Rational design and protein engineering of growth factors for regenerative medicine and tissue engineering

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Abstract
Growth factors provide key instructive cues for tissue formation and repair. However, many natural growth factors are limited in their usefulness for tissue engineering and regenerative applications by their poor retention at desired sites of action, short half-lives in vivo, pleiotropic actions and other features. In the present article, we review approaches to rational design of synthetic growth factors based on mechanisms of receptor activation. Such synthetic molecules can function as simplified ligands with potentially tunable specificity and action. Rational and combinatorial protein engineering techniques allow introduction of additional features into these synthetic growth molecules, as well as natural growth factors, which significantly enhance their therapeutic utility.

Introduction
Therapeutic induction of tissue repair or replacement growth in regenerative medicine and tissue engineering requires provision of instructive bioactive signals. Crucial among these signals are growth factors, such as VEGF (vascular endothelial growth factor), EGF (epidermal growth factor) and FGF (fibroblast growth factor), which provide vital stimulation of, as appropriate, cell recruitment, proliferation, morphogenesis and differentiation [1]. The first use of growth factors in regenerative medicine entailed bolus injections of soluble natural ligands, and this was effective to some extent in stimulating new tissue formation in animal studies, e.g. induction of blood vessel formation by direct injection of VEGF [2]. However, this early promise has generally not been realized in large controlled studies with patients [3]. It has become clear that, to induce functional tissue repair and growth, more sophisticated approaches are required, involving ways to retain growth factors at the required sites of action and more controlled spatial and temporal delivery of mixtures of growth signals [1]. There are a number of approaches being pursued to achieve these aims, including protein engineering of growth factors, synthesis and modification of scaffold material and gene therapy [4]. The present review focuses on aspects of design and engineering of regenerative growth factors.

For regenerative applications, growth factors would ideally be stable, permit retention at sites of action, be easy to produce and have specific controllable activity and no side effects. Many naturally occurring growth factors do not readily lend themselves to use in regenerative medicine as they lack, for example, native retention mechanisms, are prone to degradation or have diverse multiple activities. This has led to the use of protein engineering to construct new ligands and modify natural growth factors for therapeutic applications. In one approach, the molecular mechanisms involved in activation of growth factor receptors is exploited to design synthetic growth ligands. Such synthetic ligands can provide simpler, easier to produce and more readily adaptable alternatives to natural growth factors.

Designed synthetic growth factors
Most growth factors act through RTKs (receptor tyrosine kinases) to induce their cellular effects. Binding of the growth factor to its cognate receptor activates phosphorylation of the receptor intracellular domains on tyrosine residues and initiation of downstream signalling cascades that ultimately result in modification of cellular functions [5,6]. A key step in activating RTKs is ligand-induced oligomerization or clustering of the receptors [6,7]. This triggers transphosphorylation between the intracellular domains of receptors, resulting in marked activation of their kinase activity and permitting further intracellular domain phosphorylation [6,7] (Figure 1). The minimal activated unit for most RTKs is a dimer, although some RTKs require higher-order oligomerization for activation, e.g. the angiopoietin receptor Tie2 needs a minimum tetrameric conformation for activation in endothelial cells [8]. This mechanism of receptor activation by oligomerization implies that induction of receptor clustering by means other than the natural ligand may be sufficient to stimulate receptor activity, signalling and cellular effects.

Confirmation that receptor clustering can be utilized to design synthetic ligands capable of activating growth factor...
Figure 1 | RTK activation

(A) Schematic representation of initial stages in RTK signalling. Growth factor ligand triggers receptor dimerization and intracellular domain transphosphorylation (indicated by a black arrow). Phosphorylation of key tyrosine residues in the activation domains of the tyrosine kinase increases kinase activity and stimulates phosphorylation of tyrosine residues elsewhere in the intracellular domain, creating binding sites for recruitment of intracellular signalling proteins. Phosphorylation and/or modulation of activity of these signalling intermediates by the receptors and/or other recruited proteins initiate downstream signalling cascades which result in changes in cellular functions. (B) Schematic illustration of synthetic growth factor comprising oligomerization, linker and receptor-binding modules. In this example, the oligomerization scaffold is a dimeric coiled-coil which allows assembly of a bivalent ligand.

receptors has been obtained with the construction of synthetic agonists for FGF and insulin receptors that activate the receptors by inducing oligomerization [9,10]. These proof-of-concept studies open the way for design and construction of other regenerative synthetic ligands exploiting receptor activation by oligomerization. De novo–designed synthetic ligands, such as those described for the FGF receptor, require three component modules, a specific receptor-binding module, an oligomerization scaffold and linkers connecting binding and oligomerization domains (Figure 1).

Receptor-binding module

For many growth factors, the receptor-binding domain has been identified and represents a distinct motif which can be used as a receptor-binding module in a designed synthetic ligand. For example, the receptor-binding sequence within Ang1 (angiopoietin 1) is located in the fibrinogen-related domain of the ligand, and this domain has been placed within a heterologous oligomerization scaffold to produce a novel synthetic ligand for the Ang1 receptor [11]. The receptor-binding module need not be derived from the natural ligand. Short peptides retrieved from phage display libraries by the ability to specifically bind target receptor have been used as receptor-binding modules in synthetic ligands for FGF and insulin receptors [9,10]. Advantages of using peptides is their small size, which can help to reduce the overall dimensions of the synthetic ligand, and their often minimal or simple secondary structure which can be advantageous for expression and purification of the ligand. In addition, DNA- or RNA-based aptamers, or even small chemical moieties with specific receptor-binding characteristics, could be also used as receptor-binding modules. An advantage of utilizing a peptide or other molecule distinct from the native ligand is that it could bind outside the normal ligand-binding site on the receptor and therefore would not be subjected to competition by other receptor agonists and antagonists. The Ang1 receptor, for example, promotes blood vessel stability in response to Ang1, and this is antagonized by Ang2 (angiopoietin 2) which is expressed in remodelling situations and some disease states [12]. A synthetic ligand capable of activating the receptor even in the presence of elevated Ang2 would be of benefit in maintaining vessel stability.

Oligomerization scaffolds

As with the receptor-binding module, oligomerization scaffolds can be protein- or non-protein-based. Protein oligomerization scaffolds include coiled-coil domains, e.g. the pentameric COMP (cartilage oligomeric matrix protein) coiled-coil used in COMP-Ang1 [11] and the dimeric leucine zipper from c-Jun used to construct the synthetic FGF ligand [9]. Additional approaches to oligomerization include the use of chemical scaffolds and even disulfide links such as in the insulin receptor synthetic ligand [10]. DNA can also be used as an oligomerization scaffold to present receptor-binding domains in multivalent ligands [13]. Considerations in choosing appropriate oligomerization scaffolds include size and stability of the scaffold, ease of production and simplicity of fusion with receptor-binding modules.

Linkers

The sequence connecting the receptor-binding modules to the oligomerization scaffold has the potential to impact critically on functionality of a synthetic multivalent ligand. Clearly, the dimensions of these linkers are important in allowing the receptor-binding modules to span the distance required for
receptor oligomerization. Furthermore, flexibility of linkers would be anticipated to have a significant impact on ligand function. Use of non-flexible linker sequences would require some knowledge of the molecular dimensions required for productive receptor–receptor interactions, details of which are not known for most RTKs. Intriguingly, studies with rigid multivalent ligands clustering IgE receptors demonstrate that ligands with different fixed spacing between receptor-binding motifs activate different intracellular signalling events through the same receptor [13]. This finding of differential signal activation by rigid multivalent ligands illustrates the potential of synthetic ligands as probes for understanding molecular mechanisms of receptor activation and downstream signalling.

**Engineering improved functionality into growth factors**

As indicated above, utility of growth factors, whether synthetic or natural, in regenerative applications would be enhanced by increased retention at sites of required activity, increased stability and a number of other features. Some of these modifications can be incorporated into synthetic growth factors, as well as natural growth factors, by protein engineering and are considered below.

**Ligand retention**

Crucial for effective action of growth factors in a regenerative setting is the ability to retain the ligand at the site of repair or on the tissue engineering scaffold. One approach to this is to incorporate retention or immobilization motifs into the ligand. The effectiveness of this strategy is illustrated in studies in which the CBD (collagen-binding domain) from collagenase was fused to the N-termini of EGF and FGF [14]. Incorporation of the CBD increased retention of the growth factors at the site of injection over a 10-day period and enhanced FGF mitogenic activity in mice [14]. Similarly, fusion of the CBD from fibronectin on to VEGF enhanced the ability of the ligand to induce endothelial progenitor cell mobilization in an animal model, and CBD fusion with HGF (hepatocyte growth factor) significantly increased HGF-induced neovascularization in vivo [15,16]. Other domains or motifs that have been utilized to immobilize growth factors include hexahistidine and cysteine tags, and incorporation of Ig Fc sequences [17–19]. Some endogenous growth factors, such as basic-FGF and certain VEGF isoforms, bind extracellular matrix and are released in response to local proteolytic activity that accompanies tissue growth and remodelling [20,21]. This type of cell-induced release can be reproduced in engineered growth factors by incorporation of protease-sensitive immobilization motifs [22]. For example, addition of a plasminogen-derived peptide to VEGF 121 allows its incorporation and immobilization in fibrin while providing regulated localized release in response to cellular proteolytic activity [23].

Although growth factor immobilization motifs increase local retention and activity in tissue engineering scaffolds and in vivo repair sites, it should be noted that immobilization can also have an impact on the signalling and functional effects of growth factors. Studies, predominantly with cultured cells, have shown that immobilized growth factors often demonstrate quantitative, temporal and even qualitative differences in signalling and functional effects compared with their soluble counterparts [24]. Immobilized growth factors can often activate cell signalling and functional effects more potently than soluble factors; insulin and EGF, for example, show substantially increased mitogenic activity when immobilized [25]. This apparent increased potency is likely to be related to the high local concentrations which can be achieved by the immobilized growth factors. In contrast with soluble ligands, tethered growth factors would be unable to undergo endocytosis, and therefore, if not released by the receptor, tethering would maintain receptor signalling at the plasma membrane over a prolonged period as well as decreasing depletion of ligand [25]. This has the potential to produce qualitative differences in effects of the ligand on cell function, such as those observed for EGF action on PC12 cells as a result of its immobilization. Soluble EGF causes transient activation of MAPK (mitogen-activated protein kinase) signalling in these cells and cell proliferation; however, the immobilized ligand induces prolonged activation of signalling and longer-term MAPK activation, which results in cell differentiation [26].

**Expression**

Production of growth factors for therapeutic use can become rate-limiting if the recombinant proteins are difficult to produce as active proteins, and this is another feature that can be engineered in synthetic ligands as well as those derived from natural growth factors. Clearly, for a protein-based synthetic ligand, the expression system should be factored into the ligand design and the molecule optimized for expression where possible by appropriate codon usage and attention to any necessary post-translational modifications. It is also possible to optimize synthetic and naturally derived proteins for expression by using methods of protein engineering, such as directed evolution, which have been shown to be suitable for producing protein variants with increased expression [27]. Where particular structural features are identified as having a negative impact on expression of functional protein, it may be possible to replace these with alternative functionally equivalent modules. Native Ang1 is difficult to produce as an active recombinant protein as the N-terminal superclustering and coiled-coil domains, although essential for activity, cause excessive aggregation. A much more soluble variant of Ang1 with improved bioactivity, COMP–Ang1, has therefore been engineered by replacement of the native coiled-coil motif with an alternative motif from a different protein, COMP [11].

**Pharmacokinetics and immunogenicity**

Increased stability and resistance to proteolysis in vivo can be engineered into proteins that have identifiable proteolytically sensitive regions by replacement of those regions with functionally equivalent proteolytically resistant modules. Computational approaches have been used to help to design
increased stability of proteins [28] and, although such approaches are still somewhat limited, could contribute to design of more stable ligands. Where sequence information is available for families of proteins, this can sometimes be used to design increased stability, or indeed other activities such as enhanced binding or expression. This approach acknowledges that evolution has selected residues best fitted for stability, or other functions. Such an approach has been used to design acidic FGF with significantly improved thermal and proteolytic stability [29]. Where rational design or substitution of modules is not possible, directed protein evolution approaches or combinatorial approaches selecting for variants with increased stability can be used [30,31]. At present, there are few options to engineer low immunogenicity into proteins. Those most similar to endogenous proteins are least likely to elicit an immune response. In some cases, immunogenicity can be decreased by removal of particular epitopes [32] or obscuring potential antigenic determinants with carbohydrate by incorporating glycosylation sites, a strategy that can also enhance stability and half-life in vivo [33].

**Binding and receptor trafficking**

Binding of ligand to receptor is the initial event in activation of growth factor signalling pathways. This suggests that maximizing the receptor-binding ability of the synthetic ligand for engineered natural growth factor variants may lead to increased potency of the molecule. For synthetic ligands containing a receptor-binding module different from the natural ligand, selection for maximal receptor binding can be incorporated in the strategy for deriving the module, e.g. screening peptide display libraries for high-affinity receptor binding. With ligands incorporating receptor-binding domains from the natural ligand, these proteins can be engineered for high-affinity binding by, for example, directed evolution approaches [34]. However, it is important to note that high-affinity receptor interaction does not necessarily translate into increased biological activity. This is illustrated well by studies in which variants of growth hormone were engineered with more than two orders of magnitude increased affinity for their receptor compared with wild-type ligand, but showed no elevation in proliferative effect [35].

It is likely that increased internalization of the activated ligand–receptor complex limits its ability to signal [36].

Receptor trafficking itself can be leveraged for activity gain in the design of engineered growth factors [37]. For many growth factors, binding and activation of their cognate receptors results in increased internalization of the ligand–receptor complex [38]. Ligand released from the internalized receptor is usually recycled out of the cell, whereas ligand that remains tightly bound to the internalized receptor generally undergoes degradation with the resulting depletion in extracellular ligand concentration and loss of receptor [38]. This suggests that growth factors engineered to have enhanced binding at the cell surface and decreased binding intracellularly would be recycled and demonstrate increased potency due to decreased depletion. Indeed, granulocyte colony-stimulating factor rationally engineered to have high receptor binding at extracellular pH and decreased receptor binding at the lower pH found in the endosomal compartment has substantially increased half-life and significantly increased functional effects [39]. Recognition of the limitation on signalling imposed by receptor internalization can provide innovative engineering solutions to increase ligand activity. For example, EGF variants engineered for decreased receptor affinity demonstrate enhanced mitogenic effects because of the decrease in receptor–ligand internalization and consequently lower rate of ligand and receptor depletion [40].

**Conclusions**

Growth factors are key components of any regenerative strategy for therapeutic repair or replacement of tissue. Natural growth factors, however, are often limited in stability, ease of production and specificity of action and rationally designed synthetic growth factors offer potentially attractive alternatives. Proof-of-concept studies have established the feasibility of exploiting receptor activation by clustering in design and construction of simplified synthetic ligands for growth factor receptors. Careful selection of the receptor-binding and other modules making up synthetic ligands offer the potential for highly specific binding and activity. Furthermore, rational and combinatorial protein engineering can be applied to both natural growth ligands and synthetic agonists to generate variants with enhanced potential for use in tissue engineering and regenerative medicine. It is anticipated that these engineering approaches will contribute to an increasing number of new therapeutics rationally designed to exploit biological mechanisms.

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**References**


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