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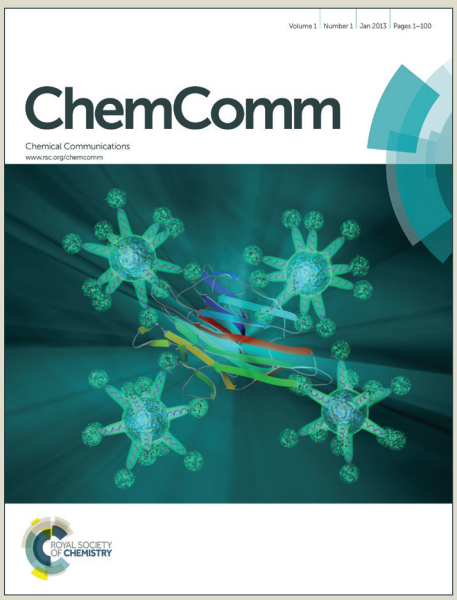

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Design of Growth Factor Sequestering Biomaterials

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Abstract

Growth factors (GFs) are major regulatory proteins that can govern cell fate, migration, and organization. Numerous aspects of the cell milieu can modulate cell responses to GFs, and GF regulation is often achieved by the native extracellular matrix (ECM). For example, the ECM can sequester GFs and thereby control GF bioavailability. In addition, GFs can exert distinct effects depending on whether they are sequestered in solution, at two-dimensional interfaces, or within three-dimensional matrices. Understanding how the context of GF sequestering impacts cell function in the native ECM can instruct the design of soluble or insoluble GF sequestering moieties, which can then be used in a variety of bioengineering applications. This Feature Article provides an overview of the natural mechanisms of GF sequestering in the cell milieu, and reviews the recent bioengineering approaches that have sequestered GFs to modulate cell function. Results to date demonstrate that the cell response to GF sequestering depends on the affinity of the sequestering interaction, the spatial proximity of sequestering in relation to cells, the source of the GF (supplemented or endogenous), and the phase of the sequestering moiety (soluble or insoluble). We highlight the importance of context for the future design of biomaterials that can leverage endogenous molecules in the cell milieu and mitigate the need for supplemented factors.

Keywords: Extracellular Matrix; Growth Factor; Sequestering; Differentiation; Tubulogenesis; Sprouting; Tissue Morphogenesis; Regenerative Medicine

Introduction

Soluble signals such as growth factors (GFs) are major regulators of cell behavior. The processes of cell differentiation[1], migration[2], multicellular organization[3–6], and survival[7] are tightly regulated by the extracellular matrix (ECM)[3,8–11], which contains high concentrations of water, structural proteins (*e.g.* collagens, fibrins)[12], and glycoproteins (*e.g.* fibronectin, vitronectin)[10,13]. In addition to these components, the ECM consists of many soluble cell-secreted[14–17] and insoluble, cell surface-immobilized proteins and proteoglycans[18,19] that can regulate GF-mediated cell function. For example, components of the ECM

(*e.g.* proteoglycans and glycoproteins) are multifunctional and capable of both promoting cell adhesion and sequestering GFs[10,20,21]. Specifically, the ECM regulates GF activity by sequestering soluble GFs and by cell-demanded release via enzymatic degradation of the ECM[8,9,22]. Both soluble (un-bound) and insoluble (ECM-bound) GFs contribute to cell signaling, and the context of these un-bound and ECM-bound GFs in relation to cells dictates the GF activity and the cell response.

Both soluble and insoluble ECM components sequester GFs and elicit differential effects on GF signaling that are dependent on the context and presentation of the GF to cells. Vascular endothelial growth factor (VEGF) provides an example of context-dependent GF signaling, as its activity is tightly regulated by both soluble and insoluble ECM components in different ways. VEGF-A, hereafter denoted "VEGF", is the most well-characterized of the VEGF family. VEGF is secreted in numerous isoforms that differ in the number of binding domains for heparan sulfate (HS) in the ECM[23–25]. Previous studies demonstrated that isoform-specific gradients of VEGF, imparted by differential binding to HS, instruct directional blood vessel sprouting in a regenerating tissue[26,27]. Signaling of VEGF through kinase insert domain receptor (KDR) elicits a pro-angiogenic response to VEGF that is regulated by membrane-bound Feline McDonough Sarcoma-related tyrosine kinase 1 (mFlt-1) on the cell surface[28]. Flt-1 has a higher affinity for VEGF than KDR[29] and competitively binds VEGF, preventing VEGF-KDR binding *in vivo*[30]. Similarly, the soluble form of Flt-1, sFlt-1[31], and soluble KDR[32,33], sKDR, competitively bind and can decrease the activity of soluble VEGF[14,32–36]. In contrast, recent evidence suggests that sFlt-1 may locally modulate VEGF activity[37] and, similarly to HS, may enhance sprout formation and guidance during angiogenesis[38,39] by sequestering VEGF and forming gradients of unbound VEGF necessary for blood vessel formation[40]. In this example, both insoluble (HS, mFlt-1) and soluble (sFlt-1, sKDR) ECM components elicit context-specific effects on VEGF regulation. Thus, sequestering of VEGF may elicit a cell response that is highly dependent not only on the identity of the sequestering moiety but also on the context of the sequestering.

VEGF context-specific regulation is one example of a more generally observed phenomenon of GF signaling in the native ECM. Specifically, molecules that bind to GFs influence their activity, and the contextual presentation of binding moieties may dictate their effects. Recent engineering approaches have used GF sequestering in multiple *in vitro* and *in vivo* contexts to modulate cell behavior. The context of GF sequestering is defined by whether the sequestering moiety is soluble or insoluble, the location of sequestering moieties relative to the cell, the source of the GF, the affinity between the GF and the sequestering moiety, and whether the sequestering moieties are presented in a 2-dimensional (2D) or 3-dimensional (3D) matrix. This Feature Article aims to introduce the reader to context-dependent GF sequestering in natural biological scenarios and with engineered materials to control cell behavior. Thereby we focus on biomaterials that contain chemically-defined GF sequestering moieties rather than

biomaterials composed entirely of native ECM components, which are reviewed elsewhere[21,41]. Specifically, we will examine engineering approaches to modulate cell behavior via GF sequestering in solution, at a 2D interface, or at 3D interfaces. We will highlight studies that have utilized these GF sequestering approaches in multiple contexts to modulate cell migration, organization, differentiation, and survival in vitro. We will discuss particular examples in which GF sequestering via the same moiety may exhibit a paradoxical role depending on the context: for instance, soluble GF sequestering may inhibit GF activity while substrate-mediated GF sequestering may enhance GF activity. We will also discuss ways in which biological GF sequestering may serve as a template to understand the context-specific nature of sequestering for dictating cell response. Finally, we will provide insight into how engineered, context-specific GF sequestering can enhance cell response to a GF and the implications of the concepts discussed as they relate to regenerative medicine.

1. Soluble Regulation of Cell Signaling Proteins

GFs are among the principal regulators of cell behavior. Upon GF stimulation, cells undergo a cascade of signaling events which result in migration and organization[6,11,42], differentiation[1,43,44], or survival. The ECM regulates GFs via sequestering[10,45], and the context of this sequestering determines the cell response to these signaling molecules. In this section, we will discuss naturally-occurring and engineered soluble approaches to modulate cell behavior using GF-binding moieties. Hereafter, we refer to "moieties" as small molecule peptides, oligonucleotide aptamers, or oligosaccharides. The guiding parameter associated with molecular sequestering is the equilibrium dissociation constant, K_D, also referred to as the "affinity constant". The K_D value indicates the affinity of a given two-species interaction and can be derived via established biochemical analytical techniques[46–50]. Similarly, the half-maximal inhibitory concentration, IC_{50}, is a measure of the potency of a moiety to inhibit a cell process. These units of measure provide a basis to compare soluble sequestering moieties and derive insights from soluble sequestering strategies.

1.1. Natural Soluble Regulators of Cell Signaling Proteins

Soluble proteins and peptides, found in soluble environments such as interstitial fluid and blood serum, bind to and regulate the activity of many GFs and thereby modulate cell behavior, including differentiation, migration, organization, and growth. For example, sFlt-1 is a known soluble inhibitor of VEGF that is produced by endothelial cells (ECs)[31], peripheral blood mononuclear cells, monocytes[15,51], and, in the case of hypoxia, cytotrophoblasts in the uterus[14]. In embryonic development, KDR antagonism by sFlt-1 [52] regulates hemogenic mesoderm specification to hematopoietic or endothelial lineages[34]. While sFlt-1 is required for several biological functions including endothelial sprout formation[38,39], elevated levels of sFlt-1 in the soluble environment contribute to endothelial dysfunction[35], for instance during pre-eclampsia[53,54] and chronic kidney disease[35], specifically increasing EC sensitivity to

inflammatory cytokiness[55]. The role of sFlt-1 in pre- and postnatal development is an example of a general phenomenon in which soluble components of the native extracellular environment, specifically a soluble receptor fragment, may regulate GF activity and control cell behavior by binding to and blocking the active site of the GF.

Soluble proteins work in concert to regulate the activity of transforming growth factor beta 1 (TGF-β1), which plays a role in many cell behaviors. Sequestering by soluble α2-macroglobulin (α2-M) protects TGF-β1 from proteolysis in blood plasma[56] and inhibits its binding to cell surface receptors[57]. In concert with active site sequestering mechanisms, soluble proteins can also regulate GF by sequestering the GF at sites distinct from the active site, termed "allosteric" sequestering. ECs and mural cells secrete inactive, "latent" TGF-β1 with a latency-associated peptide (LAP)[58], and four splice variants of latent TGF-β1 binding protein-1 (LTBP-1) bind and inhibit TGF-β1 signaling[17]. In this example, LTBP-1 acts as an "allosteric inhibitor" of TGF-β1. Cleavage of LTBP-1 from TGF-β1 by membrane type 1 matrix metalloproteinase (MT1-MMP) releases latent TGF-β1 from the ECM and also contributes to TGF-β1 activation[59]. This action of MTI-MMP requires a plasmin-dependent interaction between latent TGF-β1, ECs, and mural cells[60]. These examples highlight the complexity of soluble GF sequestering that regulates their activity. Soluble proteins can interact at the active site or at allosteric binding sites on the GF to regulate cell behavior.

Protein components of the soluble environment can also regulate GFs to modulate cell survival and proliferation. For example, soluble calcium-independent mannose-6-phosphate receptor (CIMPR) neutralizes the mitogenic effect of insulin-like growth factor 2 (IGF-2) on hepatocytes and fibroblasts, inhibits the proliferation of myeloid and lymph cell lines, and antagonizes interleukin-6 and -11 [61]. Additionally, distinct soluble portions of fibroblast growth factor receptors (FGFRs) have been identified in blood and vitreous fluid[16,62,63], and were shown to inhibit neurotrophic behavior in the regenerating retina and increase sensitivity to light-induced retinal damage[64]. GF sequestering by soluble proteins influences cell behavior in many healthy and pathological states, which motivates the design of synthetic GF sequestering moieties.

1.2. Biological Mimicry for Identifying GF Sequestering Moieties

Naturally-occurring examples of soluble GF sequestering can serve as a template for design of synthetic molecules that can sequester GFs. Soluble synthetic moieties that can sequester a GF via mechanisms similar to those used in nature can in turn regulate cell behavior *in vitro* and *in vivo*.

Researchers have explored synthetic strategies to develop and characterize peptide moieties that regulate naturally-occurring GFs by mimicking known molecular interactions (Table 1). In particular, many studies have demonstrated GF sequestering via biological mimicry (herein denoted as "biomimicry") of the interaction between α2-M and TGF-β1, between α2-M and platelet-derived growth

factor–BB (PDGF-BB) [65], and between TGF-β1 and TGF-β1 receptor III (TGFRIII)[66,67]. Others have demonstrated that peptides mimicking antithrombin III (ATIII)[68,69], platelet factor 4 (PF4)[70,71], fibroblast growth factor-1 (FGF-1)[72], and VEGF[73,74] can bind to HS, heparin, a highly sulfated form of the HS glycosaminoglycan (GAG), and both HS- and heparin-containing proteoglycans found on the cell surface and in the ECM[13]. Biomimicry can also be used to develop moieties that bind to GFs more promiscuously. Hubbell and coworkers identified peptides derived from fibronectin[75] and fibrinogen[76] that sequestered multiple GFs in solution. These studies demonstrated that moieties engineered to mimic known proteins or proteoglycans exhibited GF or heparin and HS sequestering.

Biomimicry of known protein-protein interactions can be used to down-regulate the activity of a target GF by targeting the GF active site. For example, Dobson and coworkers developed and characterized the anti-microbial properties of a soluble peptide derived from the heparin-binding domain (HBD) of the apolipoprotein E (apoE) receptor[77,78]. Bhattacharjee *et al.* further demonstrated that the peptide blocked HS-mediated pro-angiogenic GF binding to the cell surface, reduced tumor size in an *in vivo* mouse model, and inhibited ocular angiogenesis in an *in vivo* rabbit model[79,80]. Using a similar approach, Binetruy-Tournaire *et al.* identified a peptide derived from KDR that bound VEGF and inhibited VEGF-mediated angiogenesis in an *in vivo* rabbit corneal model[81]. Further, Takasaki *et al.* identified a peptide derived from tumor necrosis factor (TNF) receptor that sequestered soluble TNF-α, which is known to elicit inflammation[82]. In two studies, Aoki and coworkers showed that this TNFR-derived peptide inhibited TNF-α-mediated inflammation and bone destruction upon injection[83,84]. Finally, researchers have used biomimicry to identify oligosaccharides that sequester a target GF. Linhardt and coworkers mimicked the interaction between heparin and VEGF to develop oligosaccharides that sequestered VEGF and decreased angiogenesis[85]. These studies demonstrated that moieties derived via biomimicry reduced the activity of specific target GFs by blocking their active site. An alternative strategy to biomimicry is a screening approach that enables high throughput identification of GF sequestering moieties.

1.3. High Throughput Screening to Identify GF Sequestering Moieties

High throughput methods to identify and characterize molecular interactions have enabled the rapid discovery of small molecules that can target soluble GFs (Table 1). Phage display technology[86] and systematic evolution of ligands by exponential enrichment (SELEX) [87] are two common high throughput methods that enable rapid characterization of peptide and oligonucleotide libraries, respectively. For example, Maxwell *et al.* screened a 12-amino acid peptide library to identify peptides with varying affinity for heparin[88]. Blaskovich *et al.* utilized phage display to identify a peptide that inhibited angiogenesis *in vitro* by targeting platelet-derived growth factor (PDGF) [89]. Additionally, phage display

technology enabled development of peptides that inhibited angiogenesis[90] and tumor growth[91] by targeting VEGF and hepatocyte growth factor (HGF), respectively.

Automated synthesis and high throughput techniques enable facile screening and characterization of molecular interactions relevant to GF sequestering. Phage display technology and SELEX have been used in combination with biomimicry to identify GF sequestering moieties with high throughput. For example, Zhang *et al.* used phage display technology in combination with biomimicry of epidermal growth factor receptor 3 (ErbB3, Her3) to identify peptides that sequestered the growth factor receptor-binding protein-7 (Grb7) via the Src homology 2 domain. The identified peptides inhibited tumor growth *in vivo*[92], suggesting that the peptides inhibited tumor cell survival by down-regulating Grb7-mediated activity. Additionally, Hetian *et al.* used screening and biomimicry of FGFRI and FGFRII to identify a peptide that inhibited FGF-2-mediated angiogenesis[93]. These studies demonstrated that biomimicry together with screening technology could identify moieties that sequester a target GF.

In another biomimetic approach that used screening, Germeroth and coworkers identified a peptide sequence derived from KDR that sequestered VEGF *in vitro*[94]. The authors used an array-based peptide synthesis approach on cellulose membranes[95] to engineer, synthesize, and screen VEGF-binding peptides (VBPs) with D-amino acids substituted iteratively throughout the sequence. These substitutions enhanced VEGF inhibition and increased peptide serum stability[96]. In a series of studies, Murphy and coworkers demonstrated that the D-substituted VBP enhanced sequestering of VEGF in biological environments such as blood serum[97,98]. These studies suggest that modifications can enhance the serum stability of a sequestering peptide, which may be critical for many intended applications of target-binding peptides. This technique of substituting amino acids, as well as methods including carboxy-terminus amidation and amino-terminus acetylation, are parts of a larger theme in molecular engineering to increase peptide stability against protease-mediated degradation via terminal modifications, cyclization, or modification with carbohydrate or protein chains[96,98–101].

SELEX technology is a widely applied method to identify target-binding oligonucleotide moieties, termed "aptamers"[87]. This method has been widely applied to screen for oligonucleotide aptamers that bind to a target and to select and amplify high affinity target-binding aptamers via polymerase chain reaction (PCR)[102]. For example, oligonucleotide aptamers, identified via SELEX, inhibited angiogenesis by sequestering VEGF[103], FGF-2[104], PDGF-BB[105], angiopoietin (Ang)-1[106], Ang-2[107,108], and TGF-β1[109]. SELEX technology was also used to identify an aptamer that inhibited epithelialization by targeting keratinocyte growth factor (KGF)[110].

An important consideration to design both peptide and oligonucleotide aptamer moieties is the target-binding affinity and the serum stability, which both could affect their eventual application. Peptide moieties designed via phage display technology and phage display in combination with biomimicry

exhibited equilibrium dissociation constants (K_D) between 0.12-60 μM and 0.05-3 μM (Table 1), respectively. This suggests that biomimicry of known molecular interactions may enhance the affinity of GF sequestering. Furthermore, the oligonucleotide aptamers described herein exhibited K_D values on the scale of nM to pM, whereas the peptide and oligosaccharide sequestering moieties discussed in this study exhibited K_D values in the order of μM to nM (Table 1). Typical GF-receptor interactions, such as that between VEGF and Flt-1 or KDR, are on the order of ~10 pM[29] to ~400-800 pM[52] respectively. Thus, it may be advantageous for current peptide design strategies to use biomimicry in combination with appropriate screening techniques to identify moieties with affinities on the same order as natural biological interactions. Oligonucleotide aptamers identified via SELEX show high target-binding affinity, but several of the aptamers we feature here are RNA-based, and therefore not stable in biological environments. Strategies employing SELEX technology can be used to identify somewhat more stable DNA aptamers[108,109], and RNA aptamers can be stabilized to an extent via chemical modification[107,111] or by incorporating "locked" nucleotides[112]. Further, strategies to identify more serum-stable DNA aptamers[108,109] and to chemically modify RNA aptamers[107,111,112] have enhanced aptamer stability in biological environments.

1.4. Biochemistry of Growth Factor-Binding Peptide Interactions

Molecular recognition describes the specific binding of two species via non-covalent interactions. Models of molecular recognition, such as "lock-and-key" and "induced fit" models, describe the complementarity of two interacting species with respect to conformation and flexibility[113,114]. These models do not typically account for the fine-tuned balance of charged interactions, solvent exclusion interactions, Van der Waals interactions, and hydrogen bonding interactions required for a specific intermolecular interaction to occur[113]. Here, we focus on the biochemistry underlying GF-peptide interactions, although molecular recognition of proteins by RNA[115] and DNA[116,117] (*e.g.* by RNA and DNA aptamers) has been recently established and reviewed elsewhere. Understanding how structural and energetic characteristics impact protein-peptide interactions can aid in the design and identification of GF sequestering moieties.

Crystallographic analysis and site-directed mutagenesis studies can assist in understanding which residues or surface patches on a GF or a GF-binding peptide contribute to molecular recognition. This information can aid in the design of GF-binding peptides. Peptides recently designed to bind key growth factors provide illustrative examples of this approach. For example, TGF-β1 is known to interact with its binding partners via mostly solvent-exclusion interactions (often termed "hydrophobic interactions"), which may instruct the biomimetic design of peptides mimicking these interactions. The TGFRI binding interface with TGF-β1 contains two distinct hydrophobic patches[118], and structural analysis has revealed that TGF-β1 binds to TGFRII via hydrophobic interactions[118,119]. Similarly, hydrophobic interactions are

the primary means by which latent TGF-β1binds to the LTBP-1[120]. Indeed, peptides designed to bind TGF-β[65–67] in this review contain 55% hydrophobic residues and 38% polar residues. This peptide composition suggests that hydrophobic interactions likely contribute strongly to sequestering of TGFβ1. However, biochemical characterization of peptides mimicking known GF-binding proteins or GFRs may give insights into the chemical nature of GF sequestering when crystallographic and site-directed mutagenesis data is lacking. Peptides designed to mimic the carrier protein α2-M[65] and the type III TGF receptor, TGFRIII [66], contain 63% and 57% hydrophobic residues, respectively, suggesting that these peptides interact with TGF-β1 via mostly hydrophobic interactions. This is consistent with a previous investigation that demonstrated a hydrophobic patch on α2-M is implicated for TGFβ binding[56,121]. Taken together, this suggests that peptides designed to sequester active TGF-β1 should utilize mostly hydrophobic interactions specifically targeting unoccupied binding sites for α2-M, TGFRI, TGFRII, or LTBP-1. In contrast to TGF-β1, both polar interactions and hydrophobic interactions contribute to sequestering of VEGF[122] or FGF-2 [123], and peptides designed to bind VEGF[24,73,74,81,90,94,96] and FGF-2 [93,124,125] contain 41% and 35% hydrophobic residues and 38% and 46% polar residues, respectively. Design of GF sequestering peptides should reflect available crystallographic and biochemical data to capitalize on differences in GF structure and solvent-exposed surface chemistry.

While peptides can often bind GFs via specific molecular recognition, heparin and HS can bind numerous GF targets via less specific electrostatic interactions. Heparin and HS can promiscuously sequester GFs by virtue of the negatively charged sulfate and carboxylate groups on their constituent GAG chains[20]. Investigators have mimicked the GF-GAG interactions to design peptides that sequester heparin and HS. These peptides often include a consensus peptide sequence containing two positively charged residues flanked by uncharged residues[126]. Interestingly, not only the presence of the positively charged residues but also their spatial arrangement has been shown to influence binding of basic moieties to heparin[127,128]. Hudalla *et al.* demonstrated that a positively charged peptide, termed "HEPpep", bound substantially more heparin than a scrambled version of HEPpep[129,130], supporting the concept that the spatial arrangement of the basic residues govern the specificity of peptide-GAG interaction. Further, the heparin and HS sequestering peptides described in this review contain 29% and 55% hydrophobic and polar amino acids respectively[68–72,88], suggesting that binding of these peptides to heparin may be mediated by polar interactions. Interestingly, the heparin and HS sequestering peptides described in this review contain equal proportions of charged and uncharged polar amino acids[68–72,88], suggesting that though charged interactions are important for heparin sequestering, other polar interactions such as hydrogen bond interactions may contribute to binding. Indeed, previous literature has demonstrated that hydrogen bonding is one possible mode of intermolecular GF-heparin interactions and intra-molecular heparin interactions[20,131]. Thus, peptides designed to optimally sequester heparin or HS should be capable of

interacting via both hydrogen bonding and charged interactions while maintaining a spatial arrangement of charged amino acids, as has been demonstrated for previously identified peptides[126,127].

Protein-peptide binding is also highly dependent on the shape and flexibility of both the GF and the GF-binding peptide. Proteins and peptides are flexible in solution and can adopt conformations that are dependent upon intermolecular interaction[132]. Structural and biological characterization of a given GF-peptide or GF-protein interaction helps to determine which particular residues or motifs are important for molecular recognition. This sequence information, coupled with established peptide modifications[133], can enable the design of GF-binding moieties that have limited flexibility, and can thus present a conformationally constrained binding interface for molecular recognition of a GF. For example, peptides engineered to cyclize[134] or form stable secondary structures (*e.g.* alpha helices) may provide a defined GF-binding interface that is hypothesized to enhance target-binding affinity. Indeed, investigators have demonstrated that cyclized peptides exhibited enhanced affinity for HS[72], KDR[135], and Grb2 SH2 domains[136]. Cyclized peptides have also enhanced inhibition of PDGF-BB-mediated fibroblasts proliferation[137] relative to their linear peptide counterparts. This concept may be further explored using conformationally-constrained affibody peptides[138], wherein a peptide sequence promoting alpha helix formation can present a well-defined binding interface for molecular recognition of specific target proteins. These modifications may enhance the ability of a given GF-binding peptide to sequester its substrate by limiting peptide flexibility and presenting a defined binding interface with the GF.

An additional consideration in the design of GF sequestering moieties is the valency of the target GF or cognate GFR. Most of the GFs discussed herein are dimers, existing either as homodimers (*e.g.* TGF-β1, VEGF are typically homodimers) or heterodimers (*e.g.* PDGFs are typically heterodimers). Similarly, GFRs typically form multimeric complexes upon GF binding, resulting in multivalent GF-GFR interactions. Thus, it is perhaps logical to design multivalent GF sequestering moieties to bind one or more sites on a GF. In one example of this approach, Toepke *et al.* recently demonstrated that VBP_2, a divalent form of the KDR-mimicking peptide, sequestered VEGF with enhanced affinity relative to the monomeric form of the peptide, VBP[139]. A similar approach may be useful to engineer efficient GFR-or GF-mimicking peptides that bind to more than one site on the cognate GF or GFR. For example, using a similar approach as above, investigators have shown that dimerized erythropoietin (EPO)-mimicking peptides enhanced binding and activation of the EPO receptor (EBP)[140]. In the native cell milieu, EPO binding to EBP on the cell surface initiates EBP homodimer formation with an optimal orientation to activate downstream signaling[141], which suggests that dimerized EPO-mimicking peptides oriented the EBP dimer and enhanced activation of the receptor relative to the monomer peptide. Using a similar approach, Dyer *et al.* demonstrated that dimerized ApoE-mimicking peptides exhibited enhanced binding

to the low density lipoprotein receptor, likely by interacting with two negatively-charged repeat regions on the receptor[142]. These examples highlight the importance of valency for the design of peptide moieties.

In contrast to homodimerized GFs, FGF-2 is thought to form dimers and oligomers in the presence of heparin and heparin-like GAGs (HLGAGs)[143], and forms a signaling complex with both FGFR and surface-immobilized HLGAGs[123,144,145]. FGF-2 sequestering at the cell membrane by glypican-1, a membrane-bound HS proteoglycan (HSPG), prevents FGF-2 binding to FGFR, while sequestering to an HSPG containing syndecan-1 [45] or to the HSPG perlecan[146] enhances FGF-2 dependent signaling. This suggests that complementarity between FGFR, FGF-2, and either heparin, HLGAGs, or HSPGs, can promote or prevent FGF-2-dependent signaling, dependent on the composition of the FGF-2-bound complex. Taken together, previous studies of biomimetic GF sequestering indicate that GF-GFR complementarity and valency can instruct the design of sequestering moieties that can either up- or down-regulate GF signaling based on the composition of the signaling complex. The characteristics of the target GF (shape, conformation, flexibility, valency) and the binding interface (hydrophobicity, polarity, charge) are important considerations for future design and identification of GF sequestering moieties.

1.5. Summary

In soluble contexts, the biochemistry of the GF-moiety interaction can dictate the affinity of the GF-binding interaction and ultimately the ability to modulate GF activity. When GF-binding moieties are incorporated onto a 2D surface or within a 3D matrix, the context of sequestering can differentially modulate cell behavior based on parameters that include the spatial proximity of the sequestering to cells, the epitope of GF sequestering, the source of the GF, and the affinity of the sequestering interaction. These factors may influence the cell response to sequestering, and understanding how context influences cell migration, organization, differentiation, and survival will aid in future design of materials that may impact regenerative medicine. The following sections will discuss sequestering on solid-phase materials in 2D and 3D contexts.

Table 1. Soluble GF Sequestrants

Sequence	Seq. ID	Derivative	Function	Target	Char. Effect	Ref
Ac-GNQEQVSPK(βA)FAKLAARLYRKA	$ATIII_{121-134}$	Anti-thrombin III	---	Heparin	K_D = 87.8 nM	68,69
CGGRMKQLEDKVKKLLKKNYH LENEVARLKKLVG	PF4Zip	Platelet Factor 4	---	Heparin	K_D = 1.5 μM	70,71
-GLKKNGSCKRGPRTHYGQKA- [a]	---	FGF-1	---	Heparan sulfate	K_D = 3.1 μM	72
$SY(SO_3)DY(SO_3)G$	---	Heparin	---	VEGF	K_D = 3.1 μM	73
NH_2-GGGG-$SY(SO_3)DY(SO_3)$GGGG-OH					K_D = 0.91 μM	74
ATWLPPR	---	KDR	Anti-angiogenic	VEGF	K_D = 0.33 nM	81
NQEQVSPL – (FNIII 12-14) [b]	α_2PI_{1-8} -FN III12-14	Fibronectin	---	Multiple GFs	K_D = 0.3 – 41 μM	75
GHRPLDKKREEAPSLRPAPPPISGGGYRARPAKAAATQKKVERKAPDAGGCG	Fg $\beta 15–66_{(2)}$	Fibrinogen	---	Multiple GFs	K_D = 1.9 – 56 nM	76
KRTGQYKL	bFGFp	Phage	---	FGF-2	K_D = 122 nM	124,125
KSVRGKGKGQKRKRKKSRYK	---	HBD of VEGF	---	VEGF	Unknown	24
WDLVVVNSAGVAEVGV	---	α2-Macroglobulin	---	TGF-β, PDGF-BB	Unknown	65
TSLDASIWAMMQNA	P144	TGF-β1 Receptor 3	---	TGF-β	Unknown	66
KRIWFIPRSSWYERA	P17	Phage		TGF-β	Unknown	67
WRKWRKRWWWRKWRKRWW	ApoEdpL-W	Apolipoprotein E	Anti-biotic	Unknown	IC_{50} = 3-7 μM	78,79
Ac-LRKLRKRLLLRKLRKRLL-NH_2	ApoEdp	Apolipoprotein E	Anti-inflammatory	Unknown	CC_{50} = 103 μM	
RTELNVGIDFNWEYPASK	VBP_{WT}	KDR	Anti-angiogenic	VEGF	IC_{50} = 0.1-10μM	94
$EF_dA_dY_dL_d$IDFNWEYPASK	VBP	KDR	Anti-angiogenic	VEGF	IC_{50} ~ 1 μM	96
$(EF_dA_dY_dL_d$IDFNWEYPASK$)_2$K	VBP_2	KDR	Anti-angiogenic	VEGF	IC_{50} ~ 0.1 μM	96
PLLQATL	---	Phage (FGF-RI, FGFRII)	Anti-angiogenic	FGF-2	IC_{50} < 1 μM	93
-GDGY- [a]	GFB-111	Phage	Anti-angiogenic	PDGF	IC_{50} = 250 nM	89
VEPNCDIHVMWEWECFERL-NH_2	V114	Phage	Anti-angiogenic	VEGF	IC_{50} = 0.22 μM	90
VNWVCFRDVGCDWVL	HB10	Phage	Anti-oncogenic	HGF β chain	IC_{50} = 20 μM	91
DEEYEPYMNRRR	---	Phage (Erb3)	Anti-oncogenic	Grb7-SH2 domain	IC_{50} = 31.8 μM	92
VAVGIPTQPTTSSEPSPPSNPPWDPGRV	---	Phage (Erb3)	Anti-oncogenic	Grb7-SH2 domain	IC_{50} = 18.8 μM	92
YCWSQYLCY [a*]	WP9QY	TNFR	Anti-inflammatory	TNF-α	IC_{50} = 5 μM	82–84
CGG**AA**UCAGUGAAUGCUUAUACAUCCG	t44-OME	Random Library	---	VEGF	K_D = 49 pM	103
GGUGUGUGGAAGACAGCGGGUGGUUC	m21a	Random Library	---	FGF-2	K_D = 0.35 nM	104
GGGAGGACGAUGCGGUCCUCUCCCAAUUCUAAACUUUCUCCAUCGUAUCUGGG	14F3'T	Random Library	Anti-epithelialization	KGF	K_D = 0.8 pM	
ATGGGAGGGCGCGTTCTTCGTGGTTACTTTTAGTCCCG	20t				K_D = 0.1 nM	
CCACAGGCTACGGCACGTAGAGCATCACCATGATCCTGTG	36t	Random Library	---	PDGF-AB/BB		105
GGGCTGAGTATACTCAGGGCACTGCAAGCAATTGTGGTCCCAAT	41t				K_D = 0.1 nM	
ACUAGCCUCAUCAGCUCAUGUGCCCCUCCGCCUGGAUCAC	---	SELEX	---	Ang-2	K_D = 3.1 nM	107
ACUCGAACAUUUCCACUAACCAACCAUACUAAAGCACCGC	---	SELEX	---	Ang-1	K_D = 2.8 nM	106
TGT**C**GTTGTGT**CC**TGT**ACCCGCC**TTG**ACCA** [c]	---	Random Library	---	TGF-β	K_D = 90 nM	109
b*	Hp dp10b	Heparin	---	VEGF	K_D = 1.3 μM	85

[a] Peptide is cyclically-constrained, [a*] Peptide is constrained by disulfide linkage between cysteine residues

[b] Sequence provided in reference[147], [b*] Sequence/structure provided in column "Ref"

[c] 5' side of nucleotide is a phosphothiorate

Legend: "Ac" indicates acetylated N-terminus; (βX) indicates β-amino acid 'X'; $X(SO_3)$ indicates sulfated amino acid 'X'; "X_d" indicates D-amino acid 'X'**; Bold Text** indicates chemically modified nucleoside; Notation $(\ )_2$ indicates branched peptide

2. Growth Factor Sequestering at 2D Interfaces

In addition to, and often in concert with, soluble approaches, GF sequestering at 2D interfaces in the extracellular environment can also regulate and fine-tune cell response to GFs.

2.1. Natural Sequestering at 2D Interfaces

The cell surface contains many membrane-bound glycoproteins that sequester GFs to mediate both cell-cell and cell-matrix GF signaling[148]. Thus, the cell surface and the cell milieu can be considered an insoluble 2D interface. For example, cell membrane-immobilized heparin-binding epidermal growth factor-like growth factor (HB-EGF) enhances proliferation of adjacent cells *in vitro* upon coordination with a specific trans-membrane protein complex at the cell surface[18]. Molecular sequestering at the cell surface can also inhibit protein signaling. Reversible interactions between hepatocyte growth factor activator inhibitor type 1 (HAI-1) and hepatocyte growth factor activator (HGFA) result in inactive membrane-bound HGFA at the cell surface that becomes activated by HAI-1 cleavage by zinc-dependent MMPs during wound repair[19].

Further, the native ECM contains many insoluble components such as collagen, elastin[9], and fibrillin[149] that can self-assemble into 2D-like structures and sequester GFs and regulate their activity. Recall the aforementioned example of LTBP-1 regulation of TGF-β1 activity. Cells secrete latent TGF-β1 containing a LAP into the ECM, where fibrillin microfibrils sequester LTBP-1 in coordination with microfibril-associated glycoprotein-1 (MAGP-1). The ternary complex of LTBP-1, fibrillin, and MAGP-1 interacts with latent TGF-β1 and forms the latent TGF-β1 complex in the ECM[17,150]. Deposition of TGF-β1 in the fibrillin microfibrils thus regulates its local concentration and bioactivity[58]. Recent engineering studies have used examples of natural sequestering in the ECM as a template to design 2D sequestering interfaces that modulate cell behavior *in vitro* and *in vivo*.

2.2. Engineered Approaches for Sequestering at 2D Interfaces

Approaches that mimic the structure and function of insoluble components of the ECM can regulate cell behavior by sequestering GFs. Many of the same sequestering moieties that were identified by their ability to bind a target molecule in solution can exert a different effect when sequestering occurs at a 2D interface. Here, the context of the sequestering is defined by not only the affinity and the epitope of the sequestering interaction, but also by the spatial proximity to cells. The context of sequestering at 2D interfaces can thus regulate cell behavior using engineered bio-active substrates.

2.2.1. Sequestering on Chemically-Defined Self-Assembled Monolayers

Approaches to mimic the native ECM have enabled investigators to determine the influence of GF sequestering at engineered 2D interfaces. Surfaces presenting proteoglycans and glycoproteins in a chemically-defined monolayer can sequester proteins and modulate cell function. For example, Hudalla *et al.* investigated GF sequestering using 2D chemically-defined self-assembled monolayers (SAMs)[129,130]

terminally functionalized with HEPpep, a peptide derived from the heparin-binding domain of FGF-2 (Fig. 1D) [124,125], and Arg-Gly-Glu (RGD), a fibronectin-derived peptide sequence that promotes integrin-mediated cell adhesion (Fig. 1A). HEPpep–presenting SAMs with RGD increased HUVEC expansion relative to SAMs containing scrambled HEPpep in serum-containing medium supplemented with FGF-2. This result is consistent with the role of FGF-2 [151] to elicit increased HUVEC expansion *in vitro*[125]. Pre-treatment of serum with heparin lyase I, an enzyme that cleaves heparin with high specificity , abolished GF sequestering to HEPpep SAMs, suggesting that heparin mediated the mitogenic effect of FGF-2 on HUVECs cultured on HEPpep SAMs[129]. Further, polarization modulation-infrared reflection-absorption spectroscopy (PM-IRRAS) showed that HEPpep SAMs sequestered serum-borne molecules, and the sequestered molecules showed peaks characteristic of proteins and GAGs. Surface plasmon resonance (SPR) demonstrated that HEPpep SAMs sequestered FGF-2 only after exposure to serum or purified heparin, suggesting that sequestered heparin was sufficient to mediate FGF-2 sequestering[129]. The authors thus hypothesized that heparin-sequestering substrates could sequester endogenous, heparin-binding GFs and amplify their activity in cell culture. In a related study, heparin sequestering to HEPpep SAMs enhanced endogenous FGF signaling and endogenous bone morphogenetic protein (BMP) signaling in human mesenchymal stem cell culture. Specifically, HEPpep SAMs presenting RGD increased hMSC expansion in a FGF signaling-dependent fashion in serum-containing medium without supplemented GFs. Additionally, the same substrates increased hMSC osteogenic differentiation in a BMP signaling-dependent fashion in serum-containing osteogenic induction medium, again without supplemented GFs[130]. These studies suggested that endogenous circulating heparin, previously identified as a component of human blood plasa[153,154], harnessed at engineered 2D interfaces could enrich and enhance the activity of endogenous GFs while foregoing the need for exogenous supplemented GFs.

2.2.2. Sequestering to Engineered Self-Assembled Nanofibers

Investigators have used synthetic ECMs on engineered 2D substrates to examine the influence of GF sequestering on cell behavior. For example, engineered self-assembled nanofibers resemble fibrous structures found in the native ECM[155] and can mimic the function of GF-sequestering microfibrils. Peptide amphiphiles provide one strategy to generate self-assembled nanofibers that enable chemical modifications, such as incorporation of GF sequestering moieties. Self-assembling peptide amphiphiles contain a self-assembling hydrophobic domain and a hydrophilic domain to incorporate biological functionalities. The resulting self-assembled nanofibers can be functionalized on their outer surface with GF sequestering moieties that can sequester GFs at a 2D interface, while providing a nanofibrous matrix. Stupp and coworkers have used peptide amphiphiles to promote heparin sequestering and modulate GF-dependent cell behavior *in vitro* and *in vivo*. Atomic force microscopy (AFM) confirmed that nanofibers self-assembled upon mixing of heparin-binding peptide amphiphiles (HBPA) composed of an aliphatic

self-assembling domain (C_{15}), a spacer domain, and a bioactive heparin-binding domain. HBPA nanofibers specifically sequestered heparin when compared to self-assembled nanofibers formed with a scrambled version of the heparin-binding domain, $HBPA_{Scramble}$,[156]. Additionally, matrices composed of HBPA nanofibers increased neovascularization in a rat cornea model relative to both bolus heparin injections and collagen gels supplemented with heparin[157]. In a similar approach, investigators used a chick chorioallantoic membrane (CAM) model of angiogenesis to show that HBPA-containing matrices increased blood vessel density in the presence of heparin, hyaluronic acid, VEGF, and FGF-2 [158]. Mammadov and coworkers used a similar approach and increased tubulogenesis of cultured HUVECs in HBPA nanofiber matrices *in vitro* relative to matrices without HBPA. HBPA nanofiber matrices loaded with VEGF and FGF-2 *in situ* also increased neovascularization in a rat cornea model *in vivo* relative to bolus injections of GFs alone[159]. In another study, Chow *et al* demonstrated that HBPA nanofibers formed within a pancreatic islet enhanced FGF-2-dependent pancreatic β cell viability. Further, VEGF and FGF-2 co-delivery with HBPA nanofibers significantly increased pancreatic endothelial cell sprouting relative to GFs alone[160], suggesting that the heparin-binding nanofibers potentiated the effect of VEGF and FGF-2 by sequestering endogenous heparin or HS and supplemented GFs. Here, we refer to endogenous heparin or HS as a soluble glycosaminoglycan in blood plasma[153,154], a component of the heparin proteoglycan serglycin secreted by mast cells during an inflammatory response[161] (*e.g.* during wound healing), or a component of immobilized cell membrane heparan sulfate proteoglycans in the pericellular space[13,162]. Finally, using a novel amphiphilic peptide consisting of the HBPA sequence with a $(Arg\text{-}Ala\text{-}Asp\text{-}Ala)_{16}$ self-assembling domain, Guo and coworkers demonstrated that VEGF co-delivery via injectable HBPAs enhanced cell survival, reduced scar formation, and increased the function of an infarcted heart relative to GFs alone in an *in vivo* rat model[163].

Taken together, the above results demonstrated that heparin sequestering at 2D interfaces could enhance the pro-angiogenic activity of heparin-binding GFs such as VEGF and FGF-2. These results are consistent with previous studies demonstrating that cell surface-bound heparin and HS enhanced the activity of heparin-binding GFs by increasing the affinity of GF-GF receptor (GFR) interactions[145,164,165] and regulating the assembly of the GF-GFR signaling complex[166], thus acting as "allosteric activators" of the GF. Similar self-assembling nanofibers have been designed to enhance cell survival, multicellular organization, and differentiation in the absence of supplemented GFs. Specifically, self-assembled HBPA nanofibers enhanced the activity of endogenous GFs when implanted *in vivo*. Using a similar approach to that described above, Shah *et al.* demonstrated enhanced viability and osteogenic differentiation of hMSCs cultured within self-assembled nanofiber gels, which were composed of a self-assembly domain and a bioactive domain engineered to sequester TGFβ1. Nanofibrous HBPA gels enhanced articular cartilage regeneration in a rabbit model with and without supplemented GFs[167]. Lee *et* al further

demonstrated that heparin-sequestering HBPA nanofiber gels could enhance the activity of BMP-2 and reduce the concentration of supplemented BMP-2 needed to elicit a therapeutic effect. In the presence of heparan sulfate, nanofibrous HBPA gels enhanced bone regeneration and more effectively bridged the defect gap using a 10-fold lower BMP-2 concentration than the soluble BMP-2 dose needed for effective bone regeneration in the same model[168]. Collectively, these results suggest that sequestering to 2D nanofiber matrices may enhance the activity of both endogenous and supplemented GFs and ultimately decrease the amount of supplemented GF necessary to elicit a cell response.

2.3. Mechanisms of Sequestering at 2D Interfaces

The cell milieu consists of many ECM features that serve as a template for engineered 2D substrates. For example, self-assembled nanofibers can mimic the nanostructure and function of natural structural fibrils[155] while chemically-defined 2D SAMs can mimic proteoglycan presentation on the cell surface. We propose that 2D GF sequestering enhances sequestered GF activity via two distinct mechanisms. First, sequestering moieties at an interface may enhance the residence time of the sequestered GF via a phenomenon known as rebinding. Secondly, GF sequestering at a 2D interface may enhance or inhibit sequestered GF activity based on which site on the GF is sequestered.

GF sequestering at a 2D interface may enhance GF activity by increasing the residence time and locally enriching the GF via a rebinding mechanism previously described[169] (Fig.1A). For example, Oh *et al.* proposed a rebinding mechanism that influenced the residence times of SH2-containing proteins at surfaces containing immobilized pTyr. Within a given time frame(Δt), different SH2-containing proteins exhibited different mean square displacement (MSD) away from the pTyr-containing surface, which suggests that each protein exhibited unique rebinding characteristics at the surface[170]. Results further suggested that rebinding decreased the effective diffusion coefficient (D_{eff}) and increased the residence time of SH2-containing proteins. The probability of rebinding has been modeled as a function of the sequestering moiety concentration, the target molecule concentration, and the affinity of their interaction (Fig. 2B)[169]. Thus, the affinity of a given sequestering interaction can influence the rebinding probability and the residence time of a target protein at the sequestering interface (Fig. 2A). An illustrative example of the effect of local GF enrichment at a surface is provided by studies that have covalently immobilized a GF to a surface. Originally, GFs were believed to be active only in the soluble state; however, discovery of cell-membrane anchored GFs indicate that immobilized growth factors are capable of stimulating cells via artificial "juxtacrine" or "matricrine" mechanisms[171,172]. Previous studies indicated that covalent GF immobilization, *in vivo* and *in vitro*, provides high local concentrations of the GF, inhibits signal transduction down-regulation, and exerts different effects compared to soluble growth factors[171]. VEGF serves an example of context-dependent GF signaling as it has been immobilized onto 2D substrates for use in medical applications[171]. *In vitro*, VEGF binding to KDR induces receptor autophosphorylation and

elicits endothelial cell proliferation via activation of the mitogen-activated protein kinase (MAPK) signal transduction pathway[173,174]. Cell culture substrates containing covalently-immobilized VEGF promoted HUVEC proliferation for longer durations when compared to those cultured on substrates with non-specifically adsorbed VEGF[173]. This phenomenon was corroborated by a similar study demonstrating that KDR phosphorylation in HUVECs was prolonged when VEGF was covalently immobilized to the culture substratum, and the stability of VEGF was also enhanced[175]. The prolonged effects of covalently-immobilized VEGF can be attributed to the cell's inability to endocytose and degrade VEGF-KDR complexes, a process which normally inactivates the VEGF-dependent signal transduction pathway and suppresses over-proliferation in response to VEGF[173].

Table 2. Biomaterials that Sequester and Sustain Release of GFs

Sequence (ID)	Derivative	Target	Matrix	Function	Char. Effect	Ref
NQEQVSPNQSPNHTQNRAY (Hep-BP3) NQEQVSPQMRAPTKLPLRY (Hep-BP4) NQEQVSPSVSVKAKKSVNR (Hep-BP5)	Phage	Heparin-NGF	Fibrin	Controlled NGF release	K_D = 2.1 μM K_D = 1.3 μM K_D = 1.8 μM	17
($PF4_{ZIP}$)	PF4	FGF-2	PEG	Controlled FGF-2 release	a	71
(bFGFp)	Phage	FGF-2	PEG	Controlled FGF-2 release	a	177
$EF_dA_dY_dL_d$IDFNWEYPASKC (VBP)	KDR	VEGF	PEG	Pro- angiogenic[b] Anti-angiogenic[c]	a	97
$(EF_dA_dY_dL_d$IDFNWEYPASK$)_2$KC (VBP_2)	KDR	VEGF	PEG	Pro- angiogenic[b] Anti-angiogenic[c]	a	13
CRTELNVGIDFNWEYPASK (VBP-WT); (VBP)	KDR	VEGF	PEG	Pro-angiogenic[b] Anti-angiogenic[c]	a	178 98
(Fg β15–$66_{(2)}$)	Fibrinogen	FGF-2 PlGF	PEG	Enhanced wound closure, angiogenesis	a	76
<u>GCGATACTCC</u>ACAGGCTACGGCACGTAGA GCATCACCATGATCCTG (36t + 5' tail)	SELEX	PDGF-BB	Microparticles in Agarose	Sustained release Triggered release	K_D = 25 nM	179 180

[a] Effect listed in Table 1
[b] Pro-Angiogenic function demonstrated upon sustained release of bound VEGF (source provided in Ref)
[c] Anti-Angiogenic function demonstrated upon sequestering of soluble VEGF (source provided in Ref)
Legend: <u>Underline</u> indicates 5' tail

In contexts where the target GF is non-covalently bound to a surface via a site distinct from the active site, termed an "allosteric" sequestering site, such as the heparin-binding domain, GF-GFR signaling may increase because of GF sequestering at the surface that enhances the interaction between the GF and cell receptors (Fig. 1C,D). Conversely, materials designed to sequester a GF via the active site may decrease GF-GFR signaling by blocking the active site and decreasing GF-GFR interactions (Fig. 1B). In the native cell milieu, both heparin- and HS-mediated allosteric sequestering and active site sequestering via sFlt-1 can regulate VEGF-mediated EC function during angiogenesis. Further, *in vitro* approaches can leverage the epitope of sequestering to design bioactive substrates that promote EC pro-angiogenic function. For example, during angiogenesis, VEGF elicits a pro-angiogenic response[181] whereas high levels of TGF-β1 can inhibit angiogenesis[182,183]. Thus, surfaces that can simultaneously up-regulate VEGF activity (via allosteric sequestering) and down-regulate TGF-β1 activity (via active site

sequestering) could, in principle, promote EC pro-angiogenic function at the sequestering interface. In addition, engineered substrates should take into account the differential context of sequestering at 2D interfaces versus in 3D matrices, where additional variables such as the spatial proximity to cells may ultimately influence cell behavior in response to sequestering.

3. Influence of GF Sequestering in 3D Matrices

The context of GF sequestering in 3D scenarios can substantially change the impact on cell function. The epitope of sequestering and the affinity of the sequestering interaction may influence cell behavior, regardless of whether the cell is in a 2D or 3D environment. However, in a 3D matrix, additional parameters such as the source of the sequestered GF (e.g. cell-secreted versus supplemented in media) and the proximity of cells to the sequestering event can have a particularly significant influence on the ultimate cell response. In this section, we discuss GF sequestering in 3D matrices and examine the context-dependent influence of GF sequestering on cell behavior.

The concept of 3D sequestering of GFs mimics a key function of the native ECM, and matrices that mimic the ECM in 3D [184] have been widely used to promote cell attachment, cell-demanded degradability, and molecular sequestering[10]. Hydrogels are often used to mimic the native ECM, in part because they can recapitulate aspects of ECM physical structure and biochemical functions[185–187]. Synthetic hydrogel matrices are particularly attractive, as they provide a chemically-defined matrix to systematically incorporate moieties that can mediate cell degradability[188], cell attachment[189], and GF sequestering[186,190]. While these matrices often use simple, defined chemistries, they can mimic the biochemical and biophysical characteristics of more complex natural polymers, such as fibrin[10]. This section will introduce approaches that modulate cell function using natural and synthetic hydrogels that contain immobilized GF sequestering moieties. We discuss these materials in the context of controlled binding and release, which can promote or prevent local paracrine or autocrine signaling of adjacent cells.

3.1. Sequestering to and Controlled Release from 3D Matrices

Hydrogel formulations have increasingly used GF sequestering to control the release of GFs (Table 2) [190] for therapeutic applications including modulating angiogenesis[191]. This mechanism for sustaining GF release is distinct from drug delivery systems that rely on non-covalent interactions between GFs and a 2D substrate, which is reviewed elsewhere[192]. Specifically, studies have taken advantage of the GF-binding ability of heparin to develop heparin-sequestering matrices that sustain the release and enhance activity of heparin-binding GFs. For example, Sakiyama-Elbert and colleagues used heparin-binding peptides to sustain the release of multiple heparin-binding GFs. Three unique peptides (Hep-BP3, Hep-BP4, Hep-BP5) were shown to bind heparin with varying affinity, and fibrin matrices with tethered Hep -BP3, -BP4, and -BP5 sustained the release of nerve growth factor (NGF)[176]. In another

study, fibrin matrices with a tethered heparin-binding peptide derived from antithrombin III[68,69] ($ATIII_{121-134}$) sustained the release of β-NGF[193]. In another study, Lin and Anseth used photopolymerized hydrogels composed of polyethylene glycol (PEG) and a heparin-binding peptide (bFGFp) to sustain the release of FGF-2 *in vitro*, as measured via Förster resonance energy transfer (FRET)[177]. Leveraging a similar phenomenon, Zhang *et al.* used low molecular weight heparin and a heparin-binding peptide (PF4Zip) to demonstrate heparin-mediated hydrogel self-assembly (via interaction between heparin and PF4Zip) and sustained release of FGF-2 *in vitro*[71]. Further characterization will be required to determine whether GF sequestering with heparin-binding peptides (*e.g.* ATIII, PF4Zip, Hep-BP1-5, HEPpep) is a result of direct interactions with the GF or by indirect interactions with endogenous heparin or HS[153,154] in culture.

In order to demonstrate specific sequestering of particular growth factors, a few recent studies have developed approaches to modulate one GF of interest with specificity. For example, Murphy and co-workers developed an approach to specifically target VEGF using a peptide previously designed to mimic the extracellular domain of the VEGF receptor 2 (KDR)[94,96,122]. The authors used a thiolene chemistry[194] to generate PEG hydrogel microspheres with a covalently-immobilized, D-substituted peptide derivative (VBP) of the wild type KDR mimic (VBP_{WT})[97]. Hydrogel microspheres containing VBP or VBP_{WT} sequestered VEGF and sustained its release for longer timeframes when compared to microspheres containing a scrambled version of VBP[97,98]. VEGF sequestering using this approach significantly reduced soluble [VEGF] and associated HUVEC expansion in culture[97,98], whereas VEGF delivery significantly increased HUVEC expansion in culture[97,178]. Importantly, these effects were strongly dependent on the presence of serum, suggesting a role for VEGF-binding serum proteins in increasing VEGF release rate[98,178]. In another study, Toepke *et al.* showed that hydrogel microspheres with a covalently-immobilized, bivalent version of VBP (VBP_2) bound VEGF with particularly high affinity, resulting in efficient knockdown of VEGF signaling during sequestering and increased HUVEC expansion upon sustained VEGF release[139]. These results demonstrated that a material designed to sequester a single GF, in this case VEGF, could down- or up-regulate specific GF signaling via sequestering or release, respectively. This approach provides an interesting contrast with sequestering approaches that target heparin or mimic proteoglycans, which exhibit promiscuous GF binding and, consequently, elicit a wider array of cell responses.

Investigators have also used oligonucleotide aptamers in 3D hydrogels to sequester and sustain the release of a specific target GF. A recent study by Soontornworajit *et al.* used SELEX technology to identify a DNA aptamer that sequestered soluble PDGF-BB. The authors tethered the PDGF-BB-binding aptamer to polystyrene microparticles, embedded the microparticles in agarose, and demonstrated sustained release of PDGF-BB[180] that was dependent on the aptamer-PDGF-BB binding affinity[179]. Further, the addition of pegylated complementary oligonucleotides, designed to bind to the aptamer and

compete with aptamer-GF binding, triggered PDGF-BB release[180]. The enhanced affinity of GF-sequestering oligonucleotide aptamers compared to oligosaccharides or peptides, coupled with the ability of SELEX to efficiently identify GF-binding aptamers, suggest that they may have broad utility in GF regulation.

Table 3. Influence of GF Sequestering on Encapsulated and Invading Cells

Sequence ID	Target	Cell/Animal Model	Function	Ref
MCP BP1, BP2	MCP-1	β Islet Cells	Immuno-modulatory	195
WP9QY	TNFα	β Islet Cells, hMSC, Pheochromocytoma Cells	Immuno-modulatory	196
α_2PI_{1-8} -FN III12-14 [a]	VEGF	hECs	Increased tubulogenesis	197
	PDGF-BB	hSMCs	Increased sprouting	
	BMP-2	hMSCs	Increased osteoblastic differentiation	
$ATIII_{121-134}$	NGF	Sciatic Nerve	Increased neurite extension	198
$ATIII_{121-134}$	NT-3	Spinal Cord Model	Enhanced neural sprouting	199,200
$ATIII_{121-134}$	FGF-2	Neurite Sprouting	Enhanced neurite extension	201
Hep-BP3, -BP4, -BP5	Heparin-NGF	Dorsal Root Ganglia	Pro-neural growth	88
$(\alpha_2\text{-}PI_{1-7})^a$ $ATIII_{121-134}$ [a]	Heparin-NGF	Dorsal Root Ganglia	Pro-neural growth	202
$(\alpha_2\text{-}PI_{1-7})^a$ $PF4_{60-67}$ [a]				
$ATIII_{121-134}$, Hep-BP1, -BP2	Heparin-NGF	Peripheral Nerve	Enhanced peripheral nerve growth	203
Fg $\beta 15–66_{(2)}$	FGF-2, PlGF	Diabetic Wound	Enhanced wound healing	76
HBPA	Heparin-FGF-2/VEGF	Chick Chorioallantoic Membrane	Pro-angiogenic	158

[a] Sequence/Structure provided elsewhere[147]

3.2. Influence of GF Sequestering on Cell Behavior in 3D Matrices

The ECM provides a template to engineer 3D hydrogel matrices that can sequester GFs and thereby regulate cell function. In this section, we discuss GF sequestering that promotes or inhibits local GF availability to cells on the molecular scale in a 3D context (Table 3), which is distinct from controlled release formulations in which the material serves as a reservoir for GF storage and release into a surrounding environment. Here we discuss the impact of GF sequestering on encapsulated and invading cells in close physical proximity to the sequestering event.

3.2.1. Impact of GF Sequestering on Encapsulated Cells

GF sequestering to, and release from, the ECM tightly regulates cell behavior *in vivo.* Investigators have mimicked sequestering in the native ECM and designed materials that interact with cells to promote cell behaviors including differentiation, survival, or migration/organization in a 3D context. For example, mimicry of natural GF-receptor interactions can influence the function of encapsulated cells *in vitro*. Anseth and coworkers used molecular sequestering to modulate the immune response to implanted biomaterials. The authors used PEG hydrogels containing two distinct peptides derived from CC-chemokine receptor type 2 (CCR2) to demonstrate sequestering of cell-secreted monocyte chemotactic protein-1 (MCP-1), and to demonstrate reduced host inflammatory response to encapsulated cells. Hydrogels with tethered CCR2-mimicking peptides sequestered MCP-1 that was secreted by an encapsulated murine pancreatic β islet cell line[195]. Using a similar concept, Anseth and coworkers developed PEG hydrogels with immobilized peptides mimicking TNF receptor-1 (TNFR1).

These hydrogels sequestered supplemented TNF-α, inhibited TNF-α-induced apoptosis of encapsulated cells, and sustained the release of TNF-α[196]. TNF-α sequestering also enhanced viability and insulin secretion of encapsulated β islet cells and increased proliferation of encapsulated hMSCs upon TNF-α challenge[196]. These studies suggested that materials mimicking a GF receptor could sequester and decrease the activity of an endogenous, cell-secreted GF (Fig. 3B) or an exogenous supplemented GF (Fig. 3A) by targeting the active site of the GF.

Investigators have also studied the influence of heparin sequestering on GF-mediated cell behavior *in vitro*. Sakiyama-Elbert, Hubbell, and coworkers used GF sequestering to modulate neurite extension *in vitro*. First, Sakiyama-Elbert *et al*. demonstrated that fibrin hydrogels with immobilized heparin binding peptides, $ATIII_{121-134}$ and $PF4_{60-67}$, increased neurite extension of encapsulated dorsal root ganglia (DRGs) cultured in the presence of NGF[202]. Next, fibrin gels with immobilized $ATIII_{121-134}$ were shown to enhance neurite extension of encapsulated DRGs in the presence of FGF-2[201]. Finally, Maxwell *et al.* showed that fibrin matrices with immobilized heparin-binding peptides, Hep-BP3, -BP4, and -BP5, modulated NGF sequestering and release, and thereby increased NGF-mediated neurite extension of encapsulated DRGs[88]. Taken together, these studies suggested that molecular sequestering of endogenous heparin or HS[153,154] could enhance the activity of supplemented NGF and FGF-2 by sequestering these GFs at a site that is distinct from the receptor-binding site.

Hubbell and coworkers demonstrated that materials engineered to mimic fibronectin could sequester multiple GFs and enhance GF-mediated sprouting and differentiation of encapsulated cells *in vitro*. Specifically, Martino *et al* demonstrated that fibrin matrices - containing α_2PI_{1-8} -FN III_{12-14}, a fibronectin-mimicking peptide, and loaded with PDGF-BB - enhanced smooth muscle cell (SMC) sprouting relative to fibrin, PDGF-BB, or peptide alone[75]. Further, these same matrices enhanced retention of supplemented VEGF, PDGF-BB, and BMP-2 and elicited increased endothelial cell tube length (with VEGF), increased SMC sprout length (with PDGF-BB), and increased osteogenic differentiation of hMSCs (with BMP-2)[197]. Collectively, these studies demonstrate that sequestering of supplemented GFs via an allosteric GF-binding epitope distinct from the receptor-binding site can enhance GF-mediated sprouting and differentiation of encapsulated cells.

In contrast with the approach utilizing TNFR-1-mimicking peptides to specifically reduce TNF-α-mediated signaling, the heparin-sequestering and fibronectin-mimicking matrices described here capitalized on the promiscuous GF-binding ability of heparin and fibronectin. The strategy to mimic TNFRI and modulate TNF-α signaling relied on binding to the active site to block TNF-α binding with TNFRI on the cell surface (Fig. 3A). However, sequestering strategies using heparin-binding or fibronectin-mimicking peptide moieties may enhance GF signaling in encapsulated cells because the sequestering event leaves the receptor-binding site of the GF unblocked (Fig. 1D). This hypothesis is

consistent with literature describing heparin-mediated GF-receptor interactions that enhance the affinity of GF binding to its cognate receptor[145,164,165]. In addition, GF binding ECM moieties such as GAGs, PGs, and glycoproteins (*e.g.* fibronectin) exhibit multiple additional features that likely regulate GF signaling, including multivalent GF presentation to cells and simultaneous binding to GF receptors and other classes of receptors (*e.g.* integrins)[13]. Future studies in chemically defined contexts may provide insights into the importance of these features during GF sequestering and regulation.

3.2.2. Impact of GF Sequestering on Invading Cells or Tissues

Cell behavior is also highly dependent on the nature of the surrounding ECM. Approaches that mimic the ECM of a tissue type of interest can potentially recapitulate aspects of the extracellular space and promote cell invasion upon implantation *in vivo*. Researchers have used heparin-binding moieties to enhance cell invasion both *in vitro* and *in vivo*. In an extension of their *in vitro* studies, Sakiyama-Elbert and Hubbell used fibrin gels with immobilized heparin-binding peptides, $ATIII_{121-134}$-, Hep-BP1, and Hep-BP2, to examine the influence of NGF on neural growth *in vivo*. The authors excised 5mm segments of sciatic nerve from Lewis rats and surrounded the defect site with modified fibrin matrices encased in a cylindrical silicone nerve guidance conduit. After 6 weeks, the fibrin matrices with immobilized heparin-binding peptides Hep-BP1 and Hep-BP2 increased the nerve fiber density and the percent of neural tissue in the fibrin matrices[203]. Further, NGF sequestering to $ATIII_{121-134}$-modified fibrin matrices enhanced sciatic nerve regeneration and neurite extension in rat models[198]. In a second series of studies, the authors examined neurotropin-3 (NT-3) sequestering to fibrin matrices with immobilized $ATIII_{121-134}$ and demonstrated enhanced neurite outgrowth from a DRG model[193] and increased neural sprouting in a short-term spinal cord injury model upon NT-3 sequestering[199,200]. Collectively, these studies suggested that sequestering enhanced GF-mediated cell invasion when 3D matrices were implanted *in vivo*.

Investigators have also leveraged GF sequestering to enhance wound healing *in vivo*. Martino *et al* demonstrated that co-delivery of soluble BMP-2 and fibrin gels with an immobilized fibronectin-mimicking peptide increased the bone volume in a critical calvarial bone defect in mice. Further, the same fibronectin-mimicking matrices increased the speed of dermal wound healing and increased granulation tissue formation upon co-delivery of VEGF and PDGF-BB[197]. Finally, in an *in vivo* model of diabetic dermal wound healing, fibrin matrices containing an immobilized fibrinogen-mimicking peptide FG β15–$66_{(2)}$ enhanced wound closure and significantly increased the amount of granulation tissue via a mechanism that likely involved sequestering of supplemented FGF-2 and placental growth factor 2 (PlGF-2)[76]. Taken together, these results demonstrated that biomimetic materials, designed to mimic fibronectin or sequester heparin, enhanced *in vivo* wound healing stimulated by supplemented GFs (Fig. 4). In these studies, the materials were implanted with supplemented GFs and no supplemented heparin, suggesting that the tissue milieu contained heparin and promoted heparin-mediated GF sequestering and

GF-mediated dermal wound and bone defect healing. This provides an example of an emerging concept in biomaterial development to mimic components of the native ECM and leverage signals that are present in the soluble environment *in vivo*. This emerging paradigm in biomaterial development may be further exploited to understand the impact of sequestering on cell behavior and to limit the dependence on recombinant GFs to elicit cell response.

3.3. Modeling GF Sequestering

Modeling approaches allow us to understand GF sequestering in time and space to predict the influence on cell function. We have previously discussed modeling based on the premise that sequestering at a 2D interface is a result of protein-ligand rebinding[169,170]. Similar principles can be used to understand mass transport phenomena, and numerous models have been established to better understand the soluble environment of 3D hydrogels[190]. Such models have recently been adapted to understand the effect of GF sequestering on the cellular environment[199,201]. Protein diffusion through hydrogels is dependent on ECM properties[204] such as molecular weight of the polymer chains[205], cross-linking density[205,206], and the presence of cell adhesion peptides[207] in addition to GF sequestering interactions within the hydrogel[178,201]. For example, models of molecular sequestering in hydrogels have previously described the influence of heparin-binding peptide concentration and peptide-heparin affinity on the sustained release of both FGF-2 [201] and heparin[88]. We recently used similar modeling parameters[200,201] to demonstrate that sequestering of VEGF may generate spatial and temporal gradients of the GF (Fig. 4) that are dependent on material parameters, including the affinity of the sequestering interaction. Preliminary results demonstrated that hydrogels containing VBP enhanced EC invasion (data not shown). Thus, we hypothesize that spatial gradients of sequestered VEGF, generated over time as the hydrogel sequesters external delivered VEGF, can enhance invasion of encapsulated ECs (Fig. 4A), consistent with previous investigations implicating VEGF gradients for promoting EC invasion[208,209]. This concept may further be applied to 3D matrices with pre-loaded GFs, which likely form spatial gradients of GF upon GF release over time and can thus enhance gradient-dependent cell invasion (Fig. 4B). These spatial and temporal gradients may serve as a mechanism by which sequestering matrices pre-loaded with GFs can enhance cell invasion over time, as gradients have been shown to guide invasion of endothelial cell sprouts[27] and neurite outgrowths[210] in response to VEGF and NGF, respectively. This example may be further extended to hydrogels that mimic TNFR1 and CCR2. Mass transport models of affinity-mediated diffusion through hydrogels suggest that MCP-1 secreted by encapsulated cells would likely be sequestered to CCR2-containing hydrogels proximal to the encapsulated cells, and thus generate a spatial gradient of MCP-1 favoring enrichment at the interior of the hydrogel (Fig. 4B). However, in a different context, supplemented TNF-α from outside the hydrogel would be sequestered preferentially at the periphery of the hydrogel containing TNFR1-mimicking peptides and enriched away from encapsulated cells (Fig. 4A). This modeling provides a

potential mechanism by which TNF-α sequestering could inhibit TNF-mediated apoptosis of encapsulated cells based on the proximity of cells to the sequestering event. Taken together, these scenarios serve as examples in which GF sequestering moieties in the appropriate context can drive gradient formation that may be essential for tissue morphogenesis processes.

4. Conclusions

The components of the ECM and the soluble environment, collectively the cell milieu, play an important role to regulate the activity of GFs. The cell milieu regulates the activity of GFs via sequestering to immobilized GAGs, proteoglycans, glycoproteins[13,18,19] (in the native ECM and the cell surface) , and structural proteins like fibrin[10] and collagen found in the native ECM. These macromolecules can mediate GF sequestering that modulates cell behavior in context-specific ways. In this Feature Article, we have discussed the context of both natural GF sequestering and engineered GF sequestering, which has included sequestering in solution, at 2D interfaces, and within 3D matrices. Both natural and synthetic matrices can recapitulate one or more functions of the native ECM, and understanding the effect of sequestering on cell function is an important step for future design of implantable materials to promote tissue regeneration.

Biomimicry provides one example of a context-specific sequestering event, which can have distinct influences on cell behavior. For example, VEGF sequestering to heparin and HS or soluble receptor fragments in the native ECM can generate gradients of VEGF activity[26,38,211] which enhance endothelial cell sprouting during angiogenesis[209,212]. In contrast to allosteric GF sequestering via heparin and HS, VEGF sequestering to soluble receptor fragments can decrease VEGF activity by competitively binding to the GF active site and blocking its ability to bind to and transduce signals via KDR homodimers[28]. Using a similar mechanism, natural sFlt-1 binds to the active site of VEGF and may enhance sprout formation by forming gradients of active unbound VEGF[37–40]. Similar concepts have been applied in the engineering approaches discussed herein. For example, sequestering of supplemented TNFα via TNFR1-derived peptides decreased TNFα-mediated apoptosis *in vitro*[196] and decreased bone resorption *in vivo*[84]. In contrast, allosteric sequestering of GFs via heparin-binding and proteoglycan-mimicking peptides enhanced GF signaling in multiple *in vitro* and *in vivo* models[76,176,197,200,202]. Allosteric GF sequestering via a heparin-binding site is likely to enhance GF activity because the bound GF remains able to bind to its cognate receptor, whereas sequestering via the active site may increase or decrease GF activity depending on the binding affinity and the spatial proximity of the sequestering event to the cell milieu. These examples highlight the epitope of sequestering as one key parameter in context-dependent GF regulation. Thus, it is important to identify the context of GF sequestering in engineered materials in

order to understand the cell response to these materials and further aid in their eventual translation to biotechnology applications.

Another parameter to consider in context-dependent GF sequestering is the affinity of the sequestering interaction. Whereas many sequestering moieties described herein exhibited nano- to micromolar K_D values, typical GF-receptor interactions exhibited pico- to nanomolar K_D values. Thus, moieties that sequester GF active sites with lower affinity than the cognate cell surface receptors may enhance signaling by locally enriching GFs but maintaining GF availability, whereas moieties with comparable or higher affinity than the cognate receptor may decrease signaling by depleting GFs or locally blocking the GF active site. In one example, our lab has demonstrated that a VEGF-binding peptide with increased VEGF binding affinity (VBP) relative to another version of the peptide (VBP_{WT}) enhanced VEGF sequestering in complex serum-containing environments and reduced VEGF-dependent HUVEC proliferation by more effectively depleting soluble VEGF[98]. Thus, sequestering approaches should consider both the epitope and affinity of sequestering to fully understand and predict the influence of sequestering on cell behavior.

In conclusion, we have highlighted parameters that contribute to the context-specific effects of GF sequestering, with a particular emphasis on studies that showed a significant influence on cell behavior. The ultimate cell response to sequestering is likely influenced by parameters including, but not limited to, the affinity of the sequestering interaction, the GF-sequestering epitope, the source of the sequestered GF (supplemented or endogenous), the proximity of sequestering to cells, and the sequestering "phase" (soluble or insoluble). The context of GF sequestering plays a key role in influencing cell behavior, and understanding the sequestering parameters that influence cell behavior should be applied to future design of materials for a variety of applications, including biomanufacturing and regenerative medicine.

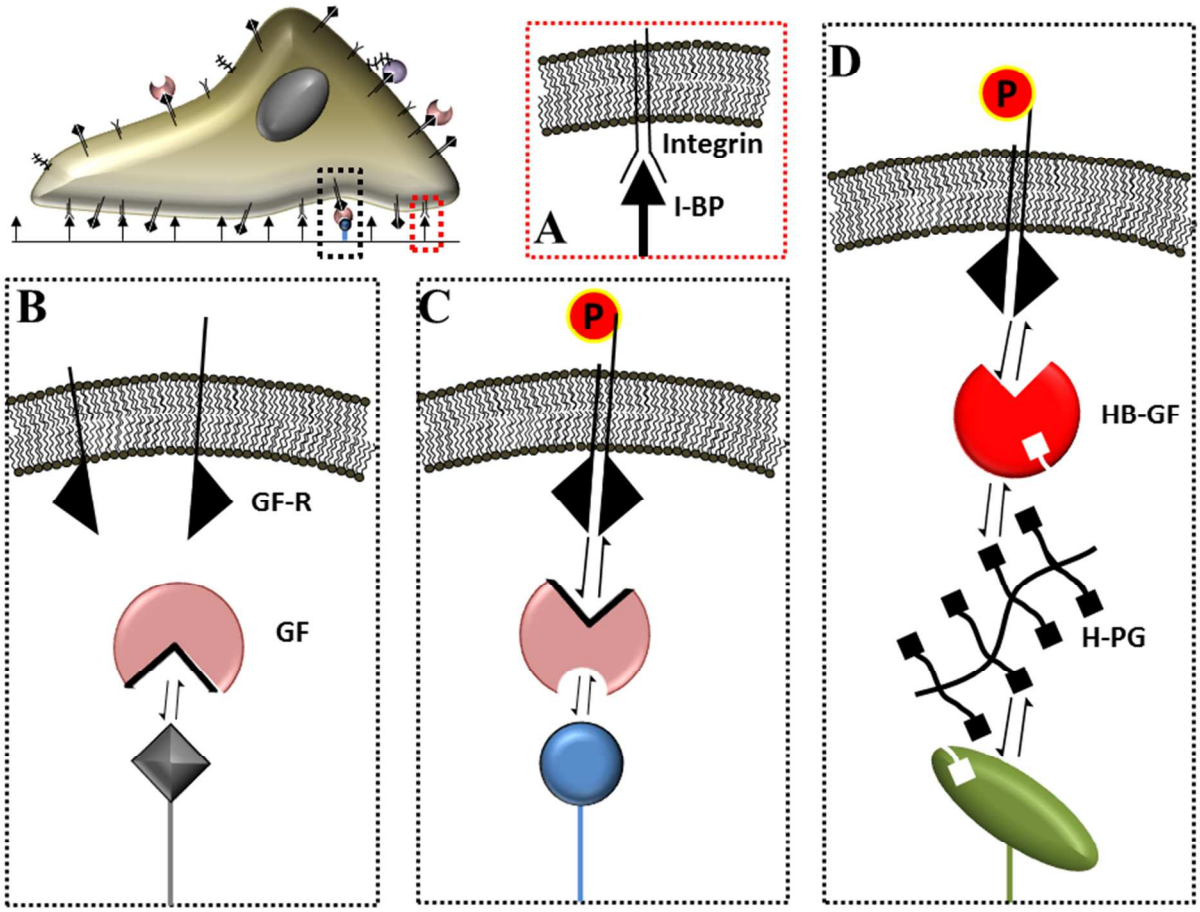

Figure 1. Influence of epitope-dependent molecular sequestering on cell signaling. A: Schematic representation of cell binding to 2D surfaces presenting integrin-binding peptides (I-BP) as demonstrated previously[129,130].B: Schematic representation of GF sequestering to a peptide whose binding epitope is the active site of the GF. Competition between the immobilized peptide (black) and GF receptor results in inhibited GF-GF receptor (GFR) binding and down-regulated GF-mediated receptor activation. C: Schematic representation of GF sequestering to surface with tethered GF-binding peptide (blue), whose binding epitope is an allosteric site away from the GF active site. Due to allosteric sequestering, GF-GF receptor binding and receptor activation are un-hindered. D: Schematic representation of GF sequestering at a 2D surface with tethered heparin-binding peptide (green), wherein GF sequestering is mediated by heparin, HS, or either heparin- or HS-containing proteoglycan (H-PGs). Heparin-mediated allosteric sequestering of heparin-binding GF (HB-GF) in the native ECM can up-regulate GF receptor activation by enhancing the affinity of the GF-GFR interaction.

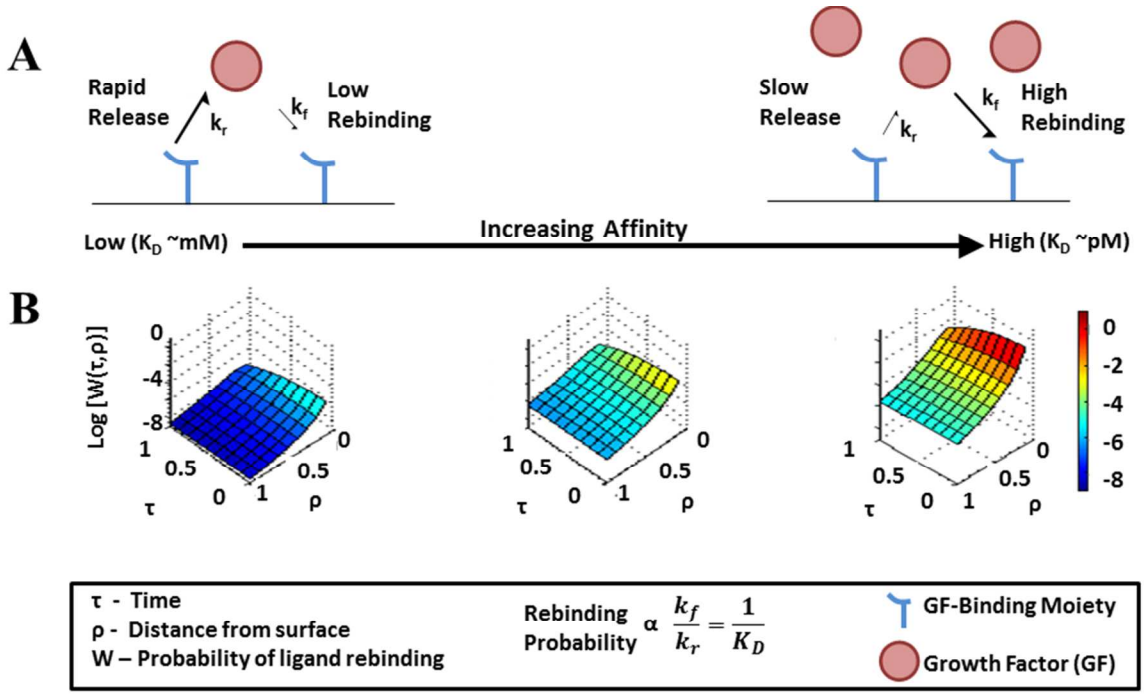

Figure 2. Rebinding probability influences GF release from and rebinding to a surface with surface-immobilized GF-binding moieties. A: Surfaces presenting low affinity GF-binding moieties exhibit rapid GF release accompanied by low GF rebinding, resulting in low enrichment of the GF at the surface. Conversely, surfaces presenting high affinity ligands exhibit slowed release rates and high rebinding, effectively enriching the GF at the surface. B: Rebinding probability (W) in dimensionless space and time as a function of dimensionless GF-binding moiety concentration (ρ) and dimensionless time (τ). With decreasing equilibrium dissociation constant (K_D) of the GF-GF binding moiety interaction (suggesting increasing affinity), the probability of GF rebinding after initial release is drastically increased in dimensionless time and space[169].

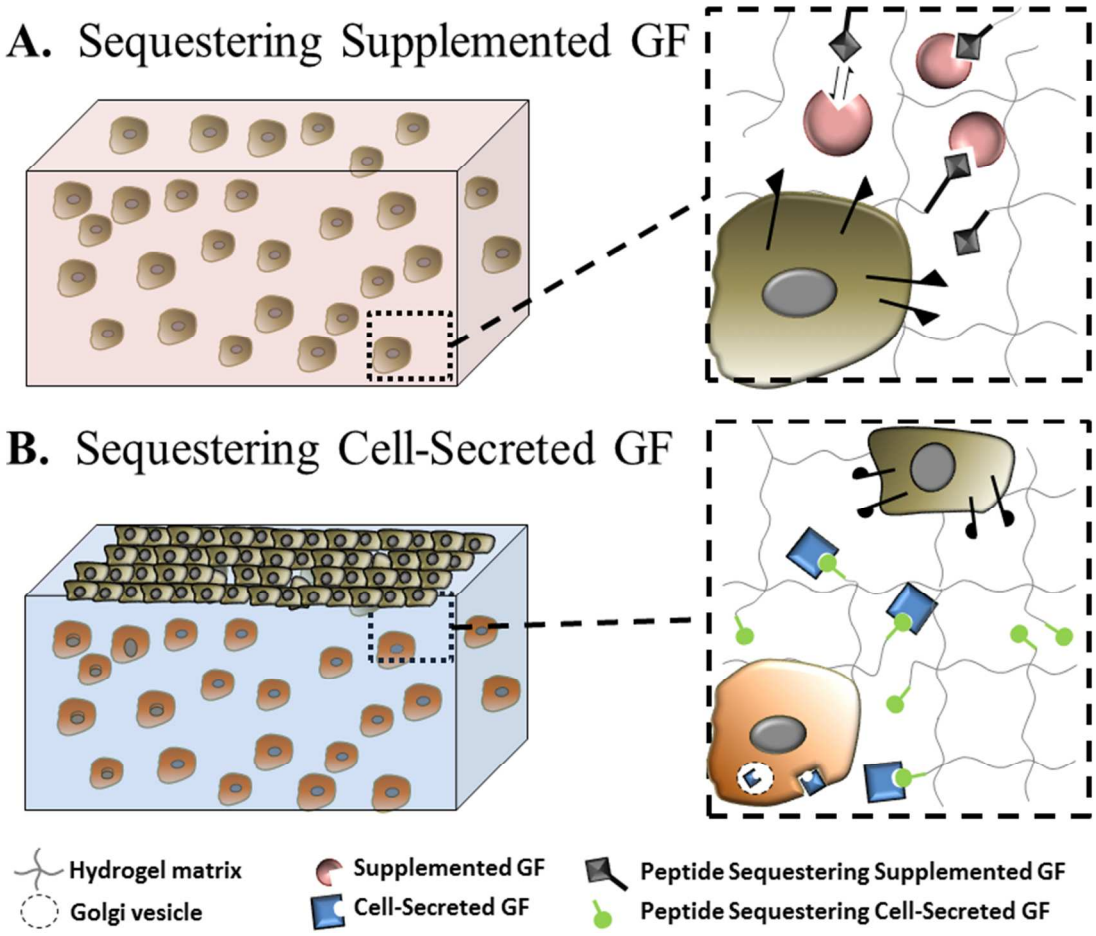

Figure 3. Context-dependent leveraging of molecular sequestering in hydrogels. A: Hydrogels containing GF receptor-mimicking peptides sequester supplemented exogenous GF and prevent receptor activation on the cell surface by blocking the GF active site. This concept has been demonstrated in hydrogels employing mimicry of CCR2 that were shown to sequester and prevent cell response to MCP-1 [195]. B: Cells encapsulated in a hydrogel with GF receptor-mimicking peptides. Hydrogels containing GF receptor-mimicking peptides sequester GFs secreted by encapsulated cells. Sequestering via the GF active site inhibits GF receptor activation on cells located outside of the hydrogel. This concept has been shown with an immunomodulatory GF to demonstrate the ability to modulate the immune response upon implanting a hydrogel containing encapsulated cells[196].

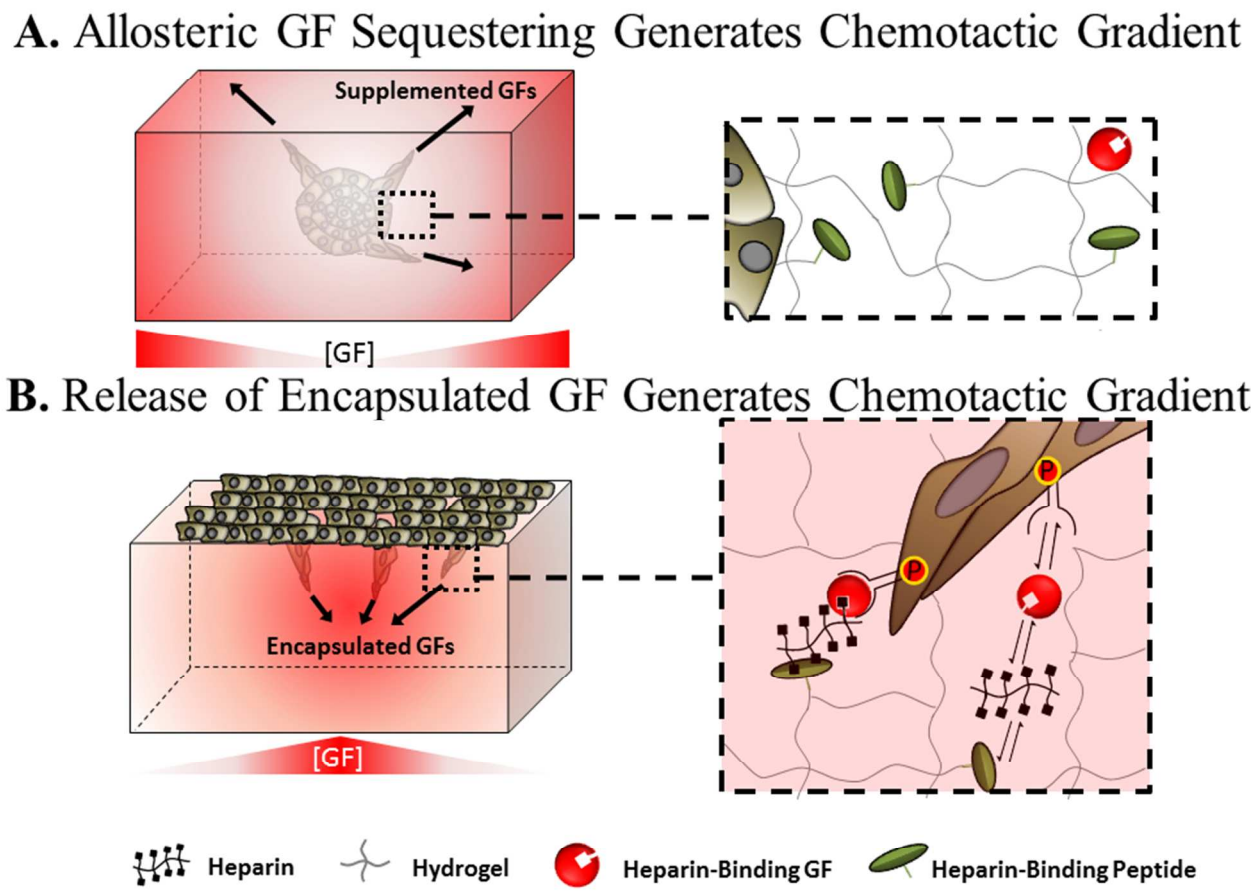

Figure 4. Enhancing cell invasion via heparin-mediated GF sequestering. A,B: Heparin-mediated GF sequestering in hydrogels with tethered heparin-binding peptide. Heparin-mediated allosteric GF sequestering in the native ECM can up-regulate GF receptor activation by i. enhancing the affinity of the GF-GF receptor interaction and by ii. generating chemotactic gradients at equilibrium during GF sequestering (A) or release of encapsulated GFs (B). This hypothesis is supported by modeling approaches which demonstrate that GF sequestering may limit the diffusion of proteins by multiple orders of magnitude through a hydrogel containing GF-binding moieites[178]. A: In 3D, heparin-mediated GF sequestering enhances GF-mediated cell sprouting. This concept has also been demonstrated with fibronectin-mimicking peptides to enhance invasion of encapsulated cells[197]. B: Within 3D constructs, heparin-mediated GF sequestering can enhance cell invasion *in vivo* by up-regulating GF-dependent cell processes such as neurite extension[203] and angiogenesis[159] upon implantation.

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Acknowledgments

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