Published in final edited form as:

Biomaterials. 2013 February ; 34(5): 1506–1513. doi:10.1016/j.biomaterials.2012.10.046.

Polyglutamate directed coupling of bioactive peptides for the delivery of osteoinductive signals on allograft bone

Bonnie K. Culpepper¹, Paul P. Bonvallet², Michael S. Reddy³, Selvarangan Ponnazhagan⁴, and Susan L. Bellis^{1,2,*}

¹Department of Biomedical Engineering, University of Alabama at Birmingham, Birmingham, AL 35294

²Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL 35294

³Department of Periodontology, University of Alabama at Birmingham, Birmingham, AL 35294

⁴Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294

Abstract

Allograft bone is commonly used as an alternative to autograft, however allograft lacks many osteoinductive factors present in autologous bone due to processing. In this study, we investigated a method to reconstitute allograft with osteoregenerative factors. Specifically, an osteoinductive peptide from collagen I, DGEA, was engineered to express a heptaglutamate (E7) domain, which binds the hydroxyapatite within bone mineral. Addition of E7 to DGEA resulted in 9× greater peptide loading on allograft, and significantly greater retention after a 5-day interval with extensive washing. When factoring together greater initial loading and retention, the E7 domain directed a 45-fold enhancement of peptide density on the allograft surface. Peptide-coated allograft was also implanted subcutaneously into rats and it was found that E7DGEA was retained *in vivo* for at least 3 months. Interestingly, E7DGEA peptides injected intravenously accumulated within bone tissue, implicating a potential role for E7 domains in drug delivery to bone. Finally, we determined that, as with DGEA, the E7 modification enhanced coupling of a bioactive BMP2-derived peptide on allograft. These results suggest that E7 domains are useful for coupling many types of bone-regenerative molecules to the surface of allograft to reintroduce osteoinductive signals and potentially advance allograft treatments.

INTRODUCTION

There are 2.2 million bone-grafting procedures performed worldwide each year to repair bone defects in orthopedics, neurosurgery, and dentistry [1]. Bone grafting is used to stimulate bone healing in delayed or non-union fractures, bone voids, spinal fusions and craniofacial reconstructions. The “gold standard” treatment involves grafting autogenous bone harvested from the patient. Optimal osteoconductive and osteoinductive properties are characteristic advantages of autograft bone transplants; however limitations include the

© 2012 Elsevier Ltd. All rights reserved

*To whom correspondence should be addressed: Susan L. Bellis, Ph.D. 982A MCLM 1918 University Boulevard Birmingham, AL 35294 bellis@uab.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

restricted amount of donor bone available, and the risk of pain and morbidity at the donor bone site [1]. Allograft bone, donated from cadaveric sources, resolves potential challenges associated with a second surgery site and is readily available commercially [1, 2]. The processing and sterilization of allograft donor bone are crucial steps to minimize the risk of pathogen transmission and immunogenicity; however, these same steps are thought to destroy many of the biological factors associated with conferring osteoinductivity on autograft bone [1, 2].

Some promising strategies aimed at increasing osteoinductivity of allograft bone include co-delivery of stem cells [3–5], platelet rich plasma [6–8], and recombinant proteins [4, 9–11]. Platelet derived growth factor BB (PDGF-BB) [12] and bone morphogenic proteins (BMP) [13] are examples of recombinant proteins that have been co-mixed with allograft bone for the treatment of osseous defects. Additionally, degradable polymer coatings have been used to introduce a more porous surface to enhance bony ingrowth on cortical allograft [14–16], and degradation of a polymer coating has been employed as a mechanism to locally deliver a small molecule compound to stimulate recruitment of endogenous cells and enhance remodeling of allograft bone [14, 17, 18]. However, in most investigations involving delivery of osteogenic factors on allograft, molecules have been passively adsorbed to the graft surface, a method that is inefficient and provides limited control over release kinetics.

Prior studies from our group have focused on coupling osteoinductive molecules to hydroxyapatite (HA). HA is a calcium phosphate molecule that comprises the principal constituent of native bone mineral, and synthetic forms of HA are widely used for bone repair. To improve the binding of osteogenic peptides to HA, we and others have modeled the process by which endogenous proteins localize to bone [19–27]. Bone-matrix proteins including bone sialoprotein (BSP) [28], osteocalcin (OCN) [29], and statherin [30] bind to the HA within bone through regions of negatively charged amino acids. Adapting this mechanism, our group has added a heptaglutamate domain (E7) to multiple distinct peptides and found that in every case the E7 domain facilitated greater loading and retention of peptide on the HA surface. This strategy was shown to be effective for the integrin-binding peptide, RGD [26], two proteoglycan-binding peptides, FHRRIKA and KRSR [25], and finally, a peptide derived from collagen I, DGEA [27].

There is currently considerable interest in using collagen-mimetic peptides to stimulate osteoregeneration, given that collagen I is a principal component of the organic bone matrix. Collagen I binds to and activates the $\alpha_2\beta_1$ integrin, a receptor that induces osteoblastic differentiation of osteoprogenitor cells [31–34]. Many studies have been directed at evaluating osteogenesis stimulated by DGEA [22, 35, 36], P15 [35, 37–40] and another collagen-derived sequence, GFOGER [41–43]. GFOGER is a triple helical peptide that binds $\alpha_2\beta_1$ [44–46], and GFOGER coatings have been shown to improve osseointegration of several biomaterial substrates including titanium [42] and polycaprolactone (PCL) [43]. The DGEA peptide was originally identified as an $\alpha_2\beta_1$ integrin ligand [34, 47] although structural studies have since brought this into question [44, 45]. However, regardless of mechanism, several groups, including ours, have shown that DGEA stimulates *in vitro* osteoblastic differentiation of mesenchymal stem cells and increased bone formation on HA substrates *in vivo* [27, 35]. Furthermore, these processes were significantly enhanced when DGEA was modified with an E7 domain to improve coupling to the HA surface [27].

In light of the disparity in clinical outcomes associated with autograft vs allograft transplantation, the goal of this study was to employ the E7 domain to couple peptides to allograft bone (via the biologic HA within bone), thus reconstituting allograft with osteoinductive factors. We compared the loading and retention of DGEA and E7DGEA peptides on allograft bone, as well as the specificity of the E7 domain for bone tissue.

Additionally, we examined the binding and retention of a BMP2-derived peptide to allograft as a proof of concept for using the E7 domain as a generic tool for anchoring diverse bioactive peptides to enhance regenerative repair.

MATERIALS AND METHODS

Peptide preparation

Collagen mimetic peptide, DGEA, and a peptide derived from bone morphogenetic protein-2 (“BMP2” KIPKASSVPTELSAISTLYL) were obtained from American Peptide Co., Inc (Sunnyvale, CA). To facilitate tracking of binding and release kinetics, variations of these peptides were synthesized to contain fluorescein (FITC) tags including: DGEA-FITC (DGEAK-FITC), E7DGEA-FITC (EEEEEEEDGEAK-FITC), BMP2-FITC (KIPKASSVPTELSAISTLYLK-FITC), and E7BMP2-FITC (EEEEEEEKIPKASSVPTELSAISTLYLK-FITC). All peptides were reconstituted in Tris-buffered saline (TBS) at 1mg/ml, aliquoted and stored at -20°C until use.

Peptide coating

Mineralized particulate cortical allograft, with a particle size ranging from 250–1000 μm (OraGRAFT), was obtained from LifeNet Health (Virginia Beach, VA) and stored under sterile conditions at room temperature until used. Peptides were coated onto allograft particles for 2 hours at room temperature while slowly rotating samples to ensure homogenous peptide coating.

Visualization of peptide binding and *in vitro* retention

FITC conjugated peptides: DGEA, E7DGEA, BMP2 and E7BMP2 (10 μM) were coated onto allograft as previously described. After 2 hours of coating, particles were washed twice for one minute with TBS to remove unbound peptide and immediately visualized by fluorescent microscopy for relative peptide binding. Additionally, to evaluate peptide retention, peptide-coated samples were washed briefly and placed into fresh TBS with agitation. Samples were washed for up to 7 days, changing TBS every 1–2 days, and then visualized using a fluorescent Nikon microscope.

Peptide binding as measured by solution fluorescence depletion

Equimolar concentrations of FITC-conjugated DGEA or E7DGEA (1 μM) were coated onto varying quantities of allograft (1–50mg) for 2 hours at room temperature. Samples were then centrifuged to precipitate the particles and the supernatants were examined using a fluorometer to quantify the amount of solution fluorescence. The amount of fluorescence remaining in the solution (the unbound peptide fraction) was then subtracted from the initial fluorescence of the starting peptide solutions to calculate percent of peptide loaded onto the surface of allograft. Additionally, to evaluate the time course of peptide loading, 1 μM concentrations of DGEA or E7DGEA were coated onto 25mg quantities of allograft for 1, 2, 6, or 24 hours. The percent bound was calculated by comparing total and residual solution fluorescence measured by fluorometry as before.

Release of peptide from surface

FITC-tagged peptides DGEA or E7DGEA were coated onto 25mg allograft particles as previously described. After initial peptide loading was measured and recorded, remaining supernatant was completely aspirated and replaced with TBS. Over a period of 5 days, release of peptide from the surface of allograft to the supernatant was assessed by monitoring the appearance of fluorescence in solution (measured on a fluorometer) and plotted as a percentage of initially bound peptide.

***In vivo* peptide retention**

Allograft particles coated with 10 μ M FITC-tagged DGEA, E7DGEA, BMP2, or E7BMP2 or uncoated particles were implanted into dorsal subcutaneous pouches in male Sprague-Dawley rats as previously described [27]. Following 1 or 3 months of implantation, rats were sacrificed and implants were retrieved. Peptide retention was analyzed qualitatively by visualizing allograft particles from each group (in the same field) using a Nikon fluorescent microscope. All animal studies were performed with prior approval from the UAB Institutional Animal Care and Use Committee.

Peptide targeting to bone tissue

FITC-tagged DGEA or E7DGEA (3mg of peptide in 200 μ l of TBS) or TBS lacking peptide was injected into the tail vein of male Sprague-Dawley rats. After 24 hours, rats were sacrificed, bones were collected, fixed in 70% ethanol, and whole bones were imaged using a Nikon dissecting microscope to evaluate the effect of the E7 domain on delivery of peptide to bone.

Binding specificity

Neonatal mice were sacrificed, fixed in 70% ethanol, embedded in OCT compound and cryo-sectioned to evaluate E7-directed peptide binding to bone in the context of surrounding soft tissue. Specifically, 10 μ M DGEA-FITC or E7DGEA-FITC was used to coat various tissue sections for 30 minutes then sections were briefly rinsed with water. Peptide binding to the sections was qualitatively evaluated by imaging these slides using a Nikon Fluorescent microscope. Each set of sections, DGEA- or E7DGEA-coated thigh, rib, or whole body was taken using the same set of parameters to allow for comparative analysis of DGEA and E7DGEA binding to bone and surrounding soft tissue.

Statistics

In vitro peptide binding kinetics and retention assays were performed with at least three independent runs, and each independent experiment was performed in triplicate. Subcutaneous implantations of peptide-coated allograft bone chips were performed in two independent runs, with each experiment performed in duplicate. Peptide labeling of mouse sections were performed in two independent runs with at least two sections analyzed per sample. Data sets were assessed using Student's t-test parametric analysis. A confidence level of at least 95% ($p < 0.05$) was considered significant and denoted by (“*”).

RESULTS

E7-peptide binding to allograft bone

To facilitate studies of peptide binding to allograft bone, DGEA and E7DGEA were engineered to express a fluorescent FITC tag on the C-terminus. This tag allowed peptide quantities to be assessed by fluorescent microscopy and fluorometry. Varying quantities (1–50mg) of allograft bone were coated with equimolar concentrations of DGEA or E7DGEA and peptide binding was measured by monitoring depletion of solution fluorescence (reflecting deposition of the peptide onto allograft). As shown in FIG 1A, greater binding of E7DGEA was observed with increasing amounts of allograft, however, less than 5% of DGEA was bound regardless of how much allograft was added. These data show that the E7 domain directs peptide binding to allograft bone in a dose dependent manner. We then evaluated binding to allograft bone over a 24 hour time course and found that the E7 domain directed significantly greater peptide binding to the surface of allograft bone than passively adsorbed DGEA at all time points (FIG 1B). Interestingly, E7-modified DGEA was loaded at near maximal concentrations after only one hour (FIG 1B). This finding suggests the

potential clinical benefit of a rapid coating with an E7-engineered biomolecule to functionalize commercially available allograft. As a qualitative assessment of initial peptide loading, we used fluorescent microscopy to analyze relative peptide loading densities. As shown in FIG 1C, substantially more E7DGEA was bound to the surface of allograft bone than passively adsorbed DGEA.

Retention of E7-peptide on allograft bone

To evaluate the strength of the interaction between the E7 domain and allograft bone, we examined retention of E7DGEA after exposure to stringent washing conditions. Allograft particles were coated with peptide as before, and then briefly washed to remove unbound peptide. The samples were resuspended in fresh TBS and incubated with agitation for 1 or 7 days. As shown in fluorescent images (FIG 2A), E7DGEA peptide was retained at high quantities for at least 7 days, whereas passively adsorbed DGEA peptide was undetectable at both 1 and 7 days. To quantitatively assess peptide retention, we coated 25 mg quantities of allograft with 1 μ M DGEA or E7DGEA, as in FIG 1, and similarly found that the E7 domain facilitated 9 times greater loading to allograft bone than unmodified DGEA (FIG 2B). Using these same samples (with known initial peptide loading), we tracked the percent of the initially bound peptide that was released over 5 days (FIG 2C). Passively adsorbed DGEA was released from the surface of allograft bone rapidly, with approximately 50% of bound peptide released within 1 hour. Conversely, E7DGEA peptide was tightly bound, releasing less than 10% of initially bound peptide after 5 days. When factoring together 9 times greater initial loading and ~ 5-fold greater retention of E7DGEA, the data suggest that engineering the DGEA peptide with an E7 domain resulted in approximately 45 times more peptide present on the surface of allograft after 5 days (FIG 2D).

In addition to measuring *in vitro* kinetics of binding and retention, we evaluated the ability of the E7-allograft interaction to withstand *in vivo* forces. To this end, we implanted peptide-coated allograft into rat dorsal subcutaneous pouches. After 1 or 3 months, we retrieved and imaged allograft particles for relative peptide density using fluorescent microscopy (FIG 3). At both 1 and 3 months following implantation, E7DGEA coated allograft exhibited a much greater fluorescent signal than DGEA coated allograft. These results highlight the strength of the E7-HA interaction and the potential of the E7 domain to anchor higher local concentrations of bioactive factors to allograft bone for longer intervals.

Bone targeting of E7DGEA

To assess the potential benefit of utilizing E7-modified peptides as a mechanism for targeting a therapeutic molecule to bone, we injected rats intravenously with FITC-tagged DGEA or E7DGEA, or with TBS as a control. After 24 hours, we sacrificed the animals and fluorescently imaged the tibia and fibula using a Nikon dissecting microscope (FIG 4). Bones retrieved from TBS-injected rats fluoresced at low levels, which are characteristic of the autofluorescence of bone tissue. Similarly, limited fluorescence was observed in bones from animals injected with DGEA. In striking contrast, bones from animals injected with E7DGEA had robust fluorescence, indicating that the E7 domain facilitated delivery of peptides to bone tissue.

Specificity of E7DGEA binding

We previously reported that the E7 domain has a high degree of selectivity for HA-containing biomaterials; for example, negligible binding is observed on other common materials including: titanium, stainless steel, polycaprolactone and tissue culture plastic [27]. To further probe specificity for HA, we evaluated the capacity of the E7 domain to bind bone within the context of surrounding soft tissues. This was achieved by coating DGEA and E7DGEA peptides onto neonatal mouse sections (FIG 5). Thigh and rib sections clearly

show specific binding of the E7 domain to bone tissue with no apparent binding to surrounding soft tissue (FIG 5A&B). Additionally whole body sections (lacking limbs) were coated and imaged, these cross sections show strong labeling of spine and rib in sections coated with E7DGEA (FIG 5C). In contrast, DGEA coated sections showed no specific binding of peptide to any tissue. In the aggregate, these data confirm that the addition of an E7 domain confers enhanced peptide binding to three sources of bone; commercial human allograft bone, rat bone (following IV injection), and intact neonatal mouse bone.

E7 binding of BMP2 peptide

To evaluate the utility of the E7 domain as a broadly applicable tool for anchoring multiple peptides to allograft bone, we engineered a BMP2 peptide known to be capable of inducing bone formation [48, 49] to contain an E7 domain. As previously described, BMP2 and E7BMP2 peptides were modified with a FITC domain to facilitate tracking of binding and retention. Using fluorescent microscopy, relative initial peptide loading was assessed (FIG 6A); the E7 domain facilitated enhanced initial coupling of BMP2 peptides. This finding is significant not only due to the increased size of the BMP2 peptide (relative to DGEA) but also because the BMP2 peptide has a different chemical composition, with several hydrophobic amino acids. Additionally, we subjected these samples to agitation *in vitro* and found that the E7-HA interaction facilitated retention of BMP2 on allograft bone for at least 7 days (FIG 6B). Consistent with studies of DGEA, BMP2 peptides lacking an E7 binding domain were loaded at low densities and retention was negligible. As a final measure, we evaluated the strength of this interaction *in vivo* using the subcutaneous implantation model. Importantly, the E7 domain facilitated retention of the BMP2 peptide in vivo for at least 1 month (FIG 6C). Collectively, these data suggest that the E7 domain strategy represents an effective mechanism for enhanced coupling of multiple bioactive peptides to allograft bone.

DISCUSSION

Allograft bone is widely used clinically, in combination with, or as an alternative to, autogenous bone [1]. One limitation of allograft is that it lacks many of the osteoregenerative factors present in autogenous bone, and thus there is substantial interest in developing methods for reconstituting allograft with such factors. Allograft substrates with passively-adsorbed BMP-7 [9], BMP-2 [50], or BMP-7 with osteoclast inhibitor zoledronate [51], have been shown to stimulate bone healing in animal implant models. Clinically, recombinant human (rh) forms of BMP-2 and BMP-7 are FDA approved for use with a collagen carrier in orthopedic and spinal fusion applications [52, 53]. While BMPs are very potent inducers of new bone synthesis, there are concerns regarding their therapeutic use including the supraphysiologic doses needed, and dissemination from the carrier. Ectopic bone formation, inflammation, and increased cancer risk are examples of potential side effects that have been associated with broad dissemination of BMPs. As an additional challenge, recombinant proteins such as BMPs are expensive to produce and require expression in a cell-based system. The use of bioactive peptide domains as an alternative to recombinant human proteins could potentially: decrease production costs; decrease sensitivity to denaturation; eliminate concern of possible contamination with cell culture products; and reduce the potential for immune response [54]. A peptide derived from the knuckle domain of BMP-2 has been identified and shown to be effective at enhancing osteoblastic differentiation [48], increasing osteoblast adhesion [55], and stimulating bone formation [56].

Given the typically low binding capacity of molecules passively adsorbed to graft surfaces, and the corresponding concerns regarding dissemination, attempts have been made to immobilize recombinant proteins or biomimetic peptides onto various materials used in bone regeneration. For instance, recombinant human BMP-2 has been immobilized on

poly(lactide-co-glycolide) (PLGA) films [57] and synthetic HA disks [58] and the tethered BMP2 was found to stimulate enhanced matrix mineralization by osteoblasts, and alkaline phosphatase activity in progenitor cells, respectively. Similarly, cobalt chromium [49] and polyethylene terephthalate (PET) [59] surfaces covalently linked with BMP-derived peptides signaled more robust *in vitro* differentiation of preosteoblasts. Collectively, these studies serve as a proof of concept that better immobilization of bioactive factors results in biologic benefit. When compared with synthetic biomaterials, immobilization of biomodifiers onto the allograft surface clearly presents substantial technical challenges, although Fmoc chemistry has been used to covalently link an antibiotic to allograft bone [60]. Furthermore, covalent coupling may interfere with the activity of some types of osteoinductive factors.

The use of polyacidic amino acid domains as an alternative to covalent immobilization offers many advantages. The E7 domain is synthesized as a continuous sequence with the bioactive part of the peptide, and thus E7-modified peptides can be produced in high quantities in a cost-effective manner using a commercial peptide synthesizer. One envisions that these peptides could be coated onto off-the-shelf commercial allograft products, avoiding the need for complex chemical coupling methods or alteration of the graft surface. As shown in this study, E7-peptides bind rapidly to allograft, achieving near maximal binding within a one-hour interval. Moreover, substantially more E7-modified peptide (up to 45-fold) was present on allograft as compared with unmodified peptide after extensive wash steps, and E7-peptides were retained for several months *in vivo* on allograft implanted subcutaneously. The concept of combining peptides with graft materials immediately before surgical placement is already being developed commercially for clinical applications. For example, BioSET offers a product that involves precoating ceramic particles with a peptide targeting the BMP-2 pathway just prior to implantation. This strategy is reportedly effective in stimulating spinal fusion rates in both rabbit [61] and sheep [62] models. It is anticipated that the E7 domain would enable greater concentrations of peptide or protein to be loaded onto the graft surface, and facilitate sustained retention, resulting in less initial peptide/protein needed to achieve a physiological benefit. As well, the E7 domain is a natural motif within bone-binding proteins, and our studies indicate that this sequence does not elicit any immune or fibrotic response when implanted into bony sites including tibiae [27] and craniae (unpublished results).

Another important implication of this study is that E7 domains may be useful as a mechanism to enhance systemic delivery of molecules influencing bone healing. E7-modified DGEA peptides injected intravenously appeared to concentrate within bone to a markedly greater degree than unmodified DGEA, although additional studies will be needed to fully characterize selective bone targeting by the E7 domain. In support of this concept, Miyamoto's group has investigated a similar polyacidic amino acid domain for targeted treatment of osteoporosis. Specifically, a six amino acid aspartate domain (D6) was conjugated to estradiol and shown to increase bone mineral density (BMD) without increasing uterine weight (a common side effect of treatment with estradiol) in both the IV [63] and nasal [64] preparations of the drug. Additionally, the D6 bone-binding domain facilitated retention of a model molecule on bone for at least 14 days *in vivo* following IV injection in rats [65]. It is plausible that this technology, utilizing a negatively charged domain to target bone, could be further developed to enhance delivery of antibiotics [66], chemotherapeutic agents, or various other drugs utilized for treatment of diverse bone pathologies.

CONCLUSIONS

There is a significant gap in the bone regenerative potential of allograft bone and the “gold standard” autograft bone. Processing and sterilization of allografts remove cellular components and denature or destroy many biologic factors that contribute to the osteoinductivity of autograft bone. The main focus of this study was to develop and evaluate a simple targeted strategy for efficient coupling of bioactive peptide domains to allograft bone. Specifically, we aimed to reintroduce osteoinductive factors onto allograft by using an HA binding domain, E7. Osteoinductive peptides modified with E7 exhibited markedly greater binding to allograft, and these peptides had increased retention both in vitro and in vivo. Additionally, the E7 technology is adaptable and can be used to enhance coupling of many types of peptides to synthetic HA biomaterials in addition to allograft bone. In sum, the E7 domain represents a promising strategy to improve the coupling of many types of bioactive factors, potentially enabling the osteoinductivity of allograft to approach the bone regenerative capacity of autograft bone.

Acknowledgments

This research was supported by NIH grant R01AR51539 (SLB), NIH-R01 AR060948 (SP), a grant from the Osseointegration Foundation (SLB), and NIH/NIDCR predoctoral fellowship 1F31DE021613 (BKC). We are grateful for technical support provided by the Bone Histomorphometry Core Facility and the High Resolution Imaging Core Facility.

REFERENCES

1. Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: An update. *Injury*. 2005; 36(Suppl 3):S20–7. [PubMed: 16188545]
2. Bostrom MP, Seigerman DA. The clinical use of allografts, demineralized bone matrices, synthetic bone graft substitutes and osteoinductive growth factors: A survey study. *HSS J*. 2005; 1:9–18. [PubMed: 18751803]
3. Lee JY, Choi MH, Shin EY, Kang YK. Autologous mesenchymal stem cells loaded in gelfoam((r)) for structural bone allograft healing in rabbits. *Cell Tissue Bank*. 2011; 12:299–09. [PubMed: 20652421]
4. Di Bella C, Aldini NN, Lucarelli E, Dozza B, Frisoni T, Martini L, et al. Osteogenic protein-1 associated with mesenchymal stem cells promote bone allograft integration. *Tissue Eng Part A*. 2010; 16:2967–76. [PubMed: 20533882]
5. Breitbart EA, Meade S, Azad V, Yeh S, Al-Zube L, Lee YS, et al. Mesenchymal stem cells accelerate bone allograft incorporation in the presence of diabetes mellitus. *J Orthop Res*. 2010; 28:942–9. [PubMed: 20058266]
6. Pantou AL, Markopoulou CE, Dereka XE, Vavouraki H, Mamalis A, Vrotsos IA. The effect of platelet-rich plasma (prp) combined with a bone allograft on human periodontal ligament (pdl) cells. *Cell Tissue Bank*. 2012; 13:81–8. [PubMed: 21120699]
7. Messoria, M.; Braga, L.; Oliveira, G.; Oliveira, LF.; Milagres, R.; Kawata, L., et al. Healing of fresh frozen bone allograft with or without platelet-rich plasma: A histologic and histometric study in rats. *Clin Implant Dent Relat Res*. 2011. Available from URL: <http://www.ncbi.nlm.nih.gov/pubmed/22176648> (DOI 10.1111/j.1708-8208.2011.00419.x)
8. Wei LC, Lei GH, Sheng PY, Gao SG, Xu M, Jiang W, et al. Efficacy of platelet-rich plasma combined with allograft bone in the management of displaced intraarticular calcaneal fractures: A prospective cohort study. *J Orthop Res*. 2012; 30:1570–76. [PubMed: 22488880]
9. Donati D, Di Bella C, Lucarelli E, Dozza B, Frisoni T, Aldini NN, et al. Op-1 application in bone allograft integration: Preliminary results in sheep experimental surgery. *Injury*. 2008; 39(Suppl 2):S65–72. [PubMed: 18804576]
10. Yasuda H, Yano K, Wakitani S, Matsumoto T, Nakamura H, Takaoka K. Repair of critical long bone defects using frozen bone allografts coated with an rhbmp-2-retaining paste. *J Orthop Sci*. 2012; 17:299–307. [PubMed: 22271007]

11. Fukuroku J, Inoue N, Rafiee B, Sim FH, Frassica FJ, Chao EY. Extracortical bone-bridging fixation with use of cortical allograft and recombinant human osteogenic protein-1. *J Bone Joint Surg Am.* 2007; 89:1486–96. [PubMed: 17606787]
12. Rosen PS, Toscano N, Holzclaw D, Reynolds MA. A retrospective consecutive case series using mineralized allograft combined with recombinant human platelet-derived growth factor bb to treat moderate to severe osseous lesions. *Int J Periodontics Restorative Dent.* 2011; 31:335–42. [PubMed: 21837299]
13. Buttermann GR. Prospective nonrandomized comparison of an allograft with bone morphogenic protein versus an iliac-crest autograft in anterior cervical discectomy and fusion. *Spine J.* 2008; 8:426–35. [PubMed: 17977799]
14. Lewandrowski KU, Bondre S, Hile DD, Thompson BM, Wise DL, Tomford WW, et al. Porous poly(propylene fumarate) foam coating of orthotopic cortical bone grafts for improved osteoconduction. *Tissue Eng.* 2002; 8:1017–27. [PubMed: 12542947]
15. Lewandrowski KU, Bondre SP, Gresser JD, Wise DL, Tomford WW, Trantolo DJ. Improved osteoconduction of cortical bone grafts by biodegradable foam coating. *Biomed Mater Eng.* 1999; 9:265–75. [PubMed: 10822482]
16. Bondre S, Lewandrowski KU, Hasirci V, Cattaneo MV, Gresser JD, Wise DL, et al. Biodegradable foam coating of cortical allografts. *Tissue Eng.* 2000; 6:217–27. [PubMed: 10941216]
17. Huang C, Das A, Barker D, Tholpady S, Wang T, Cui Q, et al. Local delivery of fty720 accelerates cranial allograft incorporation and bone formation. *Cell Tissue Res.* 2012; 347:553–66. [PubMed: 21863314]
18. Petrie Aronin CE, Shin SJ, Naden KB, Rios PD Jr, Sefcik LS, Zawodny SR, et al. The enhancement of bone allograft incorporation by the local delivery of the sphingosine 1-phosphate receptor targeted drug fty720. *Biomaterials.* 2010; 31:6417–24. [PubMed: 20621764]
19. Murphy MB, Hartgerink JD, Goepferich A, Mikos AG. Synthesis and in vitro hydroxyapatite binding of peptides conjugated to calcium-binding moieties. *Biomacromolecules.* 2007; 8:2237–43. [PubMed: 17530891]
20. Itoh D, Yoneda S, Kuroda S, Kondo H, Umezawa A, Ohya K, et al. Enhancement of osteogenesis on hydroxyapatite surface coated with synthetic peptide (eeeeeeprgdt) in vitro. *J Biomed Mater Res.* 2002; 62:292–8. [PubMed: 12209950]
21. Fujisawa R, Mizuno M, Nodasaka Y, Kuboki Y. Attachment of osteoblastic cells to hydroxyapatite crystals by a synthetic peptide (glu7-pro-arg-gly-asp-thr) containing two functional sequences of bone sialoprotein. *Matrix Biol.* 1997; 16:21–8. [PubMed: 9181551]
22. Gilbert M, Giachelli CM, Stayton PS. Biomimetic peptides that engage specific integrin-dependent signaling pathways and bind to calcium phosphate surfaces. *J Biomed Mater Res A.* 2003; 67:69–77. [PubMed: 14517863]
23. Gilbert M, Shaw WJ, Long JR, Nelson K, Drobny GP, Giachelli CM, et al. Chimeric peptides of statherin and osteopontin that bind hydroxyapatite and mediate cell adhesion. *J Biol Chem.* 2000; 275:16213–8. [PubMed: 10748043]
24. Lee JS, Lee JS, Murphy WL. Modular peptides promote human mesenchymal stem cell differentiation on biomaterial surfaces. *Acta Biomater.* 2010; 6:21–8. [PubMed: 19665062]
25. Sawyer AA, Hennessy KM, Bellis SL. The effect of adsorbed serum proteins, rgd and proteoglycan-binding peptides on the adhesion of mesenchymal stem cells to hydroxyapatite. *Biomaterials.* 2007; 28:383–92. [PubMed: 16952395]
26. Sawyer AA, Weeks DM, Kelpke SS, McCracken MS, Bellis SL. The effect of the addition of a polyglutamate motif to rgd on peptide tethering to hydroxyapatite and the promotion of mesenchymal stem cell adhesion. *Biomaterials.* 2005; 26:7046–56. [PubMed: 15964067]
27. Culpepper BK, Phipps MC, Bonvallet PP, Bellis SL. Enhancement of peptide coupling to hydroxyapatite and implant osseointegration through collagen mimetic peptide modified with a polyglutamate domain. *Biomaterials.* 2010; 31:9586–94. [PubMed: 21035181]
28. Ganss B, Kim RH, Sodek J. Bone sialoprotein. *Crit Rev Oral Biol Med.* 1999; 10:79–98. [PubMed: 10759428]
29. Hoang QQ, Sicheri F, Howard AJ, Yang DS. Bone recognition mechanism of porcine osteocalcin from crystal structure. *Nature.* 2003; 425:977–80. [PubMed: 14586470]

30. Stayton PS, Drobny GP, Shaw WJ, Long JR, Gilbert M. Molecular recognition at the protein-hydroxyapatite interface. *Crit Rev Oral Biol Med*. 2003; 14:370–6. [PubMed: 14530305]
31. Mizuno M, Fujisawa R, Kuboki Y. Type i collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-alpha2beta1 integrin interaction. *J Cell Physiol*. 2000; 184:207–13. [PubMed: 10867645]
32. Mizuno M, Kuboki Y. Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type i collagen. *J Biochem*. 2001; 129:133–8. [PubMed: 11134967]
33. Takeuchi Y, Suzawa M, Kikuchi T, Nishida E, Fujita T, Matsumoto T. Differentiation and transforming growth factor-beta receptor down-regulation by collagen-alpha2beta1 integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells. *J Biol Chem*. 1997; 272:29309–16. [PubMed: 9361011]
34. Xiao G, Wang D, Benson MD, Karsenty G, Franceschi RT. Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the osf2 transcription factor. *J Biol Chem*. 1998; 273:32988–94. [PubMed: 9830051]
35. Hennessy KM, Pollot BE, Clem WC, Phipps MC, Sawyer AA, Culpepper BK, et al. The effect of collagen i mimetic peptides on mesenchymal stem cell adhesion and differentiation, and on bone formation at hydroxyapatite surfaces. *Biomaterials*. 2009; 30:1898–909. [PubMed: 19157536]
36. Anderson JM, Kushwaha M, Tambralli A, Bellis SL, Camata RP, Jun HW. Osteogenic differentiation of human mesenchymal stem cells directed by extracellular matrix-mimicking ligands in a biomimetic self-assembled peptide amphiphile nanomatrix. *Biomacromolecules*. 2009; 10:2935–44. [PubMed: 19746964]
37. Yang XB, Bhatnagar RS, Li S, Oreffo RO. Biomimetic collagen scaffolds for human bone cell growth and differentiation. *Tissue Eng*. 2004; 10:1148–59. [PubMed: 15363171]
38. Ripamonti U, Crooks J, Khoali L, Roden L. The induction of bone formation by coral-derived calcium carbonate/hydroxyapatite constructs. *Biomaterials*. 2009; 30:1428–39. [PubMed: 19081131]
39. Gomar F, Orozco R, Villar JL, Arrizabalaga F. P-15 small peptide bone graft substitute in the treatment of non-unions and delayed union. A pilot clinical trial. *Int Orthop*. 2007; 31:93–9. [PubMed: 16761146]
40. Valentin AH, Weber J. Receptor technology--cell binding to p-15: A new method of regenerating bone quickly and safely--preliminary histomorphometrical and mechanical results in sinus floor augmentations. *Keio J Med*. 2004; 53:166–71. [PubMed: 15477730]
41. Reyes CD, Garcia AJ. Alpha2beta1 integrin-specific collagen-mimetic surfaces supporting osteoblastic differentiation. *J Biomed Mater Res A*. 2004; 69:591–600. [PubMed: 15162400]
42. Reyes CD, Petrie TA, Burns KL, Schwartz Z, Garcia AJ. Biomolecular surface coating to enhance orthopaedic tissue healing and integration. *Biomaterials*. 2007; 28:3228–35. [PubMed: 17448533]
43. Wojtowicz AM, Shekaran A, Oest ME, Dupont KM, Templeman KL, Hutmacher DW, et al. Coating of biomaterial scaffolds with the collagen-mimetic peptide gfoger for bone defect repair. *Biomaterials*. 2010; 31:2574–82. [PubMed: 20056517]
44. Knight CG, Morton LF, Onley DJ, Peachey AR, Messent AJ, Smethurst PA, et al. Identification in collagen type i of an integrin alpha2 beta1-binding site containing an essential ger sequence. *J Biol Chem*. 1998; 273:33287–94. [PubMed: 9837901]
45. Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ. The collagen-binding a-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, gfoger, in native (triple-helical) collagens. *J Biol Chem*. 2000; 275:35–40. [PubMed: 10617582]
46. Emsley J, Knight CG, Farndale RW, Barnes MJ. Structure of the integrin alpha2beta1-binding collagen peptide. *J Mol Biol*. 2004; 335:1019–28. [PubMed: 14698296]
47. Staatz WD, Fok KF, Zutter MM, Adams SP, Rodriguez BA, Santoro SA. Identification of a tetrapeptide recognition sequence for the alpha 2 beta 1 integrin in collagen. *J Biol Chem*. 1991; 266:7363–7. [PubMed: 2019571]

48. Saito A, Suzuki Y, Ogata S, Ohtsuki C, Tanihara M. Accelerated bone repair with the use of a synthetic bmp-2-derived peptide and bone-marrow stromal cells. *J Biomed Mater Res A*. 2005; 72:77–82. [PubMed: 15543633]
49. Poh CK, Shi Z, Tan XW, Liang ZC, Foo XM, Tan HC, et al. Cobalt chromium alloy with immobilized bmp peptide for enhanced bone growth. *J Orthop Res*. 2011; 29:1424–30. [PubMed: 21445991]
50. Pluhar GE, Manley PA, Heiner JP, Vanderby R Jr. Seeherman HJ, Markel MD. The effect of recombinant human bone morphogenetic protein-2 on femoral reconstruction with an intercalary allograft in a dog model. *J Orthop Res*. 2001; 19:308–17. [PubMed: 11347706]
51. Belfrage O, Flivik G, Sundberg M, Kesteris U, Tagil M. Local treatment of cancellous bone grafts with bmp-7 and zoledronate increases both the bone formation rate and bone density: A bone chamber study in rats. *Acta Orthop*. 2011; 82:228–33. [PubMed: 21434769]
52. McKay WF, Peckham SM, Badura JM. A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (infuse bone graft). *Int Orthop*. 2007; 31:729–34. [PubMed: 17639384]
53. White AP, Vaccaro AR, Hall JA, Whang PG, Friel BC, McKee MD. Clinical applications of bmp-7/op-1 in fractures, nonunions and spinal fusion. *Int Orthop*. 2007; 31:735–41. [PubMed: 17962946]
54. LeBaron RG, Athanasiou KA. Extracellular matrix cell adhesion peptides: Functional applications in orthopedic materials. *Tissue Eng*. 2000; 6:85–103. [PubMed: 10941205]
55. Balasundaram G, Yao C, Webster TJ. Tio2 nanotubes functionalized with regions of bone morphogenetic protein-2 increases osteoblast adhesion. *J Biomed Mater Res A*. 2008; 84:447–53. [PubMed: 17618492]
56. Choi JY, Jung UW, Kim CS, Eom TK, Kang EJ, Cho KS, et al. The effects of newly formed synthetic peptide on bone regeneration in rat calvarial defects. *J Periodontal Implant Sci*. 2010; 40:11–8. [PubMed: 20498754]
57. Shen H, Hu X, Yang F, Bei J, Wang S. The bioactivity of rhbmp-2 immobilized poly(lactide-co-glycolide) scaffolds. *Biomaterials*. 2009; 30:3150–7. [PubMed: 19232709]
58. Schuessele A, Mayr H, Tessmar J, Goepferich A. Enhanced bone morphogenetic protein-2 performance on hydroxyapatite ceramic surfaces. *J Biomed Mater Res A*. 2009; 90:959–71. [PubMed: 18655137]
59. Zouani OF, Chollet C, Guillotin B, Durrieu MC. Differentiation of pre-osteoblast cells on poly(ethylene terephthalate) grafted with rgd and/or bmps mimetic peptides. *Biomaterials*. 2010; 31:8245–53. [PubMed: 20667411]
60. Ketonis C, Barr S, Shapiro IM, Parvizi J, Adams CS, Hickok NJ. Antibacterial activity of bone allografts: Comparison of a new vancomycin-tethered allograft with allograft loaded with adsorbed vancomycin. *Bone*. 2011; 48:631–8. [PubMed: 21035576]
61. Smucker JD, Bobst JA, Petersen EB, Nepola JV, Fredericks DC. B2a peptide on ceramic granules enhance posterolateral spinal fusion in rabbits compared with autograft. *Spine*. 2008; 33:1324–9. [PubMed: 18496344]
62. Cunningham BW, Atkinson BL, Hu N, Kikkawa J, Jenis L, Bryant J, et al. Ceramic granules enhanced with b2a peptide for lumbar interbody spine fusion: An experimental study using an instrumented model in sheep. *J Neurosurg Spine*. 2009; 10:300–7. [PubMed: 19441986]
63. Yokogawa K, Miya K, Sekido T, Higashi Y, Nomura M, Fujisawa R, et al. Selective delivery of estradiol to bone by aspartic acid oligopeptide and its effects on ovariectomized mice. *Endocrinology*. 2001; 142:1228–33. [PubMed: 11181539]
64. Yokogawa K, Toshima K, Yamoto K, Nishioka T, Sakura N, Miyamoto K. Pharmacokinetic advantage of an intranasal preparation of a novel anti-osteoporosis drug, l-asp-hexapeptide-conjugated estradiol. *Biol Pharm Bull*. 2006; 29:1229–33. [PubMed: 16755022]
65. Kasugai S, Fujisawa R, Waki Y, Miyamoto K, Ohya K. Selective drug delivery system to bone: Small peptide (asp)₆ conjugation. *J Bone Miner Res*. 2000; 15:936–43. [PubMed: 10804024]
66. Takahashi T, Yokogawa K, Sakura N, Nomura M, Kobayashi S, Miyamoto K. Bone-targeting of quinolones conjugated with an acidic oligopeptide. *Pharm Res*. 2008; 25:2881–8. [PubMed: 18663412]

FIG 1. Enhanced loading of E7-modified DGEA to allograft bone

Equimolar concentrations of FITC tagged DGEA or E7DGEA were used to coat (a) 1–50mg of bone chips or (b&c) 25mg of bone chips. (a) Peptide binding from solution was measured by evaluating fluorescence of coating solutions after 2 hours of binding to 1–50mg of allograft. (b) The time course for binding of DGEA or E7DGEA was evaluated by coating 25mg of bone chips for up to 24 hours and measuring percent peptide bound. (c) Fluorescent microscopy was used as a qualitative measure to visualize differences in peptide loading at 2 hours.

FIG 2. Greater retention of DGEA peptide facilitated by E7 domain

(a) Peptide coated allograft bone chips were agitated for up to 7 days and imaged for retention of FITC tagged DGEA or E7DGEA. Equimolar solutions of FITC tagged DGEA or E7DGEA were used to coat 25 mg of allograft bone chips. (b) After 2 hours of coating, peptide loading was evaluated by measuring fluorescence present in coating solution. (c) Remaining coating solution was discarded, and bone chips were resuspended into fresh TBS. The TBS solution was monitored over 5 days, and fluorescence values were used to quantify percent of bound peptide released from allograft surface (of note, some error bars were too small to be visualized). (d) Percent peptide originally bound and peptide released over 5 days was used to calculate relative peptide density on the surface of allograft.

FIG 3. E7-domain facilitated enhanced retention of DGEA peptide in vivo
FITC-tagged DGEA or E7DGEA (10 μ M) was used to coat allograft bone chips for 2 hours. Bone chips were then implanted into dorsal subcutaneous skin pouches in rats for up to 3 months. Peptide-coated chips were retrieved following 1 or 3 months implantation and imaged using fluorescent microscopy to qualitatively evaluate peptide retention.

FIG 4. E7DGEA targeted to bone following IV injection

DGEA-FITC, E7DGEA-FITC or a TBS control was injected into the tail vein of rats. After 24 hours, rats were sacrificed and bones were harvested. Whole tibiae and fibulae were imaged using fluorescent microscopy to evaluate relative peptide density.

FIG 5. Enhanced E7-loading is specific to bone tissue

Neonatal mice were sacrificed, embedded with OCT, and sectioned using a cryostat. Mouse sections were then coated with equimolar FITC tagged- DGEA or E7DGEA and imaged for peptide specificity using identical parameters to allow for comparative analysis. Sections of (a) thigh, (b) rib and (c) whole body (lacking limbs) were qualitatively evaluated for peptide binding to bone and surrounding soft tissue.

FIG 6. Enhanced loading and retention of E7-modified BMP2 derived peptide

Equimolar concentrations of FITC-tagged BMP2 and E7BMP2 peptides were coated onto allograft. (a) We then imaged bone chips after 2 hours to qualitatively evaluate loading of BMP2 peptides. (b) Peptide coated bone chips were resuspended in fresh TBS, washed with agitation for 7 days and then imaged for retention of peptides. (c) To evaluate retention of BMP2 peptides in vivo, peptide coated allograft was implanted into a rat subcutaneous pouch for 1 month. Rats were then sacrificed, and bone chips were retrieved and fluorescently imaged.