the fragments could be covalently crosslinked into a fibrous matrix with the aid of transglutaminase activity of factor XIIIa during the natural fibrin polymerisation process. A series of *in vitro* evaluations confirmed that the most remarkable GF-induced effects could only be observed when the two domains of FN III9–10 and FN III12–14 were fused in a single polypeptide chain (FN III9–10/12–14), indicating the powerful synergistic signalling activities between α5β1 integrin and growth factor receptors. Moreover, this joint matrix (FN III9–10/12–14) displayed significantly improved angiogenic ability and wound healing conditions, on the contrary, the equal dose of growth factors delivered within fibrin only had no obvious effects. This research represented a key strategy for enhancing GF-mediated wound healing, which is to engineer a microenvironment supporting synergistic signalling between GF receptors and integrins [208].

Likewise, researchers designed a novel system based on PEA [poly(ethyl acrylate)] having the function of organising fibronectin into nanonetworks to expose and supply simultaneously the integrin (FN III9–10) and GF-binding (FN III12–14) domains of fibronectin. This platform could more effectively promote synergistic integrin/VEGF signalling than high-dose VEGF did, leading to remarkable vascularisation with the use of low-dose VEGF *in vitro*. Implantation of FN-VEGF in mice also successfully induced extensive vascularisation desirable for wound healing and new tissue growth [209]. In summary, engineering multi-functional domains in one fusional protein, with or without the use of polymeric materials, has proven an effective approach to harness the synergy between ECM proteins and GF receptors, which may better recapitulate the multi-dimensional interactions at the cell-matrix interface and increase the efficiency of GFs at the 'destination' of their delivery for wound healing.

5. Conclusion and outlook

In this article, we have comprehensively and critically reviewed the progress in developing molecular engineering and biomaterials-based approaches to enhance the control over the bioactivities of growth factors (GFs) delivered for wound healing. Based on these two types of strategies, a diverse range of tools, materials and approaches have been elegantly devised, to maintain and enhance the functions of GFs through their entire fate in the cell microenvironment. In particular, the investigations we have summarised effectively improved the conditions of GFs for supply, stabilised them in extracellular matrix (ECM) as well as strengthened their interaction with cell receptors, generating promising outcomes that may have the potential to overcome the major obstacles now hampering the clinical use of GFs.

Future studies can be directed in numerous aspects, aimed at further enhancing the stability, efficacy and specificity of the delivered GFs. First, microbial infection is a common challenge to wound management. The bacterial metabolites (such as enzymes) can degrade GFs, and their formed biofilms may interfere with the spreading and receptor binding of GFs. It would be beneficial to combine the use of anti-bacterial films or devices for GF delivery at the wound site [210, 211]. Second, on top of improving their stability and efficacy, more efforts should be paid to increasing the specificity of the GFs. This task has further two folds of missions – the first one is on the GFs *per se* and aims to minimise the side, unwanted effects of GFs by using molecular engineering techniques; and the second is to improve the specific affinity of GF-binding matrix or molecules, raising their selectivity to bind, accumulate and empower only one type of GF for action. Third, all the engineering approaches should be more clinically relevant, and their efficacies evaluated in specialised models. Different types of wounds, such as traumatic injuries and chronic diseases, have distinct mechanisms of degeneration and regeneration, involving cellular and immunological events to different extent. Molecular conjugation of one GF to an environmentally-responsive polymer for ulcer healing should be tested in a microenvironment with persistent inflammation; while a newly developed glycosaminoglycan (GAG) for sequestration of VEGF should be assessed for its inherent immunostimulatory risk. Finally, integration of interdisciplinary approaches, such as real-time imaging technologies and non-invasive diagnostics, will complement the existing delivery strategies by providing tools to monitor and assess the delivery process [212–214], towards a *genuinely* precise control of the GF activities throughout the healing.

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