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Journal
Biomaterials Science, 6(5)

ISSN
2047-4830

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Publication Date
2018-05-01

DOI
10.1039/c7bm01052d

Peer reviewed

Published in final edited form as:

Biomater Sci. 2018 May 01; 6(5): 1076–1083. doi:10.1039/c7bm01052d.

Multivalent Conjugates of Basic Fibroblast Growth Factor Enhance In Vitro Proliferation and Migration of Endothelial Cells

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Abstract

Growth factors hold great promise for regenerative therapies. However, their clinical use has been halted by poor efficacy and rapid clearance from tissue, necessitating the delivery of extremely high doses to achieve clinical effectiveness which raises safety concerns. Thus, strategies to either enhance growth factor activity at low doses or to increase their residence time within target tissues are necessary for clinical success. In this study, we generated multivalent conjugates (MVCs) of basic fibroblast growth factor (bFGF), a key growth factor involved in angiogenesis and wound healing, to hyaluronic acid (HyA) polymer chains. Multivalent bFGF conjugates (mvbFGF) were fabricated with minimal non-specific interaction observed between bFGF and the HyA chain. The hydrodynamic radii of mvbFGF ranged from ~ 50 to ~75 nm for conjugation ratios of bFGF to HyA chains at low (10:1) and high (30:1) feed ratios, respectively. The mvbFGF demonstrated enhanced bioactivity compared to unconjugated bFGF in assays of cell proliferation and migration, processes critical to angiogenesis and tissue regeneration. The 30:1 mvbFGF outperformed the 10:1 conjugate, which could be due to either FGF receptor clustering or interference with receptor mediated internalization and signal deactivation. This study simultaneously investigated the role of both protein to polymer ratio and multivalent conjugate size on their bioactivity, and determined that increasing the protein-to-polymer ratio and conjugate size resulted in greater cell bioactivity.

Graphical Abstract

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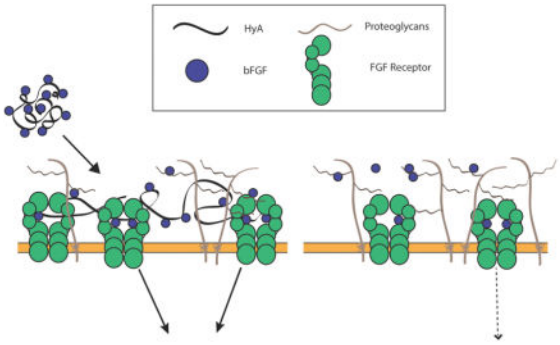
Disclosure Statement

KEH and WMJ have financial relationships with Valitor Inc., and both they and the company may benefit from commercialization of the results of this research.



Keywords

growth factor therapy; basic fibroblast growth factor (bFGF); multivalent conjugates; hyaluronic acid (HyA); regenerative engineering

Introduction

Growth factors are critical signaling molecules that control a wide variety of cellular functions including growth, migration, differentiation, apoptosis and metabolism. *In vitro* studies have repeatedly demonstrated the power of growth factors in regenerative medicine.[1,2] However, as of 2015 only two growth factor-based drugs were available for clinical use in the US market:[3–5] keratinocyte growth factor (KGF), and platelet-derived growth factor (PDGF). The lack of growth factor-based therapies that have progressed to the clinic has mainly been attributed to their short half-life, which is typically on the order of minutes, and poor retention kinetics within target tissues.[2,6] This necessitates the use of higher doses, as well as repeated dosing, all of which significantly increase drug costs and engender potential safety risks. In fact, a link between administration of 0.1% PDGF-BB (becaplermin) and an increased risk of cancer mortality resulted in a 'black-box' warning from the FDA for this product.[7] These issues and statistics clearly demonstrate that the therapeutic potential of growth factors has failed to reach the clinic.

Biomaterial-based strategies have been proposed as a means of improving the efficacy of growth factor based therapies.[8–13] Biomaterial systems can enhance growth factor activity over bolus delivery by acting as a delivery reservoir, protecting the growth factor from degradation, and increasing its retention in the microenvironment by prolonging the release kinetics.[14] A range of biomaterial systems have been developed to enhance delivery of growth factors, including solid scaffolds, hydrogels particles and composite systems.[15–17] Hyaluronic acid (HyA), a non-sulfated glycosaminoglycan (GAG) found in the extracellular matrix (ECM), has been identified as a potential material for drug delivery applications, due to its versatility and ease of modification.[18,19] HyA-based hydrogels,[20,21] particles[22] and combinations thereof[23] are capable of loading and releasing bioactive growth factors, thus enhancing their activity.

An alternative biomaterials approach involves the conjugation of multiple bioactive proteins/peptides to long-chain biopolymers. The increase in molecular weight of the therapeutic due

to biopolymer conjugation can increase residence time in the microenvironment,[24] while multivalent conjugation can also protect the conjugated protein from enzymatic degradation.[25] We have also shown that multivalent conjugation can enhance the bioactivity of conjugated proteins in comparison with unconjugated protein.[26,27] Thermodynamic modeling suggests that multivalent ligand presentation can improve the likelihood of repeated ligand-receptor binding following initial interaction of a ligand with the cell surface,[28] and that multivalent proteins conjugates can enhance receptor clustering.[27] Thus, enhancing the bioactivity of a protein via multivalent conjugation could reduce the effective therapeutic dose required and could potentially facilitate clinical translation.

In this study, we assessed the effect of multivalent conjugation on the biological function of basic fibroblast growth factor (bFGF). bFGF is an important molecule that has demonstrated its usefulness in terms of regenerative medicine in a broad range of applications.[29,30] As with previous proteins that demonstrated enhanced bioactivity after multivalent conjugation,[26,27] oligomerization of bFGF is an endogenous mechanism to modulate its function *in vivo*.[31] Dimerization is key to bFGF activity, as it leads to FGFR dimerization and activation.[31,32] In fact, a number of other dimerization strategies have led to increased bFGF activity *in vitro*[33] and *in vivo*.[34] The mechanisms of cellular activation via receptor tyrosine kinases (RTKs) and endocytosis-mediated signal deactivation have also been well characterized for the bFGF pathway.[35,36] Therefore, multivalent conjugation of bFGF could potentially enhance its bioactivity by clustering and activating FGFRs and/or by reducing the endocytosis rate of activated receptors.

To test whether multivalent conjugates of bFGF (mvbFGF) exhibited greater cellular bioactivity compared to unmodified bFGF, we synthesized and rigorously characterized mvbFGF with two different protein-to-polymer ratios by conjugating bFGF to HyA chains. The effect of conjugation valency and size was simultaneously investigated using *in vitro* assays examining endothelial cell proliferation and migration.

Materials and Methods

Protein Synthesis (Cys-bFGF)

We engineered a Cys-tagged bFGF double mutant in which the external Cys residues were substituted to Ser (i.e., C85S/C96S) based on a publicly available sequence,{1368488; 8575426} to which we appended a C-terminal cysteine for site specific conjugation (Cys-bFGF, synthesized at the QB3 Macrolab, UC Berkeley). Specifically, the bFGF sequence used was:
AAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDP HIKLQLQAEERGVVSIKGV**S**ANRYLAMKEDGRLLASK**S**VTDECFFFERLESNNYNT YRSRKYTSWYVALKRTGQYKLGSKTGPGQKAILFLPMSAKS. The bioactivity of the double mutant bFGF was shown to be no different to commercial bFGF over a range of concentrations in an in vitro HUVEC proliferation assay (Figure S1).

Multivalent conjugation of bFGF

Multivalent conjugates of bFGF (mvbFGF) were fabricated by modifying a previously described method.[25–27,37] Briefly, HyA (MW 200 kDa) was dissolved in MES buffer (0.1M, pH 6.5) at 4 °C at a concentration of 3 mg/mL. Next, 10 mg/mL EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide), 0.3 mg/mL sulfo-N-hydroxysuccinimide (NHS), and 1.2 mg/mL EMCH (*N*-(ε-Maleimidocaproic acid) hydrazide) were added to the solution, and reacted for 4 hours at 4 °C to synthesize HyA-EMCH. Samples were then dialyzed twice using 100kDa MWCO dialysis membrane (Spectrum) against PBS buffer (10% glycerol in DPBS) for a minimum of 4 hours each. Cys-bFGF was reacted with the HyA-EMCH in DPBS solution (10% glycerol) at molar feed ratios of 10:1 or 30:1 of HyA to bFGF overnight at 4 °C. The resulting high conjugation ratio (HCR) and low conjugation ratio (LCR) mvbFGF conjugates were then exhaustively dialyzed three times using 100kDa MCWO dialysis membrane for a minimum of four hours each to remove unreacted bFGF. The concentration of mvbFGF conjugates was determined using the Pierce BCA™ Protein Assay (Thermo Scientific) as per the manufacturer's instructions, before being aliquoted and frozen down to −80°C. All samples were sterilized by filtration using a 0.22-μm syringe filter immediately before using them in biochemical or cell biology assays.

SDS-PAGE analysis of bFGF conjugates

Samples were prepared with 5X SDS dye loading buffer, 2-mercaptoethanol and boiled for 5 minutes at 95°C. Precast Mini-Protean TGX 4–20% gradient gels (Bio-Rad Laboratories) were run for 90 minutes at 110 volts. Gels were stained with Bio-Safe Coomassie Stain (Bio-Rad Laboratories) for 2 hours and then imaged using a BioRad Molecular Imager ChemiDoc XRS+. The intensities of bFGF in the stacking and gradient gel were analyzed using ImageJ to determine the amount of bFGF that was conjugated versus free unconjugated bFGF that did not dialyze out of solution following the conjugation reaction.

SEC-MALS analysis of mvbFGF conjugates

Protein conjugation was characterized using size exclusion chromatography (Agilent HPLC 1100 series) with multi-angle light scattering (SEC-MALS) as described previously.[38] Shodex OH pak SB-804 HQ columns were used for separation (Phenomenex Inc.), and then we analyzied the protein and HyA contribution to mvbFGF conjugates using light scattering and refractive interferometry (DAWN-HELEOS II and Optilab T-rEX, respectively, Wyatt Technology). The radius of gyration (Rg) values were calculated by obtaining a linear regression in the form of RMS radius (R) = kM^b, where M was the molar mass, from the conformation plots for two online runs of the unmodified 200 kDa hyaluronic acid.[39] The resulting equation was used to calculate the predicted linear HyA Rg value at the M value equivalent to M_w for each of the conjugates.

Size Characterization of mvbFGF

Dynamic light scattering (DLS) was used to measure the hydrodynamic diameter of MVCs using a Goniometer & Laser Light Scattering system (BI-200SM Laser Light Scattering Instrument, Brookhaven Instruments Corporation). Data acquisitions were realized at an angle of 90 degrees, using a 637nm laser, for 2 minutes. Data analysis was carried out with

the BIC Dynamic light Scattering Software (supplied by Brookhaven) using a BI-9000AT signal processor. The intensity average particle size was obtained using a non-negative least squares (NNLS) analysis method.

HUVEC Proliferation Assay

Human umbilical cord vein endothelial cells (HUVEC) were purchased from ATCC and cultured in EGM-2 media (Lonza) in a humidified incubator at 37°C and 5% CO_2. HUVEC were seeded at a density of 5000 cells/ well in a 96-well plate. After 24 hours, the EGM-2 was removed, the cells rinsed twice with PBS and M199 media supplemented with 1% FBS. Cells were then treated with one of the following: (1) unconjugated bFGF in M199 media with 1% FBS; (2) mvbFGF 10:1 in M199 media with 1% FBS; and, (3) mvbFGF 30:1 in M199 media with 1% FBS. For each treatment (conjugated and unconjugated), a range of bFGF concentrations were tested over a range that was used to assess its bioactivity using HUVECs in previous studies (0, 5, 10, 25, 50, and 100 ng/mL).[35] After 72 hours, the media was aspirated, the plate rinsed with PBS and frozen for analysis of cell proliferation with the CyQUANT assay kit (Invitrogen), following the manufacturer's instructions. The data was normalized to the cell number in the control well after 72 hours (no bFGF or mvbFGF added).

HUVEC Metabolic Activity Assay

HUVEC were seeded at a density of 5000 cells/well in a 96- well plate. After 24 hours, the media was removed and the cells were rinsed twice with PBS and M199 media supplemented with 1% FBS. The cells were then treated with one of the following: (1) unconjugated bFGF in M199 media with 1% FBS; (2) mvbFGF 10:1 in M199 media with 1% FBS; (3) mvbFGF 30:1 in M199 media with 1% FBS. After 72 hours, cell metabolic activity was assessed using the Alamar Blue assay kit (Invitrogen, Carlsbad, CA). The data was normalized to the control well (no bFGF or mvbFGF added).

HUVEC Migration Assay

Wells of a 12-well plate were coated with 0.2% gelatin. HUVECs were seeded at a density of 150,000 cells/well 24 hours prior to starting the assay. Using a 1 ml pipette tip, crosses were scratched into the confluent layer of HUVECs. The wells were then washed with excess PBS to remove cell debris and media and replaced with M199 with 1% FBS, containing either (1) unconjugated bFGF, (2) conjugated bFGF 10:1, or (3) conjugated bFGF 30:1, all at a concentration of 50 ng/ml. Scratches were imaged at 0 and 24 hours post scratch and the area without cells was quantified using ImageJ. The percent open wound area was calculated by comparing the open scratch area at 24 hours to the open scratch area at 0 hours. The change in scratch area between 0 and 24 hours for each treatment group was compared with untreated scratches.

Statistical analysis

GraphPad Prism® (v.5 GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. Analysis of variance (ANOVA) was used followed by Tukey's *post-hoc* test to

determine statistical significance between groups. All graphical data is presented as mean ± standard deviation. p values of < 0.05 were considered statistically significant.

Results and Discussion

We employed a strategy of modifying the bFGF amino acid sequence to ensure site-specific conjugation of a double mutant bFGF to activated HyA. A similar bioorthognal approach has been used to conjugate alkyne-functionalized bFGF to azide and hydroxyl functionalized agarose beads in a site-specific manner.[40] Multivalent bFGF conjugates were synthetized using a two-step reaction (Figure 1). By varying the feed ratio of cys-bFGF to activated HyA, conjugates of two different protein-to-polymer ratios (10:1 and 30:1) were synthesized to examine the effect of bFGF valency on the biological function of conjugated bFGF. We confirmed a stable, covalent conjugation of bFGF to HyA by running the mvbFGF conjugates on a gradient acrylamide gel (Figure 2A). The migration of unconjugated bFGF into the gel was consistent with its molecular size of approximately 18 kDa. In contrast, both mvbFGF conjugates remained in the stacking portion of the gel, indicating that they were substantially larger than the unconjugated bFGF. Based on densitometry analysis, we estimated that after dialysis both mvbFGF conjugate solutions contained less than 5% unconjugated bFGF.

We then used SEC-MALS and DLS to characterize the mvbFGF conjugates as biopolymers in solution (Table 1; Figure 3). The conjugates made with a feed ratio of 10:1 had a protein-to-polymer ratio of 9.7:1 (97% conjugation efficiency), while the 30:1 conjugate had a protein-to-polymer ratio of 26.1:1 (87% conjugation efficiency). Calculation of the RMS R via SEC-MALS showed a difference in size between the two conjugates, with the 10:1 having a size of 47 nm, and the 30:1 having a larger size of 75 nm (Table 1). Data from DLS analysis demonstrated the diameter (Dh) size distribution for each sample: activated HyA pre-cursor without conjugated bFGF (HyA intermediate) at 42.6 ± 9.9 nm, mvbFGF 10:1 at 81.2 ± 14.7 nm and mvbFGF 30:1 at 155.6 ± 22.9 nm (Figure 3). These numbers broadly agree between SEC-MALS and DLS analyses. The ρ ratio ($\rho = Rg/Rh$) calculated from the SEC-MALS and DLS data is a characteristic quantity of the segment distribution,[41] such that a compact sphere would have a ratio of ~ 0.78 and linear chains in a good solvent would have a ratio ~ 1.78. MVCs had a Rg/Rh ratio of approximately 1, which was indicative of a less compacted highly branched structure. Based on the Rg/Rh measurements, these conjugate molecules are highly hydrated and flexible, and not a compact nanoparticle.

In previous studies, we have analyzed multivalent protein conjugates as branched polymers by modeling the conjugated proteins as branches emanating from the biopolymer backbone.[25,39] Based on branching analysis,[39] the number of branch units per HyA molecule was 7.5 and 14.9 for the 10:1 and 30:1 conjugates, respectively. Since MVCs behave as branched molecules, at a given molar mass, as the protein-to-polymer ratio or conjugation ratio of the growth factor increases, the molecular size (RMS radius) should decrease. This behavior is a fundamental principle behind the detection and characterization of branching,[42–44] and suggests that conjugated proteins could be treated as branches off of the polymer backbone. Based on this analysis, increasing protein protein-to-polymer ratios should have decreased the molecule size by increasing the compactness of the macromolecule. With mvbFGF, we

observed an increase in conjugate size with increasing protein-to-polymer ratios. Since the polydispersity of the mvbFGF molecules is low, the distribution of mvbFGF sizes in our observations may have included interchain interactions between conjugates mediated by the conjugated bFGF, which has a propensity to form dimers that are mediated through their heparin-binding domains.[45,46] Alternatively, the large molar mass of bFGF may have prevented compaction of the MVCs as predicted by branching analysis. Independent from any effects of bFGF dimerization, the small standard deviations observed in the DLS measurements and low polydispersity indicate a relatively homogeneous population of mvbFGF, and we did not observe any evidence of uncontrolled aggregation that would compromise their bioactivity or bioavailability.

We chose to assess the effect of multivalent bFGF conjugation using *in vitro* assays of cell proliferation and migration, two of the key processes that are critical to both angiogenesis and wound healing. The addition of bFGF, from 5 ng/ml to 100 ng/ml, significantly enhanced HUVEC proliferation compared to the control (Fig. 4A). More importantly, mvFGF showed significant improvement over the bFGF control (Fig. 4A). The 10:1 mvbFGF enhanced HUVEC proliferation at a concentration of 25 ng/ml and 100 ng/ml. In addition, the 30:1 mvbFGF enhanced proliferation over unconjugated bFGF at concentrations between 10 ng/mL and 100 ng/mL. These data emphasize the importance of valency on conjugate function, as the higher protein-to-polymer ratio (30:1) consistently increased proliferation.

Metabolic activity was also measured with HUVEC following treatment with bFGF as well as mvbFGF. In comparison with the unconjugated bFGF, conjugates at both protein-to-polymer ratios significantly enhanced metabolic activity at every concentration from 5 ng/ml to 100 ng/ml (Fig. 4B). The difference between the proliferation and the metabolic assays was attributed to the role of bFGF in upregulating glucose metabolism.[47] Although able to encourage cell proliferation, measured on the basis of total DNA content, the cells treated with unconjugated bFGF cells had low overall metabolic activity. By contrast, the signaling by mvbFGF conjugates was able to stimulate both cell proliferation and metabolism. Thus, the combination of both assays provided a more accurate analysis to compare the level of cellular activation by bFGF or mvbFGF.

To further assess the effect of conjugation on bFGF function, we utilized a scratch assay that would emphasize the role of bFGF on migration over 24 hours. As seen in Figure 5, mvbFGF had a significant effect on cell migration, as observed by increased closure of the scratch area. Again, the effect of protein-to-polymer ratios was observed in terms of the efficacy of the conjugates: both mvbFGF conjugates showed increased wound closure over the control, while the unconjugated bFGF did not. However, only the higher protein-to-polymer ratio (30:1) significantly increased closure compared to the unconjugated bFGF. This further emphasizes the importance of bFGF valency, as only the higher protein-to-polymer ratio mvbFGF showed a statistically significant effect on cell migration compared to the unconjugated bFGF.

Our findings are consistent with previous observations that multivalent growth factors can also be more potent than their corresponding monovalent forms.[26,27,37,48] The enhanced

bioactivity is attributed to the effects of receptor clustering, where multiples of the same ligand bind to cell surface receptors in proximity to one another. This type of receptor clustering is abundant in cell signaling transduction,[49,50] and some cell signaling pathways require receptor clustering in order to be activated.[51] Previously, we have shown that multivalent sonic hedgehog (Shh) exhibited greater bioactivity than the same concentration of Shh,[26] but the role of receptor clustering for the Shh cell activation pathway is currently not well understood.[47] By contrast, clustering of FGF RTKs is known have an effect specific for bFGF to amplify its cellular response, and multivalent protein conjugates have previously been shown to enhance receptor clustering.[27] Therefore, we propose that conjugation of bFGF to a HyA chain facilitates multiple bFGF binding events on the cell surface and enhances receptor clustering.

In addition to receptor clustering, multivalent conjugation of bFGF to HyA may also slow down the endocytosis-mediated attenuation of the bFGF receptor,[35,52] thereby contributing to a longer duration of cellular activation Zhang *et al.*[53] mathematically modeled endocytosis as a function of ligand-conjugated particle size and demonstrated that particles with a radius above 60nm significantly slowed cellular uptake. From the SEC-MALS and DLS data (Table 1, Fig. 3), the 1:30 conjugates have radii of above the 60 nm threshold, while the 1:10 and unconjugated bFGF have radii below 60nm. Thus, higher valency conjugates may have exhibited greater biological activity since their macromolecular size was sufficiently large to inhibit endocytosis-mediated signal attenuation, thus prolonging bFGF activity.

Based on our previous studies, we assume multiple mechanisms related to the potency and stability of MVCs contribute to their enhanced bioactivity. For example MVCs are capable of resisting enzymatic digestion of the cargo protein, which likely also plays a role in increased bFGF activity.[25] For the two mvbFGF conjugates in this study, the difference in bioactivity was statistically significant, but smaller than the differences in their macromolecular size or protein-to-polymer ratios. Thus, over the range of protein-to-polymer ratios tested in this study, the ability of mvbFGF to coordinate higher bFGF bioactivity may have been above a critical threshold for enhanced signaling, either via receptor clustering or by slowing receptor-mediated endocytosis. Identifying this threshold for higher bioactivity would have economic value in minimizing the number of bFGF molecules per chain needed to produce an enhanced effect.

Conclusion

Multivalent bFGF conjugates on HyA biopolymers, at different protein-to-polymer ratios, resulted in greater endothelial cell proliferation and migration compared to unconjugated bFGF. Increasing the protein-to-polymer ratio and conjugate size resulted in greater cell bioactivity. Based on these findings, multivalent conjugation appears capable of increasing the bioactivity of bFGF and potentially lowering its therapeutic concentration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge funding from the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number R21AR063940, the Jan Fandrianto and Selfia Halim Chair Fund (KEH), and the Irish Research Council via the ELEVATE Fellowship (ELEVATEPD/2014/33) (SB). This content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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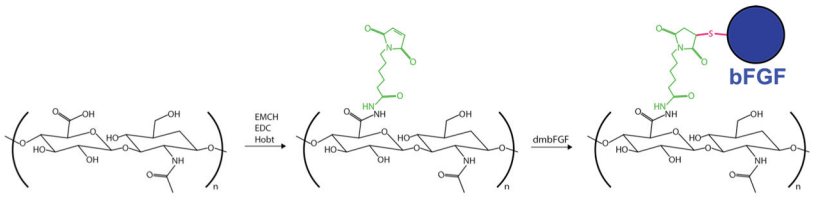

Figure 1. mvbFGF synthesis reaction

The first step is the HyA activation by adding an EMCH linker (green). The second step is the addition of the double-mutant Cys-bFGF (blue) at high conjugation ratio (HCR) or low conjugation ratio (LCR) to form the mvbFGF, followed by exhaustive dialysis to remove unreacted bFGF.

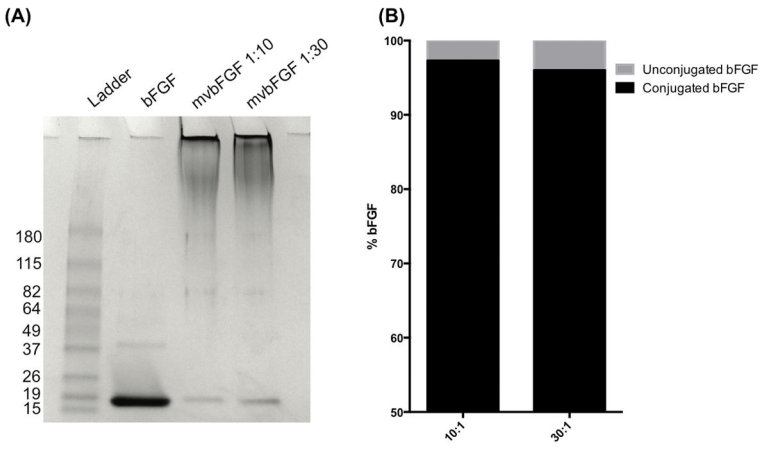

Figure 2. Minimal non-specific bFGF interaction with HyA

(A) SDS-PAGE gel visually shows that a large proportion of the bFGF is retained at the top of the gel, indicating increased size due to conjugation to HyA. A minimal proportion of the total bFGF runs into the gel. (B) Quantification of the gel indicates that less that 5% of the total bFGF runs into the gel, and thus over 95% of the bFGF is specifically bound to the HyA.

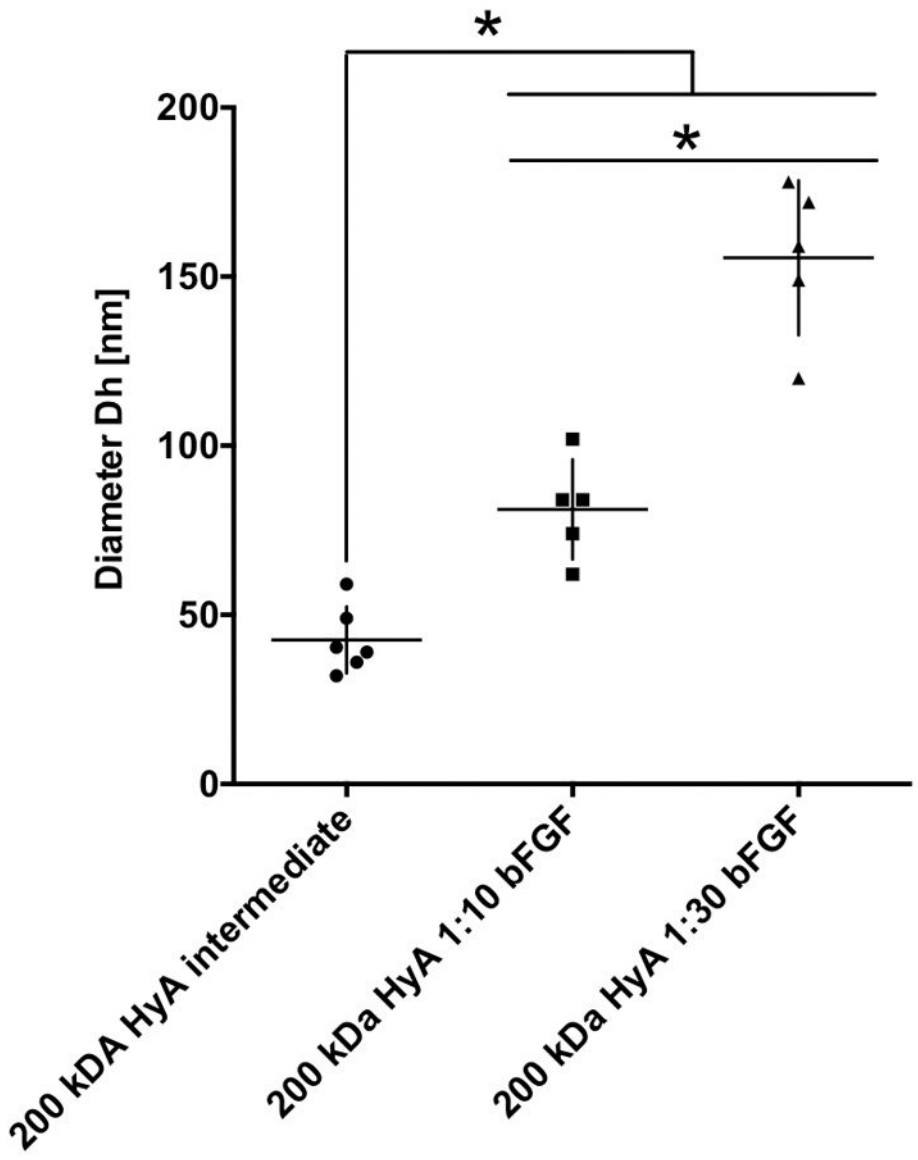

Figure 3. Change in diameter of hyaluronic acid chain due to bFGF conjugation

Measurement of multivalent conjugate diameter at two ratios (1:10 and 1:30) as compared to unconjugated Hyaluronic acid (HyA) intermediate, as measured by dynamic light scattering (DLS). * indicates statistical significance ($p<0.05$)

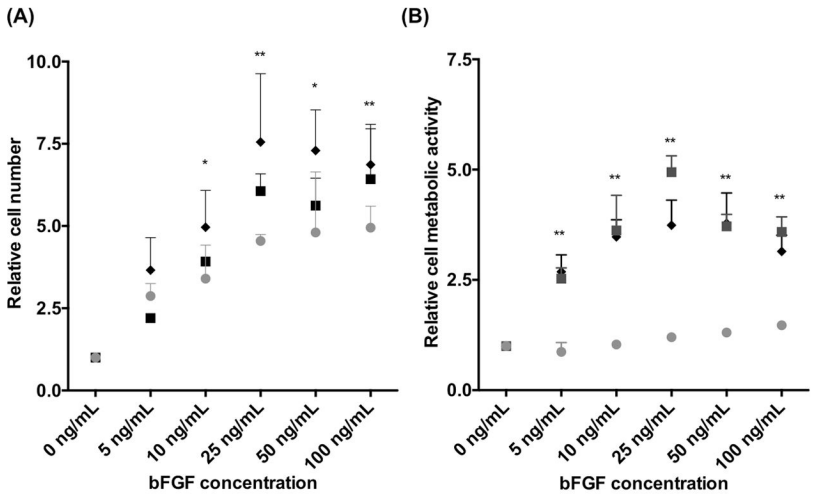

Figure 4. mvbFGF enhances HUVEC proliferation and metabolic activity

(A) Normalized cell proliferation as a function of concentration and conjugation ratio. (B) Normalized cell metabolic activity as a function of concentration and conjugation ratio. * indicates 200kDa 1:30 bFGF is significantly different than the unconjugated bFGF; ** indicates 200kDa 1:10 & 200kDa 1:30 bFGF are significantly different than the unconjugated bFGF ($p<0.05$) ●bFGF, ■1:10 mvbFGF ◆1:30 mvbFGF.

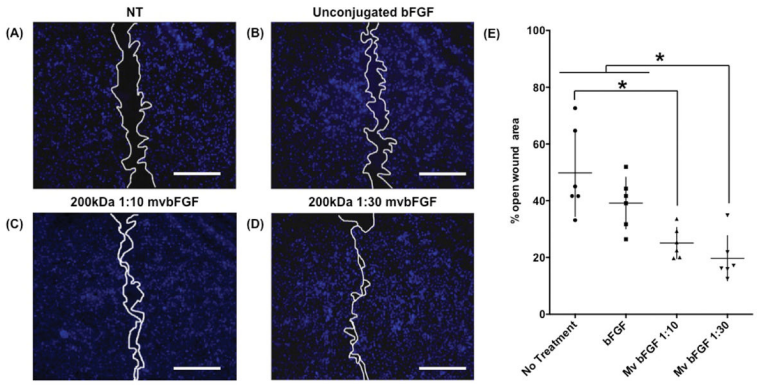

Figure 5. mvbFGF enhances scratch closure in a manner dependent on the protein-to-polymer ratio

Representative images of DAPI stained nuclei of HUVEC 24 hours after scratch and application of the following treatments: (A) No treatment, (B) unconjugated bFGF, (C) 200kD 1:10 mvbFGF and (D) 200kDa 1:30 mvbFGF. (E) Quantification of wound closure reveals that both 200 kDa bFGF at protein-to-polymer ratios of 1:10 and 1:30 significantly reduce open wound area compared with control, while 1:30 significantly enhances wound closure compared with unconjugated bFGF. * indicates statistical significance ($p<0.05$) Scale bar = 200 μm.

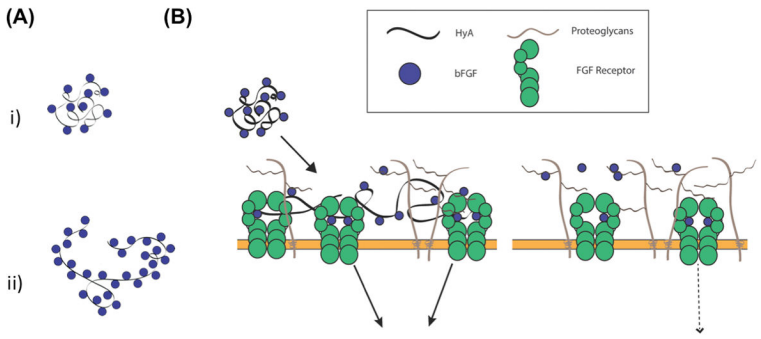

Figure 6. Schematic representation of mvbFGF form and function

(A) mvbFGF conjugates with protein-to-polymer ratios of (i) 1:10 and (ii) 1:30. (B) mvbFGF interacts with the bFGF receptors on the cell surface and following binding acts to increase local bFGF concentration, promoting increased binding of bFGF and receptor clustering, resulting in enhanced cell proliferation and migration compared to the case of unconjugated bFGF.

Table 1

Characterization of mvbFGF using SEC-MALS.

	10:1 bFGF:HyA	30:1 bFGF:HyA
Total M_n (g/mol)	4.07E5	7.50E5
Total M_w (g/mol)	4.30E5	8.89E5
Đ	1.06	1.19
RMS radius (nm)	47	75
HyA M_n (g/mol)	1.92E5	2.26E5
HyA M_w (g/mol)	1.98E5	2.62E5
bFGF per HyA chain M_n (g/mol)	2.07E5	5.21E5
bFGF per HyA chain M_w (g/mol)	2.33E5	6.27E5
bFGF:HyA	9.7	26.1
Conjugation Efficiency	97%	87%
Branch Units per Molecule	7.5	14.9