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Biomimetic approaches to protein and gene delivery for tissue regeneration

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Novel therapeutic strategies that promote wound healing seek to mimic the response of the body to wounding, to regenerate rather than repair injured tissues. Many synthetic or natural biomaterials have been developed for this purpose and are used to deliver wound therapeutics in a controlled manner that prevents unwanted and potentially harmful side-effects. Here, we review the natural and synthetic biomaterials that have been developed for protein and gene delivery to enhance tissue regeneration. Particular emphasis is placed on novel biomimetic materials that respond to environmental stimuli or release their cargo according to cellular demand. Engineering biomaterials to release therapeutic agents in response to physiologic signals mimics the natural healing process and can promote faster tissue regeneration and reduce scarring in severe acute or chronic wounds.

Introduction to wound healing

Disruption of the skin by injury is a vital breakdown of the barrier protecting the body. Because of the potential seriousness this threat might pose, the body prevents blood loss and further exposure to pathogens by forming a fibrin-rich clot, which is sourced from fibrinogen monomers in the blood [1]. Soon after, the extracellular matrix, growth factors and cytokines released from the degranulating platelets act in tandem to attract inflammatory leukocytes to cleanse the wound of bacterial contamination [2]. The wound healing cascade comprises four sequential but overlapping processes: inflammatory response, re-epithelialization, granulation tissue phase and finally remodeling of the healed wound. Infiltrating monocytes differentiate into macrophages that aid the removal of harmful debris; these cells are crucial to the production and release of the growth factors that initiate cellular responses from nearby endothelial cells, fibroblasts and keratinocytes of the epidermis. This begins the onset of the granulation phase of wound healing, in which epithelial and mesenchymal cells become heavily involved in the reformation of a viable and functional tissue. Fibroblasts continue to remodel the wound by depositing extracellular matrix such as collagen and elastin. Once the new epithelium is established, the blood vessel density in the wound decreases [3]; however, further remodeling of the dermis continues for a period of several months. The morphology of mouse-skin tissue is described in Box 1.

The complex sequence of molecular and cellular events that are carefully orchestrated during wound healing are difficult to emulate. Nevertheless, administration of growth factors has been shown to promote healing by affecting cell proliferation and migration, and extracellular matrix molecules and carefully designed biomaterials have been shown to guide tissue ingrowth [4–6]. Gene delivery has also been explored as an alternative to growth factor delivery because infiltrating cells uptake the genes and produce the therapeutic protein(s) in the local environment continuously (Box 2). The following sections focus on the application of natural and synthetic biomaterials for growth factor and gene delivery to promote tissue regeneration.

Biomaterials for growth factor delivery

Desired properties of biomaterials for wound healing

Biomaterials are used for promoting wound healing by providing scaffolds for cell attachment, growth and differentiation, and they act as vehicles for protein and gene delivery to regenerate functional tissue. For wound healing, biomaterials must possess enough elasticity to conform to the size and shape of the wound and endow temporary mechanical support to withstand *in vivo* forces; they should provide some level of bioactivity to accommodate cellular attachment and migration; they should act as reservoirs for the controlled delivery of wound healing factors; and, finally, they should be non-immunogenic and absorbed by the body once tissue regeneration is complete.

Delivery of therapeutics, such as growth factors or genes, from biomaterials must be performed in a controlled manner to prevent deleterious side-effects. For example, high doses of transforming growth factor beta-1 (TGF-β1) can cause serious systemic effects in animals, including fibrosis in the kidneys and liver [7], and uncontrolled delivery of vascular endothelial growth factor (VEGF) could induce angiogenesis in non-target tissues or even aid the growth of tumors [8,9]. Furthermore, growth factors are susceptible to harsh proteolytic environments and are more effective if delivered in a biological matrix that provides suitable protection [10]. Ideally, several growth factors should be released sequentially, in a manner that mimics the time profile of the healing process *in vivo*, resulting in wound regeneration rather than repair. Finally, the matrix material must be

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Box 1. Skin structure and physiology

The skin is the largest organ in the body and it acts as a barrier to pathogens, water loss and mechanical injury. It is composed of two layers: epidermis and dermis. The epidermis is a highly organized, multilayered tissue that is maintained by undifferentiated cells in the basal layer and differentiated cells in the suprabasal, granular and cornified layers (Figure I). Homeostasis of the epidermis is maintained by a small population of stem cells and their progeny of rapidly dividing transit-amplifying cells. Stem cells are located in the basal layer of the epidermis and the bulge region of hair follicle [94]. Follicular stem cells are multipotent and they can give rise to epidermis, sweat glands, neurons, glia and smooth muscle cells [95–97]. The progeny of stem cells, transit-amplifying cells, proliferate a few times before they detach from the basement membrane and propagate upwards to the suprabasal compartments. Upon detachment from the basement membrane, keratinocytes downregulate important anchoring molecules, such as integrins, and begin the process of terminal differentiation [98]. These spinous or suprabasal cells maintain cell–cell connections through integrins and cadherins in a Ca^{2+}-dependent manner [99]. In the last stages of differentiation, cells extrude lipids into the intercellular space to form the permeability barrier. At the same time, cells break down their nuclei and form a highly cross-linked protein envelope, immediately beneath their cellular membranes. This envelope is connected to a network of keratin filaments that provide much of the physical strength of the epidermis. The cells of the stratum corneum are eventually sloughed off and replaced with new cells coming from the lower layers. The entire renewal process takes approximately 30 days.

The dermis is rich in extracellular matrix such as collagen, elastin, vitronectin and fibronectin. It also contains mesenchymal cell types, such as fibroblasts and endothelial cells (i.e. blood vessels), which are responsible for maintaining the foundation for the epidermis and the overall integrity of the skin structure. A subcutaneous layer below the dermis consists mainly of fat cells and blood vessels, which are vital to providing stores of energy and nutrition to the cells in the epidermis and dermis. Finally, the dermis also contains sebaceous glands, sweat glands for thermoregulation and nerve endings for sensing touch and pain.

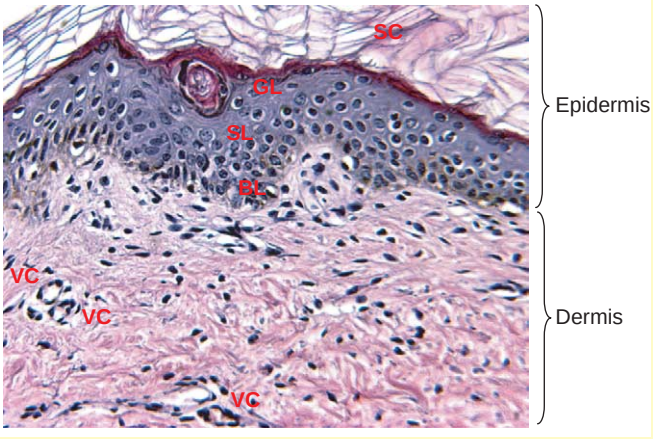

Figure 1. Morphology of mouse skin tissue. Skin tissue was harvested from an athymic mouse and processed for histology. Paraffin-embedded tissue sections were stained with hematoxylin and eosin following standard protocols (magnification x40). Abbreviations: BL, basal layer; SL, suprabasal layer; GL, granular layer; SC, stratum corneum (cornified layer); VC, vessel capillaries.

degraded and absorbed by the body without accumulation of toxic by-products.

There are several modes of drug delivery using bioengineered matrices (Table 1). They can be broadly classified into three categories: (i) diffusion-controlled; (ii) chemically controlled or environmentally responsive systems; and (iii) biologically inspired release systems. Diffusion-controlled systems exist in two geometries, namely a reservoir, in which the drug is surrounded by a polymer, or a matrix, where the drug is uniformly distributed through the polymer [11]. Environmentally responsive hydrogels release the drug when swelling of the matrix occurs because of changes in temperature, pH or the ionic strength of the solution. Finally, the relatively new paradigm in drug delivery is the development of systems that are engineered to enable cell-triggered drug release and matrix remodeling by selective display of protein domains in natural or synthetic materials [6].

Diffusion-controlled systems

Diffusion-controlled systems exhibit an initial burst release phase, which is followed by a phase of slower drug release from the matrix. This is typical of hydrophilic matrices that degrade by bulk erosion – water enters the matrix, and degradation occurs throughout the structure. By contrast, hydrophobic matrices degrade by surface erosion, exhibiting zero-order release kinetics without a burst release phase [12].

Several approaches have been developed to better control the release kinetics and avoid burst release of the encapsulated factor(s). For example, chemical modification of hydrophilic alginate with long alkyl chains resulted in stronger gels, which were hydrophobic and had significantly different release profiles [13]. Others demonstrated that the rate of hepatocyte growth factor (HGF) release from gelatin hydrogels could be modulated by the degree of matrix crosslinking by varying the amount of gluteraldehyde, indicating that HGF release was controlled by hydrogel degradation [14]. Importantly, subcutaneous implantation of HGF-containing crosslinked microcapsules significantly enhanced the number of newly formed capillaries around the implanted site, in marked contrast to free HGF of the same dose. Others exploited the natural affinity of fibroblast growth factor (FGF)-2 for type I collagen, to slow its release and protect it from proteolytic degradation [15]. Subcutaneous implantation of FGF-2 in a collagen-sponge sheet resulted in sustained release according to the biodegradation of the sponge matrix and induced local angiogenic activity in a dose-dependent manner. Also, intramuscular injection of FGF-2-containing collagen microsponges significantly increased blood flow in the murine ischemic hindlimb, whereas bolus injection of FGF-2 had no effect [15]. Similarly, release of FGF-1 and FGF-2 from fibrin hydrogels accelerated the healing of large excisional wounds in the rabbit ear model [16,17], whereas delivery of FGF-2 from a photocrosslinkable chitosan gel resulted in significant acceleration of wound closure in healing-impaired diabetic (*db/db*) mice [18].

Although most studies deliver single growth factors, the wound healing process is complex and affected by multiple growth factors that are released in a well-orchestrated manner. To address this challenge, Richardson *et al.* engineered a poly(lactide-co-glycolide) (PLG) polymer scaffold for the dual delivery of VEGF and platelet-derived growth factor (PDGF)-BB to promote

Box 2. Gene therapeutics

Gene delivery to the cells surrounding the wound has the potential to promote wound healing or reduce healing complications that lead to scarring, keloid formation or chronic ulceration. In general, two classes of methods are employed to deliver genes into target cells. They are broadly classified as viral and non-viral.

Viral methods, which use recombinant viruses as gene delivery vehicles, are used in the majority of clinical trials. The genome of recombinant viruses has been modified by deletion of some or all viral genes and replacement with foreign therapeutic or marker genes. The recombinant viruses most widely used to date include retrovirus, lentivirus (HIV-based), adenovirus and adeno-associated virus.

Non-viral methods include delivery of DNA using physical and chemical means. Physical methods, such as electroporation and particle acceleration (gene gun), facilitate entry into target cells but compromise cell viability; therefore, they might not be appropriate for use in tissue engineering. Delivery of DNA complexed with lipids or cationic polymers has met with some success *in vitro*, and is used in a significant fraction of current clinical trials. However, *in vivo* gene delivery requires a scaffold to release DNA in a controlled manner and protect plasmids or recombinant viruses from the harsh environment of the wound. To this end, natural hydrogels and synthetic polymeric materials are used as scaffolds for DNA delivery to promote tissue regeneration *in vivo*.

growth of stable blood vessels [19]. This system was composed of PDGF-BB-containing PLG microspheres suspended in a VEGF-containing PLG scaffold. VEGF was released first, to stimulate the growth of endothelial channels, followed by PDGF-BB, to stabilize the nascent vessels by recruiting smooth-muscle cells. This study clearly demonstrated the importance of growth factor delivery in a timed manner, mimicking the natural release profile of growth factors in the healing process. A similar design used encapsulation of insulin-like growth factor (IGF)-1 in gelatin microspheres, which were then suspended in oligo(poly(ethylene glycol) fumarate) containing TGF-β1 [20]: this system enabled fast delivery of TGF- β1 and more sustained delivery of IGF-1 to promote cartilage repair [20]. Finally, dual delivery of HGF and FGF-2 from collagen microspheres enhanced blood vessel formation and produced more mature vasculature in an ischemic mouse hindlimb model at lower doses than either factor alone [21]. Engineering natural and/or synthetic scaffolds to release several growth factors in a sequential manner might affect more than one phase of the wound healing process, for example, neovascularization and epithelialization ultimately leading to wound regeneration rather than repair.

Environmentally responsive systems

An emerging group of hydrogels that might be useful in tissue regeneration are those that respond to environmental changes such as pH, moisture or temperature [22]. This class of biomaterials includes polymers with ionic pendant groups that release their drug load upon swelling in response to an environmental stimulus [11]. Hydroxyethylcellulose is one such biomaterial that swells in aqueous media, enabling diffusion of the encapsulated antibiotics to disinfect wounds and reduce the toxic effects of systemic delivery [23]. Poly(*N*-isopropyl acrylamide) (PNIPAAm) shows sol-to-gel transformation – the transition of a system from a liquid to a solid phase – at the physiologic temperature of the wound site and can also exhibit 'on–off' behavior, enabling oscillatory drug release [24]. Such thermoresponsive polymers have been used as wound dressings to prevent bleeding and deliver antibiotics or growth factors to the wound site [25–28]. Another thermoresponsive tri-block copolymer of poly (ethylene glycol) (PEG) and poly(lactic-co-glycolic acid)

Table 1. Examples of controlled delivery of growth factors to cells *in vitro* and *in vivo*

Growth factor	Hydrogel(s)	Wound model	Healing process	References
In vitro				
EGF	Collagen sponge	HaCaT	Cytoprotective	[81]
FGF-1	PLGA microspheres and/or fibrin	NIH-3T3	Proliferation	[82]
FGF-2	Fibrin glue (Baxter)	HUVECs	Proliferation	[83]
KGF (FGF-7)	PGA–PLA blends	Release studies	Modulated release	[84]
PDGF	EVAc	Human osteoblasts	Proliferation	[85]
PDGF–BB	PVA	Human fibroblasts	Proliferation	[86]
TGF-β1	OPF and/or gelatin microspheres	Release studies	Modulated release	[20]
VEGF	SIS matrix	HMEC	Tube formation	[87]
VEGF	NiPAAm –NtBAAm	HAEC	Proliferation	[28]
In vivo				
EGF	Gelatin microspheres	Rabbit	Re-ep, gran	[88]
EGF	Collagen	Mouse (*db*/*db*)	Gran	[40]
FGF-1	Fibrin, collagen	Pig	Re-ep, angio	[16,17]
FGF-2	Chitosan	Mouse (*db*/*db*)	Re-ep, gran, angio	[18]
FGF-2	Poly(galactone)	Rat	Re-ep, gran, angio	[89]
FGF-2	Gelatin microspheres	Pig	Gran, angio	[90]
KGF	Fibrin	Athymic mouse	Re-ep, gran, angio	[50]
PDGF, VEGF	PLGA microspheres	Mouse	Angio	[19]
PDGF	PLG	Rat	Angio	[63]
PDGF	Collagen–fibrin blend	Dog	Re-ep, gran	[91]
PDGF–BB	PEG	Mouse (*db*/*db*)	Re-ep	[92]
TGF-α	PEG	Mouse	Re-ep	[92]
TGF-β1	PEO–PPO block copolymer	Rat	Re-ep, gran	[93]
VEGF121	Fibrin hydrogels	Mouse	Angio	[48]
VEGF165	Fibrin glue (Baxter)	Chick embryo	Angio	[83]

Abbreviations: HaCaT, a cervical carcinoma cell line; NIH-3T3, mouse fibroblast cell line; HUVECs, human umbilical vein endothelial cells; OPF, oligo(poly(ethylene glycol) fumarate); HMEC, human dermal microvascular endothelial cell; HAEC, human aortic endothelial cells; SIS, small intestinal submucosa; NiPAAm, N-isopropylacrylamide; NtBAAm, N-tert-butylacrylamide; PEO–PPO, poly(ethylene oxide)–poly(propylene oxide) copolymer; angio, angiogenesis; Re-ep, re-epithelialization; gran, granulation tissue phase.

(PLGA), PEG–PLGA–PEG, was used to deliver TGF-β1-encoding DNA to a wound site, promoting extracellular matrix formation, granulation tissue phase and wound closure [29]. Finally, other polymers, such as hydroxypropylmethacrylamide, poly(ethylene oxide) (PEO) or poly(vinyl alcohol) (PVA), have also been used to develop environmentally sensitive hydrogels with potential applications in wound healing [11].

Investigators have combined synthetic polymers with peptides, protein motifs or antibodies to engineer hydrogels that respond to biological signals [30–32]. Others have synthesized protein-based hydrogels that respond to physiological signals (e.g. temperature or calcium concentration) by conformational change of the constituent protein [33,34]. Such systems translate biological signals into mechanical action, demonstrating their significant potential as drug delivery devices. These biomolecule-sensitive hydrogels can be engineered using recombinant DNA technology and provide self-regulated drug delivery vehicles for several applications, including wound healing and tissue regeneration.

Biologically inspired systems

Perhaps some of the most interesting release systems are biologically inspired materials that use the biochemistry of the wound to control delivery [6]. These materials achieve cell-mediated delivery through biological signals that are recognized by the cells actively repairing the injury. An early demonstration of this concept was the delivery of a chemotherapeutic drug (5-fluorouracil) through the use of oligopeptide sequences that were recognized by enzymes within the lysosomal compartment of the target cells [35]. Since then synthetic biomaterials have been engineered to contain biological cues, such as the domains of extracellular matrix molecules, growth factors or protease substrates, to promote a cell response or deliver therapeutics upon cellular demand. To this end, hybrid materials have been designed to contain cell recognition domains in a polymer backbone. Specifically, PEG hydrogels were modified by conjugation of VEGF and the cell adhesion peptide RGD, and subsequently cross-linked by a peptide sequence that was recognized by the matrix metalloproteinase MMP-2 [36]. The resulting matrix released VEGF upon degradation by MMP-2, which was secreted by activated endothelial cells. Other investigators have used genetic engineering to synthesize multi-domain peptides that might function as extracellular matrix analogs and promote cell migration and tissue regeneration [33,37,38].

Genetic engineering and chemical methods have also been used to immobilize peptide signals and growth factors to natural hydrogels such as collagen and fibrin. In one study, epithelial growth factor (EGF) was engineered to have high affinity for collagen by fusion of the collagen-binding domain of fibronectin (FNCBD) to its N-terminus [39]. Application of FNCBD–EGF-containing collagen gels in skin, arterial and hindlimb wounds of diabetic mice accelerated wound closure and improved granulation tissue formation [40]. Similarly, Hubbell's laboratory used bi-domain peptides to covalently attach small cell-adhesion peptides [41,42] or heparin-binding growth factors, such as nerve growth factor (NGF) or brain-derived neurotrophic factor, into fibrin hydrogels [43]. To do this, one domain of the peptide was recognized by factor XIIIα and incorporated into fibrin during polymerization, whereas the heparin-binding domain immobilized heparin, which, in turn, interacted with the heparin-binding growth factor. This approach was later extended to engineer fusion proteins containing the factor XIIIα-recognition domain at the N-terminus and was used successfully to deliver β-NGF and VEGF in a manner that was controlled by cellular activity [44,45]. The investigators then used this system for inducing and controlling angiogenic responses *in vitro* and *in vivo* [46–49].

We have adopted this method to conjugate keratinocyte growth factor (KGF) into a fibrin matrix to achieve a localized delivery that is in tune with cellular demand at the wound microenvironment [50] (Figure 1). Fibrin is a particularly attractive scaffold because it is a natural biomaterial that promotes wound healing of epidermal keratinocytes and three-dimensional skin substitutes [51,52]. The factor XIIIα-recognition domain was covalently attached to KGF, and the peptide–KGF complex (P-KGF) was conjugated to fibrin during polymerization through the action of factor XIIIα. Plasmin digestion of the fibrin gels yielded KGF devoid of fibrinogen fragments and promoted epithelial cell proliferation to the same extent as native KGF. We evaluated this delivery system, *in vivo*, using a hybrid model of wound healing that was created by transplantation of human bioengineered skin onto athymic mice [53]. At six weeks post-grafting, the transplanted tissues were subjected to full-thickness wounding and treated with fibrin gels containing bound KGF. By contrast to topical application, fibrin-bound KGF persisted in the wounds for several days and was released gradually as the fibrin matrix was degraded by the infiltrating cells, resulting in significantly enhanced wound closure. Taken together, our data showed that active KGF can be released from fibrin hydrogels in tune with cellular demand, providing localized treatment and enhancing tissue regeneration.

Biomaterials for gene delivery

Despite high expectations, growth factor delivery has met with limited clinical success. A notable exception, PDGF-BB, was successful in promoting wound healing in phase I/II clinical trials, resulting in the first growth factor delivery product, Regranex, approved by the Food and Drug Administration for treatment of diabetic foot ulcers [54,55]. Lack of success with other growth factors can be attributed, at least in part, to short half-lives [56] and loss of protein bioactivity in the protease-rich environment of the wound [57]. Alternatively, delivery of the gene(s) encoding for therapeutic molecules might overcome this problem: the infiltrating cells uptake the genes and continuously produce the therapeutic protein(s) in the local environment (Box 2). To this end, biomaterials could be used for delivery of viral and non-viral vectors encoding therapeutic genes [58,59]. In this setting, biomaterials must be designed to protect plasmid DNA or viral particles from the proteases [60,61], reduce immunogenicity and

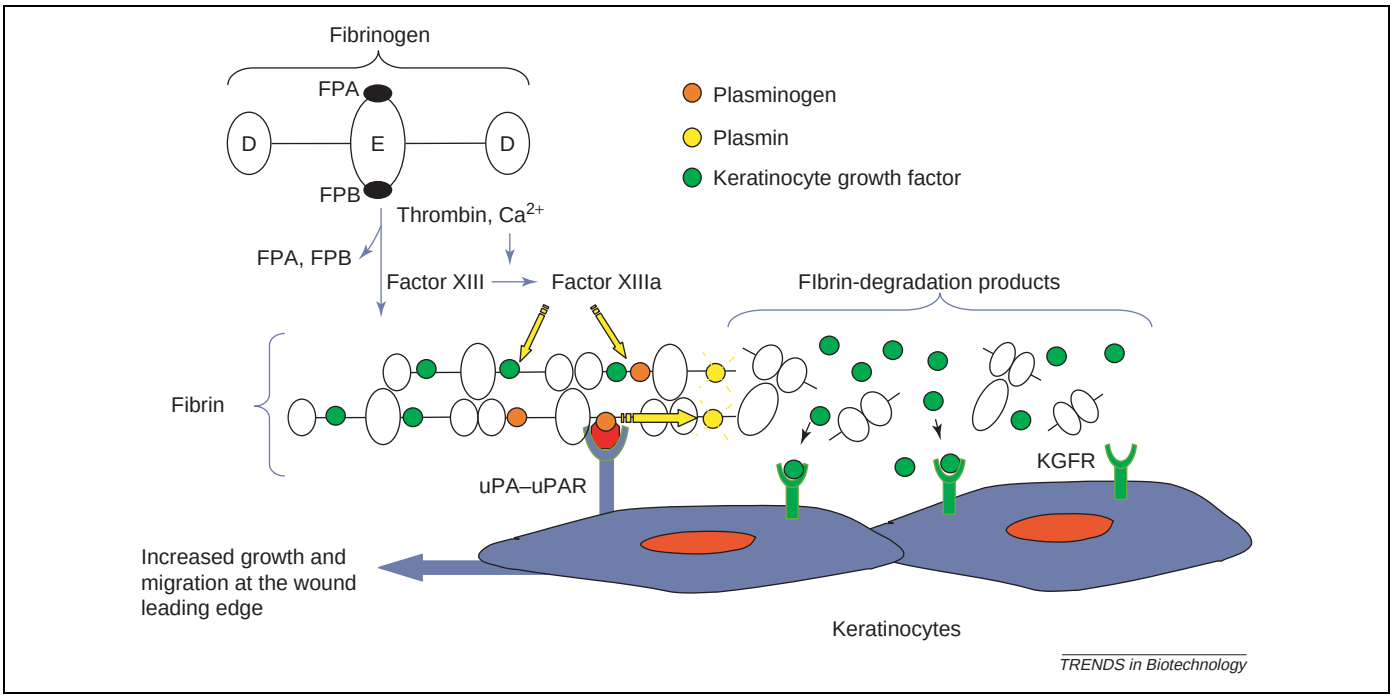

Figure I. Enzymatic conjugation into fibrin hydrogels and cell-controlled release of KGF into the wound microenvironment. Thrombin cleaves fibrinopeptides A and B from fibrinogen monomers, which then polymerize to form a hydrogel. During this process thrombin also mediates the conversion of factor XIII into its active form, XIIIα, which strengthens the matrix by crosslinking lysine and glutamine residues. At the same time, factor XIIIα crosslinks bioactive molecules, such as plasminogen, into the fibrin matrix. KGF is modified to possess a peptide sequence that is recognized by factor XIIIα and incorporated into the fibrin matrix during polymerization. Upon contact of migrating keratinocytes with fibrin, plasminogen is converted to plasmin on the cell surface by the urokinase plasminogen activator system (uPA–uPAR). Plasmin degrades the fibrin matrix, locally releasing KGF into the wound microenvironment, where it binds to KGF receptor and accelerates cell proliferation and migration at the leading edge of the wound.

improve safety through targeted gene transfer only to the cells that infiltrate the wound bed.

Natural and synthetic biomaterials have been used to deliver plasmid DNA and recombinant viruses in the wound microenvironment. Collagen-embedded DNA encoding for platelet-derived growth factor (PDGF-A or -B) increased granulation tissue, re-epithelialization and wound closure in an ischemic rabbit ear model [62]. Poly(lactide-co-glycolide) matrices were also used to deliver the PDGF gene into skin wounds, resulting in significantly increased vascularization and granulation tissue formation up to four weeks post-wounding [63]. PLGA nanoparticles efficiently encapsulate DNA and provide sustained release during a four week period [64]. More recently, matrix-mediated delivery of two genes, IGF-1 and KGF, enhanced wound healing compared with each gene delivered individually [65]. PGA scaffolds were also used to deliver PDGF-B and VEGF121 overexpressing human umbilical vein endothelial cells to the ischemic rabbit ear model, resulting in significantly increased rates of wound closure and granulation tissue formation [66,67].

Bioactive matrices have also been used to deliver recombinant viruses to the site of injury. Encapsulation in gelatin or alginate microspheres protected adenoviral particles from degradation, and the release kinetics could be controlled by modulating the composition of the microspheres [68]. Adenoviral delivery of PDGF-BB increased granulation tissue formation and neo-vascularization of full-thickness wounds. When adenoviral particles were conjugated with FGF-2, cellular uptake through the FGF receptors increased, ultimately increasing the efficacy of the treatment [69,70]. Additionally, encapsulation of adenovirus in PLGA matrices reduced immunogenicity and decreased inactivation by neutralizing antibodies, thus facilitating the repeating virus administrations that might be required for a therapeutic effect [71–73]. Adenoviral delivery of PDGF-B has been shown to significantly enhance epithelial gap closure, granulation tissue formation and vessel density in *db/db*, streptozotocin, and non-obese diabetic (NOD) mice [74]. In another study, KGF-expressing keratinocytes were placed in a nylon-mesh matrix and delivered onto acute porcine wounds, resulting in significant acceleration of epidermal closure [75]. Interestingly, lentiviral delivery of the gene encoding PDGF-B also enhanced angiogenesis and collagen deposition in diabetic wounds but did not affect re-epithelialization [76]. By contrast to adenovirus, both retroviral and lentiviral vectors are not immunogenic and have the potential for permanent genetic modification, which might be advantageous in the treatment of chronic wounds such as diabetic ulcers.

Outstanding issues

Biomaterials have been used successfully for the delivery of small organic molecules, growth factors and, more recently, genes for wound healing and other diseases. Despite such progress, further advances in biomaterial design are required to achieve prolonged release, protect proteins or DNA from degradation, target certain cell types in the wound space or guide DNA transport to specific cellular compartments. For example, design of

pH-responsive biomaterials to release the DNA from the endosomes and avoid lysosomal degradation might increase the efficiency of gene transfer. In addition, non-viral delivery of genes is still inefficient, and viral technologies are hampered by safety issues that need to be resolved. To this effect, hybrid materials that contain nuclear-localization signals would increase gene transfer to slowly dividing cells [77–80]. Biologically inspired materials containing cell-recognition peptides could promote DNA or growth factor uptake by activated cells in the wound microenvironment, improving targeting and possibly reducing virus immunogenicity. Finally, simultaneous delivery of multiple growth factors and/or genes in a timed manner and at the right concentration might enhance the therapeutic effect by targeting more than one step in the wound healing cascade.

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