Published in final edited form as:
Biomaterials. 2007 July ; 28(21): 3228–3235.

Biomolecular surface coating to enhance orthopaedic tissue healing and integration

Catherine D. Reyes[1,2], **Timothy A. Petrie**[1,2], **Kellie L. Burns**[1,2], **Zvi Schwartz**[2,3,4], and **Andrés J. García**[*,1,2]

1*Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA, USA*

2*Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, USA*

3*Coulter Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA, USA*

4*Department of Periodontics, Hebrew University, Jerusalem, Israel*

Abstract

Implant osseointegration is a prerequisite for clinical success in orthopaedic and dental applications, many of which are restricted by loosening. Biomaterial surface modification approaches, including calcium-phosphate ceramic coatings and macro/microporosity, have had limited success in promoting integration. To improve osseointegration, titanium surfaces were coated with the GFOGER collagen-mimetic peptide, selectively promoting $\alpha_2\beta_1$ integrin binding, a crucial event for osteoblastic differentiation. Titanium surfaces presenting GFOGER triggered osteoblastic differentiation and mineral deposition in bone marrow stromal cells, leading to enhanced osteoblastic function compared to unmodified titanium. Furthermore, this integrin-targeted coating significantly improved *in vivo* peri-implant bone regeneration and osseointegration, as characterized by bone-implant contact and mechanical fixation, compared to untreated titanium in a rat cortical bone-implant model. GFOGER-modified implants also significantly enhanced osseointegration compared to surfaces modified with full-length type I collagen, highlighting the importance of presenting specific biofunctional domains within the native ligand. In addition, this biomimetic implant coating is generated using a simple, single-step procedure that readily translates to a clinical environment with minimal processing and cytotoxicity concerns. Therefore, this study establishes a biologically active and clinically relevant implant coating strategy that enhances bone repair and orthopaedic implant integration.

Keywords

biomimetic material; cell adhesion; collagen; osseointegration; integrin

INTRODUCTION

Biomaterial surface properties regulate cellular and host responses to implanted devices, biological integration of biomedical prostheses and tissue-engineered constructs, and the performance of biotechnological arrays and cell culture supports [1-4]. Upon implantation,

*Corresponding Author: Andrés J. García, Ph.D. Woodruff School of Mechanical Engineering Georgia Institute of Technology 315 Ferst Drive, Room 2314 IBB Atlanta, GA 30332-0363 andres.garcia@me.gatech.edu Phone: 404-894-9384; Fax: 404-385-1397

synthetic materials elicit an inflammatory response that results in a foreign body reaction and fibrous encapsulation [1]. The foreign body reaction severely limits device integration and *in vivo* performance of numerous biomedical devices, including chemical biosensors, electrical leads/electrodes, therapeutic delivery systems, and orthopaedic and cardiovascular prostheses. Extensive efforts have concentrated on surface treatments and coatings to improve host tissue-implant integration. For instance, current orthopaedic and dental implant surface technologies focus on micro/macroporous coatings for bone ingrowth and calcium-phosphate ceramic coatings to promote integration with the surrounding bone [5,6]. However, while these approaches are generally successful, they are restricted by slow rates of osseointegration and poor mechanical anchorage in challenging clinical cases, such as those associated with large bone loss and poor bone quality [7,8].

Recent surface modification approaches to improve bone formation and osseointegration center on the immobilization of extracellular matrix components, including cell adhesive proteins or synthetic peptides derived from matrix molecules such as type I collagen and fibronectin [9-12]. The rationale for these strategies is that binding of cellular integrin receptors to these bioactive adhesive motifs activates signaling pathways that promote osteoblastic differentiation and matrix mineralization [13]. While full-length extracellular matrix proteins represent attractive targets for functionalizing biomaterial surfaces because of their inherent bioactivity, these whole-protein strategies are limited by immunogenicity and complexities associated with purification and processing as well as the risk of pathogen transmission [14]. In addition, native extracellular matrix proteins often have binding sites for other biological ligands, such as fibrinogen, complement, or von Willebrand factor, which may trigger sub-optimal healing responses to the implanted biomedical device. To address these limitations, significant efforts have focused on short synthetic analogs that present the bioadhesive motif. The most common peptide-based strategy involves the surface deposition of peptides containing the argineglycine-aspartic acid (RGD) sequence, which mediates cell attachment to several matrix proteins, including fibronectin, vitronectin, osteopontin, and bone sialoprotein. However, these bio-inspired strategies have only yielded marginal increases in implant osseointegration and mechanical fixation [12,15,16]. An explanation for the disappointing results with RGD-functionalized implants is that this peptide, while specific for integrins, lacks selectivity among integrins and therefore triggers non-discriminatory cell attachment. Therefore, engineering peptides that selectively target integrin signaling cascades implicated in specific tissue responses, for example osteogenesis, would allow the optimization of surface coatings for enhanced integration and biological performance.

The $\alpha_2\beta_1$ integrin is highly expressed on osteoblasts and is one of the predominant adhesion receptors for type I collagen [17]. $\alpha_2\beta_1$ integrin-type I collagen interactions provide crucial signals for the induction of osteoblastic differentiation and matrix mineralization [18-24]. For example, $\alpha_2\beta_1$-mediated osteoblast adhesion to type I collagen activates Runx2/Cbfa1 [25], a transcription factor that regulates osteogenesis. Furthermore, the collagen-$\alpha_2\beta_1$ integrin interaction induces osteoblastic differentiation in multipotent bone marrow stromal cells [23, 24].

Integrin $\alpha_2\beta_1$ recognizes the glycine-phenylalanine-hydroxyproline-glycine-glutamatearginine (GFOGER) motif in residues 502-507 of the α1[I] chain of type I collagen [26,27]. This sequence fully supported $\alpha_2\beta_1$-dependent cell adhesion and exhibited divalent cation-dependent binding to isolated $\alpha_2\beta_1$ and recombinant α_2 I-domain. Importantly, recognition of this sequence is entirely dependent upon the presence of a triple helical conformation, emphasizing the crucial role of collagen's tertiary structure in $\alpha_2\beta_1$ integrin binding [28]. In the present analysis, we generated biomaterial coatings presenting a triple-helical, collagen-mimetic GFOGER peptide. We hypothesized that coating titanium implants

with this peptide enhances peri-implant bone formation and mechanical osseointegration, thus providing a simple, clinically relevant strategy for improving implant osseointegration.

METHODS

Cell isolation and culture

Primary bone marrow stromal cells were harvested from the long bones of young adult male Wistar rats in accordance with an IACUC-approved protocol [29]. After excision, hindleg femora and tibiae were cleared of soft tissue and rinsed in growth medium (α-minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and 0.3 μg/ml amphotericin B). The ends of the bones were removed and the marrow space was flushed with culture medium (3-5 ml), using a syringe with an 18-gauge needle. Marrow isolates were pooled, centrifuged, resuspended in growth medium, and seeded for adhesion-dependent selection on tissue culture polystyrene dishes. Non-adherent hematopoietic cells were removed during subsequent medium exchanges, which occurred every other day. Cells were subcultured every two days according to standard techniques. For *in vitro* osteogenic assays, cells were seeded at 10,000 cells/cm^2 in growth medium. After 24 h, cultures were maintained in osteogenic medium consisting of growth medium supplemented with 50 μg/ml L-ascorbic acid and 3 mM sodium β-glycerophosphate.

GFOGER peptide surface coating

The peptide GGYGGGPC$(GPP)_5$GFOGER$(GPP)_5$GPC [O=hydroxyproline] was prepared by the Emory University Microchemical Facility using solid phase t-Boc synthesis [30]. For *in vitro assays*, glass chamber slides (16-well Lab-Tek Chamber Slides, Nalge Nunc) or tissue culture-treated polystyrene dishes were coated with 300 Å of pure titanium using an electron beam evaporator at a chamber base pressure between 1-2 × 10^{-6} torr with a deposition rate of 1.5 Å/second. The GFOGER peptide was diluted to 20 μg/ml in Dulbecco's phosphate-buffered saline (PBS) and incubated on the titanium surfaces for 1 h at 22°C. Surface density measurements were obtained by surface plasmon resonance using a Biacore X instrument [31].

Cell adhesion assay

Cell adhesion to functionalized and untreated titanium surfaces was measured using a centrifugation assay that applies controlled detachment forces [32]. Titanium-coated glass chamber slide wells were reassembled using a silicone-based adhesive and coated with 20 μg/ml GFOGER peptide or 20 μg/ml GRGDSPC peptide (BACHEM). Control titanium slides were coated with 10% fetal bovine serum (to model serum protein adsorption) or blocking buffer (5% non-fat dry milk in PBS to produce a non-adhesive support). Stromal cells were loaded with the fluorescent dye calcein-AM (2 μg/ml, Molecular Probes), detached using trypsin + EDTA, and resuspended serum-free in PBS with 2 mM dextrose. Cells were seeded onto the substrates (10,000 cells/well) for 1 h at 37°C. For blocking experiments, cells were incubated in the presence of 20 μg/ml anti-rat α_2 antibody (hamster anti-rat CD49b monoclonal antibody, clone Ha1/29, BD Pharmingen) or 20 μg/ml anti-rat α_v antibody (mouse anti-rat integrin α_v chain monoclonal antibody, clone 21, BD Pharmingen). Isotype control antibodies had no effect on cell adhesion (data no shown). Initial fluorescence intensity was measured to quantify the number of adherent cells prior to application of centrifugal force. After filling the wells with PBS and sealing with transparent adhesive tape, substrates were inverted and spun at a fixed speed in a centrifuge to apply a centrifugal force corresponding to 12*g*. After centrifugation, media was exchanged and fluorescence intensity was read to measure remaining adherent cells. For each well, adherent cell fraction was calculated as the ratio of post-spin to pre-spin fluorescence readings.

Osteoblast-specific gene expression

Gene expression was analyzed by qRT-PCR. Total RNA was isolated at 7 days in culture using the Qiagen RNeasy RNA isolation kit. During RNA isolation and purification, samples were treated with DNaseI (27 Kunitz units/sample) for 15 min at room temperature to eliminate any genomic DNA contamination. The concentration of purified RNA was quantified using a NanoDrop (NanoDrop Technologies) and 1 μg of total RNA was used to synthesize cDNA templates by oligo(dT) priming using the Superscript First-Strand cDNA Synthesis System.

Quantitative RT-PCR (qRT-PCR) was performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems; 40 cycles; melting for 15 s at 95°C; annealing and extending for 60 s at 60°C) using SYBR Green DNA intercalating dye. Gene transcript concentration in the sample cDNA template solutions was quantified by preparing a functional range of dilutions from an absolute standard for each gene. Linear standard curves were then generated by plotting the log of the known concentration versus the C_T value (the cycle number at which the fluorescence reached a threshold level). Oligonucleotide primers were designed using Primer Express software (Applied Biosystems). The following forward and reverse primers (accession number in parenthesis) were used: Runx2 (NM009820): 5'-GGCCTTCAAGGTTGTAGCCC-3', 5'-CCCGGCCATGACGGTA-3'; OCN (X04141): 5'-ACGAGCTAGCGGACCACATT-3', 5'-CCCTAAACGGTGGTGCCATA-3'; BSP (J04215): 5'-TGACGCTGGAAAGTTGGAGTT-3', 5'-GCCTTGCCCTCTGCATGTC-3'.

Alkaline phosphatase (ALP) biochemical activity and calcium incorporation assays

ALP activity was quantified at 7 days in culture using a modification of the Sodek and Berkman method [33]. Briefly, cells were rinsed with PBS and scraped in cold 50 mM Tris-HCl. After sonication and centrifugation, the protein concentration was quantified using a Pierce Micro BCA protein assay kit. Equal amounts of protein (2.5 μg) were added to 60 μg/ml 4-methyl-umbelliferyl-phosphate fluorescent substrate in diethanolamine buffer (pH 9.5). After a 60 min incubation at 37°C, the fluorescence was read at an excitation of 360 nm and an emission of 465 nm on an HTS 7000 Plus BioAssay Reader (Perkin Elmer). Enzymatic activity was standardized using purified calf intestinal alkaline phosphatase.

Calcium content was determined by dissolving mineralized deposits with 1 N acetic acid overnight. Appropriately diluted sample (25 μl) was added to 300 μl of arsenazo III-containing Calcium Reagent (Diagnostic Services Ltd). The absorbance of the resulting samples was measured at 650 nm and compared to a linear standard curve of $CaCl_2$ in 1 N acetic acid.

Implantation procedure

Custom-machined, commercially pure titanium implants were sonicated in de-ionized water for 20 min to remove surface debris. Implants were then dipped in 4% HF for 30 sec to remove the existing oxide layer and then incubated in 35% HNO_3 for 30 min at 50 °C to regenerate a new oxide coating. Samples were transferred to 1.8 N NaOH for 1 min to terminate the oxidation reaction. Implants were then rinsed and boiled in de-ionized water for 1 h. To create the bioactive coating, the implants were incubated in 20 μg/ml GFOGER peptide or purified bovine type I collagen solution (Vitrogen-100; Cohesion, Palo Alto) for 1 h. Control titanium rods were incubated in PBS.

Implantations were conducted in accordance with an IACUC-approved protocol. Both hind legs of anesthetized, mature Sprague-Dawley male rats (250-350 g) were shaved and scrubbed with alcohol. The medial aspect of the proximal tibial metaphysis was exposed through an antero-medial skin incision, leaving the medial collateral ligament intact. Using a saline-cooled drill, two defects were created in each tibia. Sterile implant rods were press fit into the defects. Periosteum was mobilized and sutured over the implantation site, and the skin was closed with

wound clips. Subjects were euthanized after 4 weeks and proximal tibiae were fixed in neutral buffered formalin for histology or recovered without fixation and maintained in PBS-moistened gauze for immediate mechanical testing.

Based on power calculations, we estimated that a minimum of eight implants per experimental group are required to detect differences of 10% in mechanical testing and a minimum of four implants per experimental group are required for histomorphometry for a total of 11 implants per experimental group. In this model, each animal receives four implants, two in each tibia. The sample conditions were distributed according to a randomized block design, in which the three conditions were randomized according to proximal/distal and left/right tibia placement, but were constrained into blocks containing one each of the conditions. We used a total of 12 animals with 16 implants per condition (histology [7], mechanical testing [9]). An additional animal with one implant per condition was included as an extra.

Histomorphometry analyses and mechanical testing

Excised tibiae were fixed in 10% neutral buffered formalin for 1 week. The formalinfixed tibiae were dehydrated in a graded series of alcohol incubations and then embedded in poly(methyl methacrylate). Ground sections of 50-80 μm were generated using the Exakt Grinding System. Two longitudinal ground sections were generated per tibia, each containing two titanium plugs inserted transverse to the tibia's long axis. Sections were then stained with Sanderson's Rapid Bone Stain™ and a van Gieson counter stain. Bone-implant contact (BIC) was measured as the percentage of implant's circumference that was in direct contact with bone tissue.

Implant mechanical fixation to the bone was measured with a pull-out force test using a biomechanical testing apparatus (EnduraTEC Bose ELF 3200). The ends of each excised tibia were secured in a custom designed holding apparatus with the exposed head of each implant facing in the direction of the pull motion and centered along the axis of motion. A 0.014” diameter piano wire was threaded through the implant head and both wire ends attached firmly to an 11 lb. INTERFACE load cell. Samples were pre-loaded with 2 N to ensure proper and identical wire tautness among implants. Tests were performed at a constant force rate of 0.2 N/sec using WINTEST application software. The direction of the pull was parallel to the long axis of the implant. The pull-out force (N) was the maximum load achieved before failure and was determined from the recorded load vs. displacement data.

Statistics

Data are reported as mean ± standard error. Results were analyzed by one-way ANOVA using SYSTAT 8.0 (SPSS). If treatment level differences were determined to be significant, pair-wise comparisons were performed using Tukey post-hoc test. A 95% confidence level was considered significant. All of the *in vitro* assays were performed as two separate experiments in triplicate.

RESULTS

GFOGER peptide promotes $\alpha_2\beta_1$ integrin-specific cell adhesion to titanium supports

We previously designed a triple-helical, collagen-mimetic peptide [GGYGGGPC $(GPP)_5$GFOGER$(GPP)_5$GPC] that contains the GFOGER adhesion motif from type I collagen that is recognized by the $\alpha_2\beta_1$ integrin [30]. Circular dichroism analysis demonstrated that this peptide adopts a stable triple-helical conformation similar to the native structure of type I collagen. This collagen-mimetic peptide specifically targets the $\alpha_2\beta_1$ integrin receptor and promotes density-dependent cell adhesion, focal adhesion kinase signaling, and osteoblastic differentiation in the MC3T3-E1 immature osteoblast cell line [30,34].

To reproduce titanium implant surfaces *in vitro*, polystyrene culture dishes were coated with a 300 Å titanium layer via electron beam evaporation. The GFOGER peptide was then passively adsorbed onto the titanium at a concentration of 20 μg/ml, creating the integrin-targeted bioactive coating. We selected this concentration because it produced a saturated coating of GFOGER. Surface plasmon resonance spectroscopy revealed a surface density of 123.2 ± 6.2 ng/cm^2. Primary rat bone marrow stromal cells were used to validate this surface treatment strategy *in vitro* since this heterogeneous population contains osteoprogenitors and human bone marrow stromal cells are currently used in clinical applications. A centrifugation cell adhesion assay demonstrated greater stromal cell adhesion on the GFOGER-peptide surfaces compared to titanium surfaces pre-exposed to linear RGD peptide or 10 % serum (Fig. 1). Because this adhesion assay is performed under serum-free conditions, the serum-exposed titanium surface was included to correspond to the untreated titanium surfaces in subsequent *in vitro* assays performed in the presence of serum. Control experiments demonstrated no differences in adhesion between cells plated under serum-free conditions on surfaces pre-exposed to 10% serum and cells seeded on untreated titanium in the presence of serum. Hence, the untreated (in the presence of serum) and serum-exposed surfaces are equivalent in terms of cell adhesion. Cell adhesion to the RGD-treated surface was equivalent to background levels observed on titanium blocked with non-adhesive proteins, reflecting the inability of this short peptide to passively adsorb onto titanium. Surface plasmon resonance measurements confirmed that low levels of RGD passively adsorbed onto titanium (equivalent to detection limit of instrument, 2 ng/cm^2; for serum-exposed surfaces, the density of adsorbed biomolecules was 195 ng/cm^2). Importantly, a blocking anti-α_2 antibody completely eliminated cell adhesion to GFOGER-treated surfaces, verifying the peptide's specificity for the $\alpha_2\beta_1$ integrin. However, this $\alpha_2\beta_1$ blocking antibody had no effect on adhesion to serum-exposed titanium, demonstrating that stromal cell adhesion to untreated titanium is not mediated by $\alpha_2\beta_1$ integrin. Since untreated titanium adsorbs abundant RGD-containing serum proteins, such as vitronectin, adhesion to these surfaces most likely involves the $\alpha_v\beta_3$ integrin, which recognizes RGD in a wide variety of proteins and synthetic peptides [35]. Indeed, a function-perturbing anti-α_v antibody had no effect on adhesion to the GFOGER peptide but completely blocked adhesion above background on the serum-exposed titanium. These results demonstrate that the bioactive GFOGER peptide specifically targets the $\alpha_2\beta_1$ integrin. These adhesion results also show that untreated titanium surfaces, which directly adsorb serum proteins, preferentially engage the $\alpha_v\beta_3$ integrin. Because GFOGER peptide-coated and control titanium surfaces each interact with unique integrins, these surfaces may recruit different cell populations at the implant site and/or have diverse effects on cellular maturation and bone formation *in vivo*.

GFOGER peptide triggers enhanced osteoblastic differentiation and mineralization

To investigate the osteoblastic differentiation potential of these surfaces, we used qRTPCR to probe osteoblast-specific gene expression in 7 day cultures of bone marrow stromal cells (Fig. 2). Expression levels of Runx2/Cbfa1, a transcription factor essential for bone formation and osteoblastic differentiation [36], were elevated on the GFOGER-treated surfaces compared to untreated titanium (Fig. 2). The upregulation of this key osteoblast-specific transcription factor demonstrates the ability of the bioactive GFOGER-peptide surface to trigger the transcriptional machinery necessary for osteoblastic differentiation. To determine whether this pattern of increased Runx2 gene expression parallels similar increases in the expression of other osteoblast-specific genes, the transcript levels of osteocalcin and bone sialoprotein were also examined. For both bone-specific markers, qRT-PCR revealed greater levels of gene expression on the GFOGER-peptide surfaces compared with untreated titanium (Fig. 2a). These results indicate that the $\alpha_2\beta_1$ integrin-targeted peptide promotes the expression of multiple genes specifically associated with a mature osteoblastic phenotype.

Osteoblastic differentiation is also characterized by the activation of multiple proteins, including alkaline phosphatase (ALP). The ALP enzyme is often used as a marker for osteoblastic metabolic activity and an early indicator of osteoblastic differentiation [37]. An ALP biochemical assay revealed elevated levels of activation on the GFOGER-peptide coating compared to untreated titanium (Fig. 3A). Because ALP is the enzyme responsible for hydrolyzing phosphate esters and inducing bone mineralization [37], these results suggest that this bioactive surface treatment may also be capable of promoting enhanced bone matrix mineralization.

Matrix mineralization was examined as an *in vitro* end-point indicator of the osteoblastic phenotype in the bone marrow stromal cells. Calcium phosphate mineral deposition was examined after 14 days in culture via calcium content analysis. Cultures on GFOGER-treated surfaces displayed a three-fold enhancement in calcium-based mineral deposition compared to untreated titanium (Fig. 3B). This enhanced capacity for mineralization on the GFOGER peptide-treated surfaces is in excellent agreement with the observed up-regulation in osteoblast-specific gene expression and ALP activity. These results verify the advantageous effects of controlled $\alpha_2\beta_1$ integrin-binding on cell function, in this case osteoblastic differentiation and matrix mineralization.

GFOGER coatings enhance bone formation and osseointegration of orthopaedic implants

To evaluate the performance of the bioactive GFOGER peptide treatment *in vivo*, we quantified osseointegration in a rat tibia cortical bone model using quantitative histomorphometry and pull-out mechanical testing [38]. We designed a cylindrical titanium implant rod with a tapered stop collar (Fig. 4A). The tapered head ensures that all implants are inserted into the bone at the same depth, guaranteeing uniform bone contact among treatments. Using a saline-cooled drill, two defects (2 mm diameter) were created in the medial aspect of the proximal tibial metaphysis. Implant rods consisting of GFOGER peptide-functionalized, full-length type I collagen, or untreated (control) titanium were press fit into the cortical defects (Fig. 4B). We have previously demonstrated that the purified bovine type I collagen used in these experiments adopts a triple helical conformation in solution and engages the $\alpha_2\beta_1$ integrin. It is included as a reference surface comparing the GFOGER peptide to the full-length matrix protein.

After four weeks, the rat tibiae were harvested and evaluated for bone apposition by histological staining and mechanical integration by pull-out testing. Histological sections revealed substantial and contiguous bone matrix (orange stain) along the periphery of GFOGER-coated titanium implants (Fig. 4C). Less bone mineral was visible on surfaces treated with full-length type I collagen, and the adjacent mineral appears more porous. Significantly less mineral staining was present on untreated titanium and the mineral deposits appear in isolated patches along the surface of the implants. Image quantification to determine the percentage of the bone-implant apposition (bone implant contact, BIC) demonstrated a nearly two-fold enhancement in bone apposition on the GFOGER peptide-coated surfaces compared to untreated titanium or implants coated with the full-length type I collagen (Fig. 4D). No evidence of foreign body giant cell persistence or fibrous capsule was observed in any of the histological sections. Importantly, pull-out mechanical testing indicated significantly higher mechanical fixation of the GFOGER peptide-functionalized implants compared to type I collagen-coated or untreated titanium (Fig. 4D). These results demonstrate a greater quantity and continuity of peri-implant bone formation on the integrin-targeted GFOGER peptide surfaces *in vivo* as well as enhanced mechanical integrity and osseointegration. In addition, the biomimetic peptide induced greater bone formation and apposition than the native full-length extracellular matrix protein coating, demonstrating the benefit of integrin-target mimetic peptides over whole biomolecules.

DISCUSSION

This work proposes a specific biomolecular strategy to improve bone regeneration and osseointegration by exploiting the cell adhesive activity of type I collagen, the most abundant matrix component in bone. In particular, type I collagen modulates intracellular signal transduction by binding to the $\alpha_2\beta_1$ integrin, which enhances the expression of the osteoblastic phenotype [23,39,40]. It also exhibits low immunogenicity and high conformational stability, making it suitable for implantation applications. However, designing surface treatments using full-length matrix molecules, such as type I collagen, is often limited by a lack of specificity for particular integrins and thus exhibit minimal control over cellular responses. In addition, native matrix proteins often have binding sites for other ligands, which may trigger antagonistic signaling cascades that may ultimately interfere with desired healing responses. The GFOGER peptide strategy described in this study targets the $\alpha_2\beta_1$ integrin-ligand interaction that is crucial for the development and maintenance of the osteoblast phenotype as well as the mineralization of the extracellular matrix. *In vitro* assays using bone marrow stromal cells verified that a GFOGER peptide coating enhances expression of multiple osteoblast-specific genes and alkaline phosphatase activity when compared to untreated titanium controls. This bioactive treatment also improved calcification of the extracellular matrix, demonstrating functional osteoblastic differentiation. Notably, the cortical bone implantation studies revealed greater bone tissue formation on the surface of GFOGER-treated titanium implants, in terms of both quantity and connectivity. Most significantly, we have shown that the GFOGER peptide coating improved the implant's mechanical fixation and functional osseointegration as determined by a quantitative pull-out test. Faster integration of these GFOGER coated implants would result in sooner and more reliable loading in a clinical setting, improving device function and patient outcomes. Not only does this bioactive coating enhance bone formation and implant integration, but it is also created using a single-step procedure conducted under physiological conditions, thus eliminating the cytotoxicity and biocompatibility concerns associated with covalent immobilization methods. As such, this GFOGER peptide surface treatment represents a simple, clinically relevant approach to improving orthopaedic and dental titanium implant integration. Due to the fundamental character of receptor-ligand principles and the significance of cell-collagen interactions in multiple tissues, this material coating strategy may also have the potential to improve implant integration in non-orthopaedic tissue systems.

CONCLUSION

Titanium surfaces passively coated with the GFOGER collagen-mimetic peptide promoted integrin $\alpha_2\beta_1$-mediated cell adhesion and osteoblastic differentiation. Moreover, GFOGER coating significantly enhanced osseointegration of titanium implants in rat cortical bone. This study establishes a biologically active and clinically relevant implant coating strategy that enhances bone repair and orthopaedic implant integration.

ACKNOWLEDGMENTS

This work was funded by the NIH (R01 EB-004496), Arthritis Foundation, and Georgia Tech/Emory NSF ERC on the Engineering of Living Tissues (EEC-9731643). C.D.R. was supported by a Whitaker Foundation Graduate Fellowship. The authors gratefully acknowledge the Emory University Microchemical Facility for peptide synthesis, T. Couse and Wasatch Histo Consultants for histology, and M. Mathews for implant machining.

REFERENCES

1. Anderson JM. Biological responses to materials. Annu Rev Mater Res 2001;31:81–110.
2. Hench LL, Polak JM. Third-generation biomedical materials. Science Feb 8;2002 295(5557):1014–7. [PubMed: 11834817]
3. Hubbell JA. Biomaterials science and high-throughput screening. Nat Biotechnol Jul;2004 22(7):828–9. [PubMed: 15229544]

4. Vreeland WN, Barron AE. Functional materials for microscale genomic and proteomic analyses. Curr Opin Biotechnol Apr;2002 13(2):87–94. [PubMed: 11950557]
5. Bauer TW, Schils J. The pathology of total joint arthroplasty. I. Mechanisms of implant fixation. Skeletal Radiol Aug;1999 28(8):423–32. [PubMed: 10486010]
6. Ducheyne P, Qiu Q. Bioactive ceramics: the effect of surface reactivity on bone formation and bone cell function. Biomaterials Dec;1999 20(2324):2287–303. [PubMed: 10614935]
7. Bauer TW, Schils J. The pathology of total joint arthroplasty.II. Mechanisms of implant failure. Skeletal Radiol Sep;1999 28(9):483–97. [PubMed: 10525792]
8. Sporer SM, Paprosky WG. Biologic fixation and bone ingrowth. Orthop Clin North Am Jan;2005 36 (1):105–11. [PubMed: 15542129]vii
9. Bernhardt R, Van Den DJ, Bierbaum S, Beutner R, Scharnweber D, Jansen J, et al. Osteoconductive modifications of Ti-implants in a goat defect model: characterization of bone growth with SR muCT and histology. Biomaterials Jun;2005 26(16):3009–19. [PubMed: 15603796]
10. Elmengaard B, Bechtold JE, Soballe K. In vivo effects of RGD-coated titanium implants inserted in two bone-gap models. J Biomed Mater Res A Nov 1;2005 75(2):249–55. [PubMed: 16106438]
11. Alsberg E, Anderson KW, Albeiruti A, Rowley JA, Mooney DJ. Engineering growing tissues. Proc Natl Acad Sci U S A Sep 17;2002 99(19):12025–30. [PubMed: 12218178]
12. Ferris DM, Moodie GD, Dimond PM, Gioranni CW, Ehrlich MG, Valentini RF. RGD-coated titanium implants stimulate increased bone formation in vivo. Biomaterials Dec;1999 20(2324):2323–31. [PubMed: 10614938]
13. Garcia AJ, Reyes CD. Bio-adhesive surfaces to promote osteoblast differentiation and bone formation. J Dent Res May;2005 84(5):407–13. [PubMed: 15840774]
14. Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol Jan;2005 23(1):47–55. [PubMed: 15637621]
15. Schliephake H, Scharnweber D, Dard M, Rossler S, Sewing A, Meyer J, et al. Effect of RGD peptide coating of titanium implants on periimplant bone formation in the alveolar crest. An experimental pilot study in dogs. Clin Oral Implants Res Jun;2002 13(3):312–9. [PubMed: 12010163]
16. Barber TA, Ho JE, De Ranieri A, Virdi AS, Sumner DR, Healy KE. Peri-implant bone formation and implant integration strength of peptide-modified p(AAM-co-EG/AAC) interpenetrating polymer network-coated titanium implants. J Biomed Mater Res A. Sep 7;2006
17. Gronthos S, Stewart K, Graves SE, Hay S, Simmons PJ. Integrin expression and function on human osteoblast-like cells. J Bone Miner Res Aug;1997 12(8):1189–97. [PubMed: 9258748]
18. Lynch MP, Stein JL, Stein GS, Lian JB. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: modification of expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. Exp Cell Res Jan;1995 216(1):35–45. [PubMed: 7813631]
19. 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 Nov 14;1997 272(46):29309–16. [PubMed: 9361011]
20. 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 Dec 4;1998 273 (49):32988–94. [PubMed: 9830051]
21. Jikko A, Harris SE, Chen D, Mendrick DL, Damsky CH. Collagen integrin receptors regulate early osteoblast differentiation induced by BMP-2. J Bone Miner Res Jul;1999 14(7):1075–83. [PubMed: 10404007]
22. Suzawa M, Tamura Y, Fukumoto S, Miyazono K, Fujita T, Kato S, et al. Stimulation of Smad1 transcriptional activity by Ras-extracellular signal-regulated kinase pathway: a possible mechanism for collagen-dependent osteoblastic differentiation. J Bone Miner Res Feb;2002 17(2):240–8. [PubMed: 11811554]
23. 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 Aug;2000 184 (2):207–13. [PubMed: 10867645]

24. Mizuno M, Kuboki Y. Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. J Biochem (Tokyo) Jan;2001 129(1):133–8. [PubMed: 11134967]
25. 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 Dec 4;1998 273 (49):32988–94. [PubMed: 9830051]
26. 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 Jan 7;2000 275(1):35–40. [PubMed: 10617582]
27. 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 Dec 11;1998 273(50):33287–94. [PubMed: 9837901]
28. Emsley J, Knight CG, Farndale RW, Barnes MJ, Liddington RC. Structural basis of collagen recognition by integrin alpha2beta1. Cell Mar 31;2000 101(1):47–56. [PubMed: 10778855]
29. Byers BA, Garcia AJ. Exogenous Runx2 expression enhances in vitro osteoblastic differentiation and mineralization in primary bone marrow stromal cells. Tissue Eng Nov;2004 10(1112):1623–32. [PubMed: 15684671]
30. Reyes CD, García AJ. Engineering integrin-specific surfaces with a triple-helical collagen-mimetic peptide. J Biomed Mater Res 2003;65A:511–23.
31. Petrie TA, Capadona JR, Reyes CD, Garcia AJ. Integrin specificity and enhanced cellular activities associated with surfaces presenting a recombinant fibronectin fragment compared to RGD supports. Biomaterials Nov;2006 27(31):5459–70. [PubMed: 16846640]
32. Reyes CD, Garcia AJ. A centrifugation cell adhesion assay for high-throughput screening of biomaterial surfaces. J Biomed Mater Res Oct 1;2003 67A(1):328–33.
33. Byers BA, Pavlath GK, Murphy TJ, Karsenty G, García AJ. Cell-type-dependent up-regulation of in vitro mineralization after overexpression of the osteoblast-specific transcription factor Runx2/Cbfal. J Bone Miner Res Nov;2002 17(11):1931–44. [PubMed: 12412799]
34. Reyes CD, Garcia AJ. Alpha2beta1 integrin-specific collagen-mimetic surfaces supporting osteoblastic differentiation. J Biomed Mater Res Jun 15;2004 69A(4):591–600.
35. Garcia AJ. Get a grip: integrins in cell-biomaterial interactions. Biomaterials Dec;2005 26(36):7525–9. [PubMed: 16002137]
36. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell May 30;1997 89(5):747–54. [PubMed: 9182762]
37. Aubin, JE.; Liu, F. The osteoblast lineage. In: Bilezikian, JP.; Raisz, LG.; Rodan, GA., editors. Principles of Bone Biology. Academic Press; San Diego: 1996. p. 69-88.
38. Branemark R, Ohrnell LO, Nilsson P, Thomsen P. Biomechanical characterization of osseointegration during healing: an experimental in vivo study in the rat. Biomaterials Jul;1997 18(14):969–78. [PubMed: 9212192]
39. 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 Nov 14;1997 272(46):29309–16. [PubMed: 9361011]
40. 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 Dec 4;1998 273 (49):32988–94. [PubMed: 9830051]

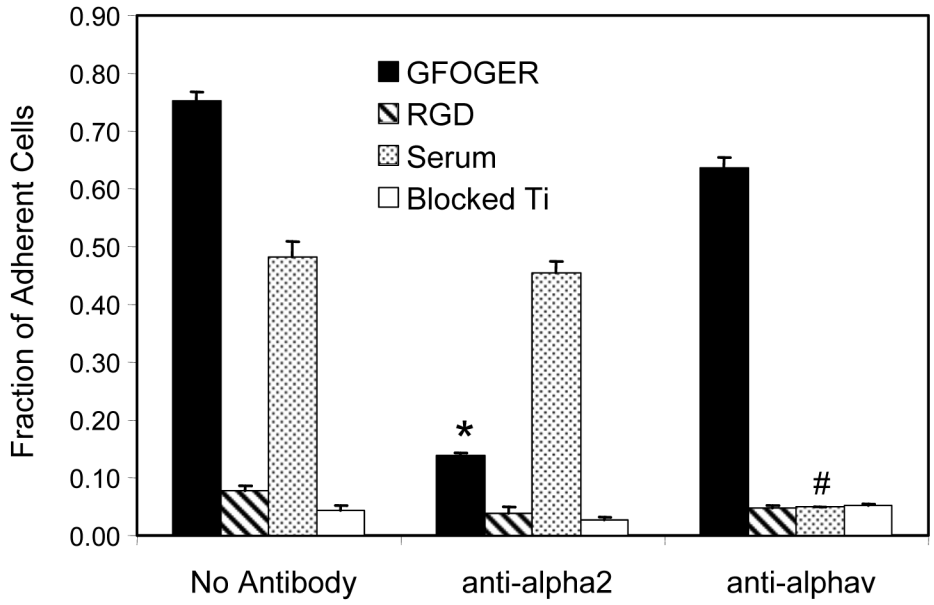

Fig. 1.

Cell adhesion is greater on adsorbed GFOGER surfaces than untreated titanium (Ti) and is specific for the $\alpha_2\beta_1$ integrin. Data represent 1 h serum-free bone marrow stromal cell adhesion and subsequent centrifugation at 12*g* for 5 min. Surfaces are adsorbed GFOGER peptide on Ti, adsorbed linear RGD peptide, adsorbed fetal bovine serum (10% in PBS), and non-adhesive blocked Ti. Cells were seeded without antibody or in the presence of either anti-α_2 or anti-α_v integrin blocking antibodies. ANOVA: $p<1E-9$; *GFOGER w/o Ab > GFOGER with anti-α_2 ($p<6E-6$); #serum w/o Ab > serum with anti-α_v ($p<6E-6$).

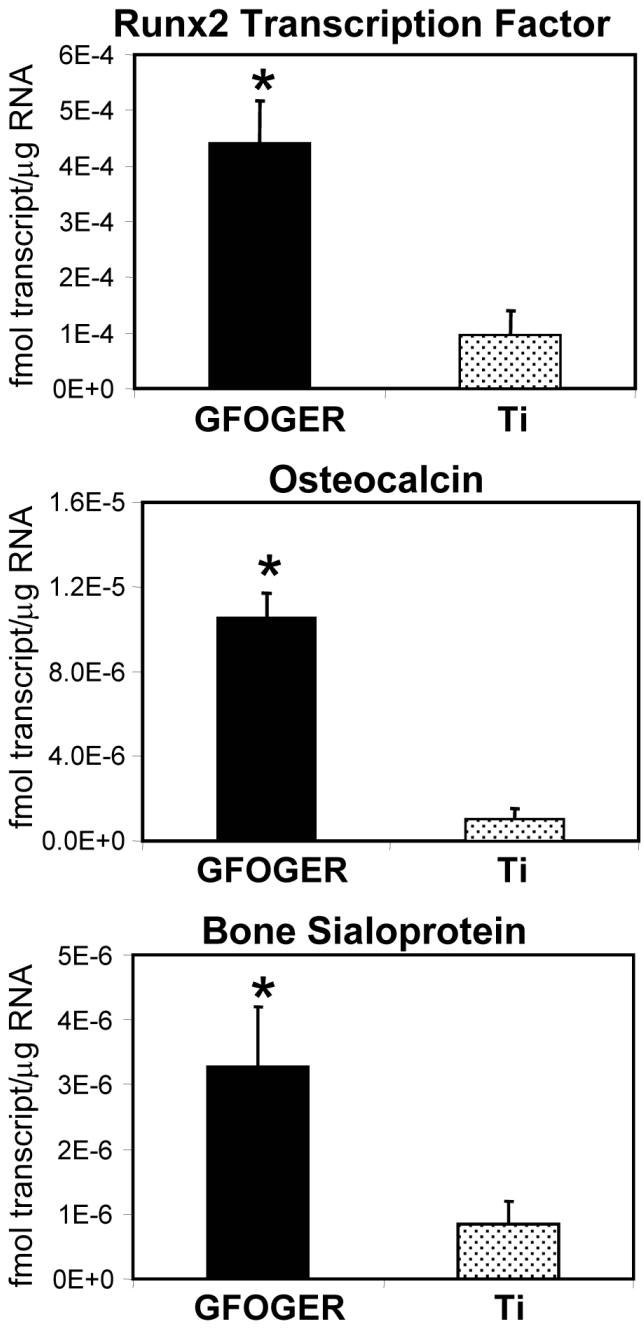

Fig. 2.

GFOGER surfaces promote osteoblast-specific gene expression in bone marrow stromal cells. mRNA transcripts were measured by qRT-PCR for Runx2 transcription factor, osteocalcin (OCN), and bone sialoprotein (BSP) in rat bone marrow stromal cells seeded for 7 days on GFOGER surfaces or untreated Ti. Runx2 ANOVA: *GFOGER > Ti ($p<0.02$); OCN ANOVA: *GFOGER > Ti ($p<0.002$); BSP ANOVA: *GFOGER > Ti ($p<0.05$).

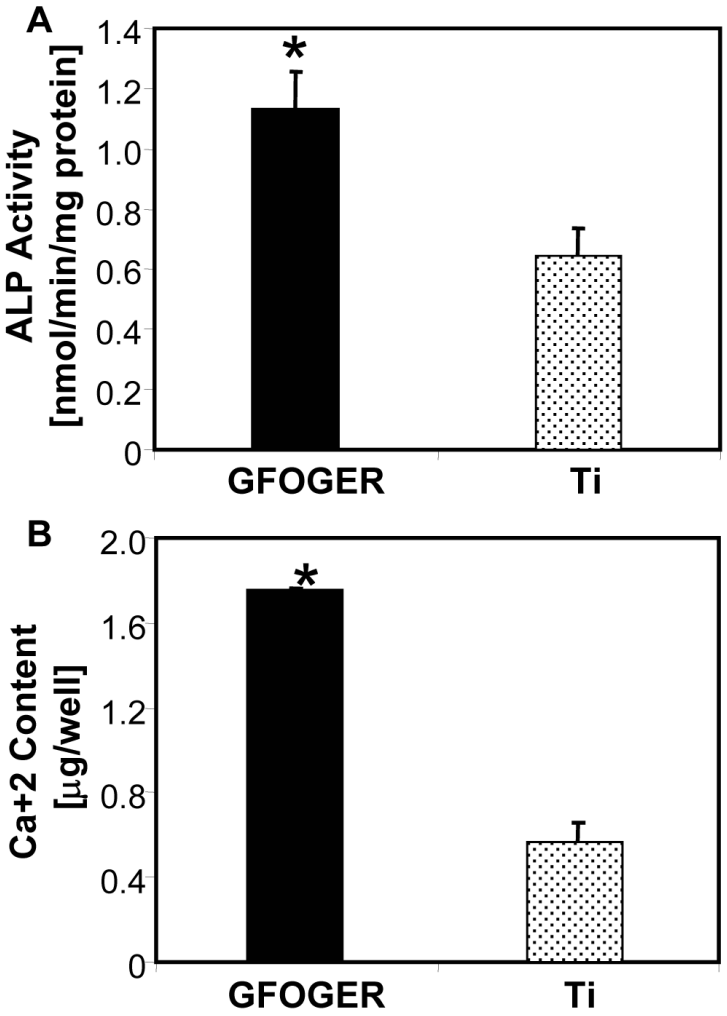

Fig. 3.

GFOGER surfaces enhance osteoblastic differentiation in bone marrow stromal cell cultures. **(A)** Alkaline phosphatase (ALP) activity and **(B)** matrix calcification in rat bone marrow stromal cultures compared to untreated titanium (Ti). ALP ANOVA: *GFOGER > Ti ($p<0.02$); Ca^{+2} ANOVA: *GFOGER > Ti ($p<2E-4$).

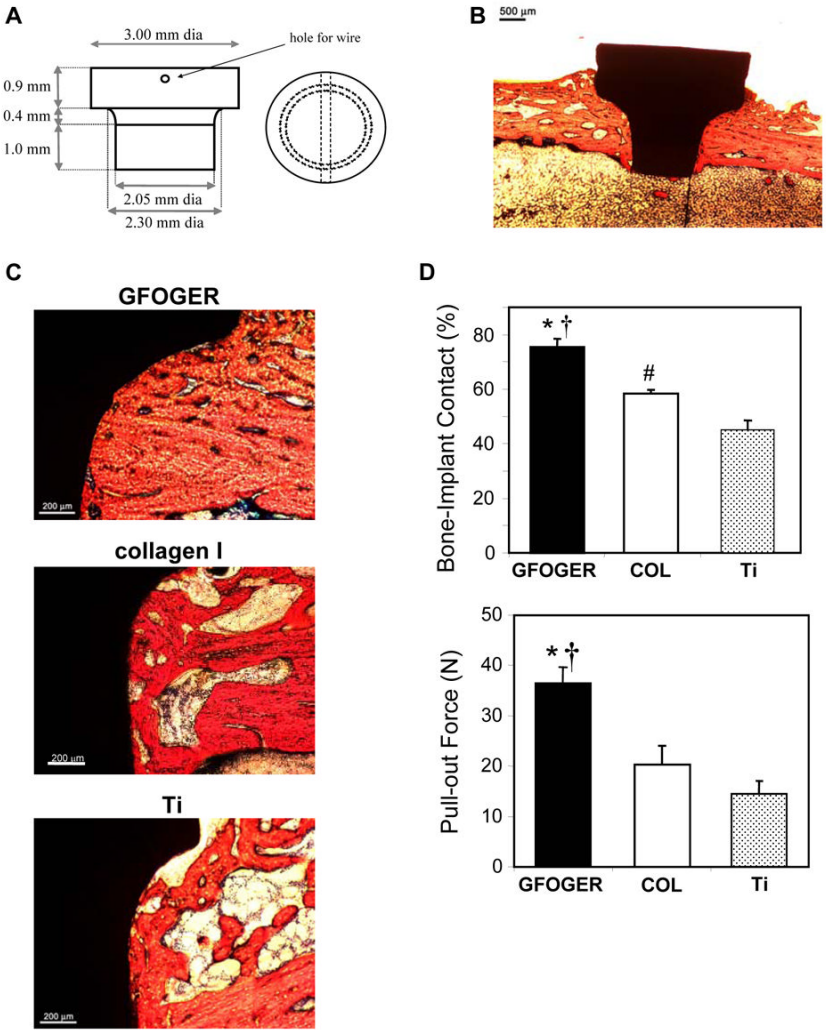

Fig. 4.

GFOGER peptide improves peri-implant bone formation and osseointegration in an *in vivo* rat tibia cortical bone implantation model. **(A)** Schematic diagram of tapered titanium implant. **(B)** Low magnification micrograph of implantation site showing implant placement. Longitudinal ground sections of rat tibiae stained with Sanderson's Rapid Bone Stain™ and van Gieson counterstain. Cells stain dark to light blue, soft tissue elements stain blue-green, and bone matrix stains yellow orange to autumn orange. **(C)** Representative micrographs of bone formation around titanium implants. GFOGER-coated implants exhibit greater amounts of newly formed bone at the implant surface compared to collagen-coated or untreated Ti. **(D)** Implant osseointegration as determined by bone-implant contact and mechanical fixation. Bone apposition is measured as the percentage of implant's circumference that is in direct contact with bone mineral in the histological sections. ANOVA: $p<4E-6$, *GFOGER > Ti ($p<0.002$), †GFOGER > COL ($p<0.01$), #COL > Ti ($p<0.04$). GFOGER surfaces demonstrate greater mechanical integration with the surrounding tissue compared to collagen-coated or untreated Ti. Osseointegration is measured as the maximum force [N] necessary to dislodge the implant in a pull-out test. ANOVA: $p<9E-7$, *GFOGER > Ti ($p<0.0009$), †GFOGER > COL ($p<0.01$).