Modular Peptide Growth Factor

DOI: 10.1002/anie.200901618

Modular Peptide Growth Factors for Substrate-Mediated Stem Cell Differentiation**

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Natural proteins are often multifunctional, and therefore capable of activating cell surface receptors, and also binding with high affinity and specificity to natural extracellular matrices (ECMs). To achieve these diverse functions, a strategy commonly employed by nature involves creating modular proteins, in which distinct domains within a single protein are designed to enable either cell signaling or ECM binding. For example, modular proteins such as osteocalcin (OCN) and bone sialoprotein (BSP) contain a domain that binds to hydroxyapatite (HA)—the major biomineral component in the ECM of bony tissues—and a distinct domain that interacts with integrin receptors to mediate cell adhesion.[1] Therefore, these proteins are capable of influencing cell behavior in particular locations within an organism by virtue of their noncovalent linkage to a specific ECM material.

The mechanisms that enable the binding of signaling molecules to ECM in nature can potentially be extended to synthetic biomaterials as well. For example, a recent study indicates that it is possible to mimic nature's modular cell adhesion proteins (e.g. OCN, BSP) by engineering synthetic modular peptide molecules that bind to synthetic HA, yet remain capable of affecting cell adhesion.[2] This modular design approach has been used to promote cell adhesion to HA materials, which are now used in a wide range of common clinical orthopedic applications. However, previous studies have not yet extended this type of modular design strategy in order to noncovalently immobilize growth factors, which are capable of actively regulating stem cell phenotype.

We hypothesized that modular peptides inspired by portions of natural proteins could provide a mechanism to attach growth factors to common biomedical materials. We reasoned that the surface-immobilized peptide growth factor could then promote stem cell differentiation on the material surface. Specifically, we synthesized modular peptide growth factors, which mimic the HA-binding ability of OCN and the ability of bone morphogenetic protein-2 (BMP-2) to promote stem cell differentiation. OCN is a 5.7 kDa protein that binds to calcium in the HA crystal lattice (600 nM dissociation constant).[3] HA–OCN binding can be largely attributed a 9-mer sequence on the N terminus, which contains three γ-carboxylated glutamic acid (Gla) residues that coordinate with Ca^{2+} ions in the HA crystal.[4] Therefore, we reasoned that the N-terminal helix derived from OCN could be used as a linker to attach BMP-2 to a HA surface. BMP-2 is a 26 kDa protein that exerts its effects by stimulating differentiation of progenitor cells toward an osteoblastic lineage.[5,6] Recently Tanihara and co-workers discovered that a 20 amino acid peptide sequence from the "knuckle" epitope of BMP-2 retains the biological activity of the full-length BMP-2 protein.[7–10] Therefore, we hypothesized that a modular peptide containing an OCN-inspired portion and the 20-mer derived from the BMP-2 knuckle epitope could be used to promote substrate-mediated osteogenic differentiation of a stem cell.

We created modular peptide growth factors that contain the BMP2-derived sequence and a series of mineral-binding sequences inspired by OCN (Table 1). These modular peptides were synthesized by standard solid-phase synthesis, using Fmoc-protected amino acids, purified by HPLC, and analyzed by MALDI spectrometry (Figure S1 in the Supporting Information).

Our modular peptide growth factors contained either all three Gla residues present in native OCN, or contained substitutions of Gla residues with either Glu or Ala. We hypothesized that Glu and Ala substitutions would influence the charge density and secondary structure of the peptide molecules, and would therefore influence peptide–HA bind-

Table 1: Sequences of modular peptide growth factors and natural template.

Peptide	Amino acid sequence
human BMP-2	KIPKACCVPTELSAISMLYL (AAs: 73–92)
human OCN	γEPRRγEVCγEL (AAs: 17–25)
eBMP2[a]	KIPKASSVPTELSAISTLYL
eBGa3[b]	KIPKASSVPTELSAISTLYLAAAAγ**E**PRRγ**E**VAγ**E**L
eBGa2	KIPKASSVPTELSAISTLYLAAAAγ**E**PRRAVAγ**E**L
eBGa1	KIPKASSVPTELSAISTLYLAAAAγ**E**PRRAVAAL
eBGu3	KIPKASSVPTELSAISTLYLAAAA**E**PRR**E**VA**E**L

[a] The eBMP2 peptide sequence was originally synthesized by Tanihara and co-workers. Cys and Met from human BMP-2 sequence were replaced by Ser and Thr. [b] Cys from human OCN sequence was replaced by Ala in modular peptides to avoid complicating disulfide linkages. Amino acid residues different from the native sequences of hBMP-2 or hOCN are underlined.

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[**] We acknowledge funding from the U.S. National Institutes of Health (R03AR052893) and the Wallace H. Coulter Foundation (Translational Research Partnership).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200901618.

ing. The two components of the peptides were separated by an $(Ala)_4$ sequence, which served as both a spacer and an "extension", based on the known propensity of poly(Ala) sequences to form α helices[11] and the α-helical structure of the OCN-derived sequence in native OCN.

OCN-inspired peptide sequences within modular peptides remained capable of binding to the surface of HA, and the binding affinity was highly sequence-dependent (Figure 1 a). Binding isotherms showed concentration-dependent increases, with saturation at high peptide concentrations. The eBGa3 peptide, which included all three Gla residues present at the N terminus of native OCN, displayed by far the highest level of binding to HA (maximum (467.31 ± 64.80) nmol m^{-2}). Replacement of one or two Gla residues with Ala significantly decreased the maximum bound peptide to (163.35 ± 34.72) nmol m^{-2} or (105.2 ± 14.33) nmol m^{-2}, respectively. We also synthesized a control peptide, eBGu3, in which all the Gla residues in eBGa3 were substituted with Glu, which is a structurally similar residue containing only one side-chain carboxylic acid. Binding of eBGu3 was similar to the binding of the 20-mer BMP2-derived peptide sequence alone (eBMP2), indicating that Gla–Glu substitution hindered HA binding. Taken together, these data indicate that the Gla residues present in the OCN-inspired sequence γEPRRγEVAγEL were primarily responsible for peptide binding to HA. Interestingly, the surface coverage of the eBGa3 modular peptide on HA (3×10^{13} molecules cm^{-2}) was six times higher than the coverage of native OCN protein on HA measured previously (0.5×10^{13} molecules cm^{-2}).[12]

The sequence specificity of the binding of the peptide growth factor to HA may be attributed to a combination of the charge density and secondary structure of the engineered modular peptides. Structural analysis of eBGa3 and eBGu3 demonstrated that both molecules primarily have random coil structure in solution, even in the presence of soluble calcium ions (Figure 1 and Figure S2 in the Supporting Information). However, in the presence of HA particles the eBGa3 molecule appears to show evolution of α-helical structure; minima in the circular dichroism (CD) spectrum develop at 207 nm and 222 nm (Figure 1 b), and these minima are stronger in the eBGa3–HA spectrum than in the the eBGu3–HA spectrum (Figure S2 in the Supporting Information). Taken together, these results suggest that the combination of enhanced α-helical secondary structure and enhanced charge density of eBGa3 may stabilize binding to HA, resulting in particularly high levels of binding relative to eBGu3–HA binding. It is noteworthy that the face of the N-terminal helix in native OCN exposes three negatively charged Gla side chains, which register with calcium ions in the HA crystal lattice during binding.[13] Our binding isotherms and CD analysis suggest that the mechanism of eBGa3–HA binding may be analogous to natural OCN–HA binding.

The eBGa3 modular peptide also bound strongly to HA cell culture substrates (slabs) formed by a solid free-form fabrication process,[14] in which an "ink" consisting of low-binder-content colloidal hydroxyapatite particles is extruded through a nozzle in a predefined trajectory to give a continuous slab (Figure 1 c,d). To confirm that modular peptides could bind to HA slabs in a similar manner to the HA particles described above, we performed an experiment in which eBGa3 and eBGu3 peptides were added onto the surface in localized "spots" (Figure 1 e) or throughout the surface (Figure S3 in the Supporting Information), allowed to incubate for 4 h, and then washed copiously with phosphate-buffered saline (PBS). The eBGa3 peptide bound strongly

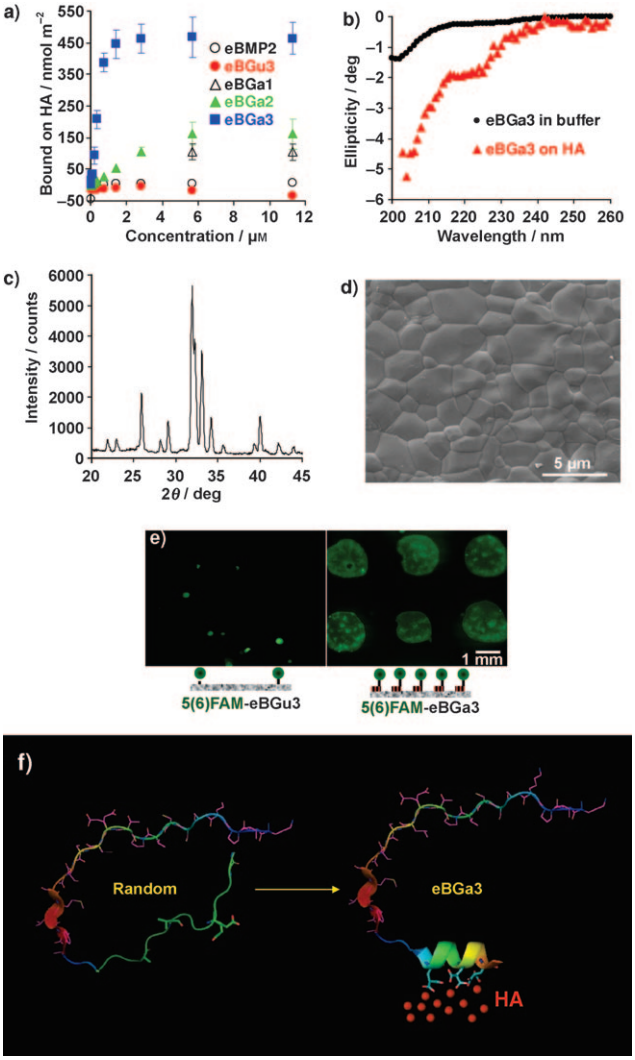

Figure 1. OCN-inspired peptide sequences in modular peptide growth factors bind to HA in a sequence-dependent manner. a) Binding isotherms of modular peptides to HA particles in 50 mM PIPES buffer (pH 7.4) at 37 °C. b) CD spectra of eBGa3 peptides in the buffer solution or bound to HA particles. c) XRD pattern and d) SEM image of HA slabs formed by directed deposition. e) Images of 5(6)-carboxyfluorescein(FAM)-labeled modular peptides bound to HA slabs. eBGu3 does not bind appreciably to the surface (left) while eBGa3 binds strongly to HA on the entire surface (Figure S3b in the Supporting Information), or in localized spots on the surface (right) and was not removed from the surface after repeated washing. f) Schematic representation of secondary-structure formation of modular eBGa3 peptides. We hypothesize that eBGa3 peptide forms a secondary α helix when bound to HA, which allows for registration of Gla residues in the peptide (cyan, stick representation) with calcium atoms (orange) in the HA crystal lattice, analogous to native OCN–HA binding.

and stably in the spotted areas to the HA slabs (Figure 1e, right), while the eBGu3 peptide showed no apparent binding (Figure 1e, left). Therefore, the observed binding was consistent with binding isotherms for these peptides on HA particles.

The BMP2-derived peptide sequence within modular peptide growth factors retained its biological activity in solution, as measured by its ability to promote osteogenic differentiation when added to the culture medium of human mesenchymal stem cells (hMSCs). Osteogenic differentiation was characterized by two markers associated with differentiating osteoblasts, upregulated alkaline phosphatase (ALP) activity, and increased production of mineralized tissue (measured by alizarin red S staining; ARS). In the absence of osteogenic supplements (OS), the eBGa3 and eBGu3 peptides promoted enhanced ALP activity (Figure 2b and Figure S4 in the Supporting Information) and mineralized tissue formation (Figure 2d and Figure S5 in the Supporting Information).

In the presence of OS, the effects of eBGa3 and eBGu3 were more dramatic, as ALP activity was enhanced more than 11-fold (Figure 2a,b), and alizarin red S staining was enhanced by more than 3-fold (Figure 2c,d). There was no significant difference between the effects of the eBGa3 and eBGu3 peptides when added in solution, indicating that the

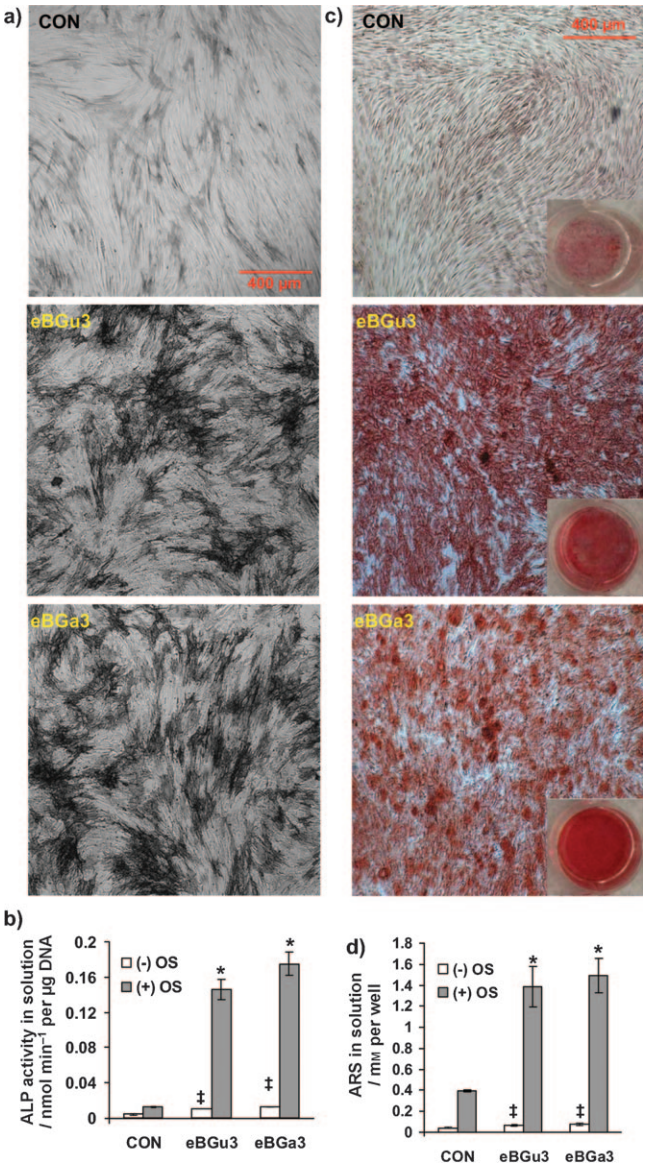

Figure 2. Biological activity of soluble modular peptide growth factors in hMSC culture. a) Images of hMSCs cultured in the presence of eBGu3, eBGa3, or no peptide growth factor ("CON") in OS medium, and stained for ALP activity at day 8. b Quantitative analysis of ALP activity at day 8 in culture. c) Formation of mineralized tissue was detected by ARS staining of hMSCs cultured with OS at day 8. d) Quantitative analysis of ARS staining at day 8 (detection at 405 nm). Statistically significant differences between control and modular peptide growth factors in the absence (‡) or presence of (*) OS are indicated. Images ALP and ARS of hMSCs cultured in the absence of OS are included in Figures S4 and S5 in the Supporting Information.

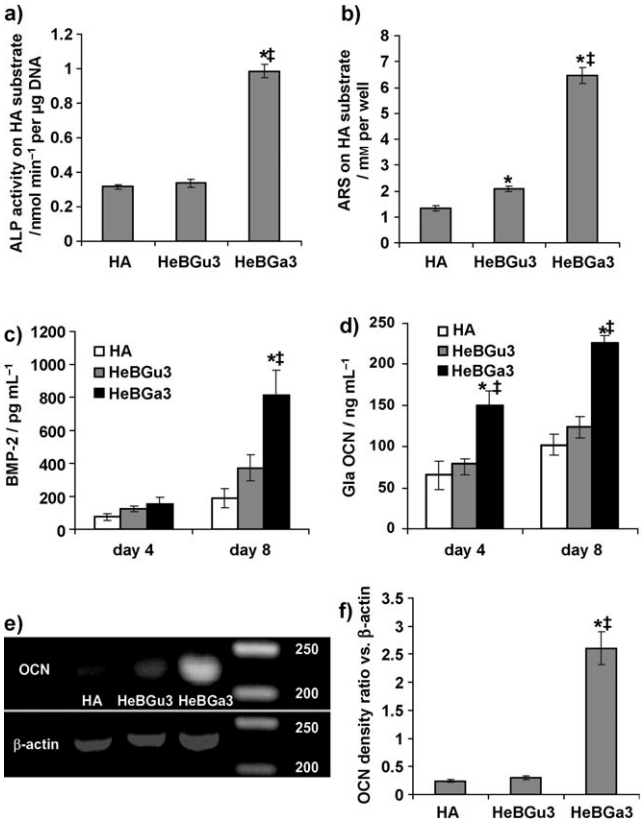

Figure 3. Influence of immobilized modular peptide growth factor on biological activity of hMSCs cultured on HA substrates in the presence of OS. a) ALP activity of hMSCs cultured on untreated (HA), HeBGu3, or HeBGa3 substrates for 8 days. b) Formation of mineralized tissue was detected by ARS staining at day 8 (detection at 405 nm). c,d) BMP-2 and OCN collected in the culture media were quantified by ELISAs at days 4 and 8. e) The mRNA expression levels of OCN in hMSCs cultured on HA, HeBGu3, or HeBGa3 at day 8. Total RNA was extracted and then amplified by RT-PCR using primer sets indicated in the methods described in the Supporting Information. β-Actin levels were used as an internal control. f) Quantitative analysis of densitometry, demonstrating a tenfold increase in mRNA expression of OCN on HeBGa3. * and ‡ indicate statistically significant differences among substrates (HA, HeBGu3, and HeBGa3) and between HeBGu3 and HeBGa3 substrates, respectively.

specific sequence of the mineral-binding portion of these modular peptide growth factors does not strongly influence the biological activity BMP2-derived portion in standard cell culture conditions.

Importantly, the modular peptide growth factor eBGa3 was also able to promote osteogenic differentiation of hMSCs when bound to HA cell culture substrates. hMSCs were cultured on either untreated HA substrates or HA substrates preincubated in eBGu3 or eBGa3 solution (termed HeBGu3 or HeBGa3, respectively), and the cultures were then analyzed for markers of osteogenic differentiation. hMSCs cultured on HeBGa3 showed significantly increased ALP activity (Figure 3a) and mineralized tissue formation (Figure 3b). In addition, hMSCs on HeBGa3 substrates showed significantly enhanced production of full-length BMP-2 protein (Figure 3c) and OCN protein on day 8 (Figure 3d), which are also markers associated with osteogenic differentiation.

OCN expression by hMSCs cultured on HeBGa3 substrates was enhanced more than 10-fold relative to that of hMSCs on untreated HA or HeBGu3 substrates (Figure 3e,f). Taken together, these data indicate that the eBGa3 peptide is multifunctional, as it is capable of binding strongly to HA while also promoting MSC differentiation. Therefore, our approach indirectly mimics the variety of natural modular growth factors that are capable of binding to particular natural ECM.[15] Interestingly, the eBGu3 control peptide promoted slight, but not significant, increases in the production of osteogenic differentiation markers when compared to untreated HA substrates (Figure 3f). The absence of significant effects of eBGu3 can likely be attributed to its weak and limited substrate binding relative to eBGa3 as demonstrated in binding isotherms (Figure 1a) and fluorescence analysis (Figure 1e).

The modular peptide molecules created in this study may be useful in current clinical orthopedic applications. The delivery of BMP-2 has become an important component of clinical strategies for bone regeneration. The modular peptides described here may help to address emerging issues associated with suboptimal delivery of several growth factors, incuding BMP-2.[16] The modular nature of this general approach may ultimately be expanded to achieve noncovalent immobilization of other biologically active molecules to HA or other clinically important biomaterials.

Received: March 24, 2009
Published online: July 16, 2009

Keywords: biomineralization · hydroxyapatite · osteocalcin · osteogenesis · protein modifications

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