Published in final edited form as:

Biomaterials. 2007 April ; 28(10): 1847–1861. doi:10.1016/j.biomaterials.2006.12.009.

Formation of Osteogenic Colonies on Well-Defined Adhesion Peptides by Freshly-Isolated Human Marrow Cells

Ada Au

Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Cynthia A. Boehm

Department of Orthopaedic Surgery and Biomedical Engineering, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

Anne M. Mayes

Department of Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

George F. Muschler

Department of Orthopaedic Surgery and Biomedical Engineering, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

Linda G. Griffith*

Department of Biological Engineering and Department of Mechanical, Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Abstract

Bone graft performance can be enhanced by addition of connective tissue progenitors (CTPs) from fresh bone marrow in a manner that concentrates the CTP cell population within the graft. Here, we used small peptide adhesion ligands presented against an otherwise adhesion-resistant synthetic polymer background in order to illuminate the molecular basis for the attachment and colony formation by osteogenic CTPs from fresh human marrow, and contrast the behavior of fresh marrow to many commonly-used osteogenic cell sources. The linear GRGDSPY ligand was as effective as tissue culture polystyrene in fostering attachment of culture-expanded porcine CTPs. Although this GRGDSPY peptide was more effective than control peptides in fostering alkaline phosphatase-positive (AP) colony formation from primary human marrow in 5 of the 7 patients tested, GRGDSPY was as effective as the control glass substrate in only one patient of 7. Thus, the peptide appears capable of enabling osteoblastic development from only a subpopulation of CTPs in marrow. The bone sialoprotein-derived peptide FHRRIKA was ineffective in fostering attachment of primary culture-expanded pig CTPs, although it was as effective as GRGDSPY in fostering AP-positive colonies from fresh human marrow. This study provides insights into integrin-mediated behaviors of CTPs and highlights differences between freshly-isolated marrow and culture-expanded cells.

*Corresponding Author, Linda Griffith MIT 16-429 77 Massachusetts Ave. Cambridge, MA 02139 Fax: 617-253-2400 Email: griff@mit.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.

Introduction

Autograft bone remains a gold standard for most clinical procedures requiring a bone graft, but the morbidity associated with harvest of bone grafts has prompted intense efforts to develop alternative graft strategies involving natural or synthetic scaffolds supplemented with growth factors or cells capable of forming bone. Bone marrow, a significant component of autograft bone, contains connective tissue progenitors (CTPs), defined as a heterogeneous population of stem cells and progenitor cells in native tissues which are individually capable of contributing to the formation of one or more connective tissues [2,3]. CTPs include osteogenic cells, which are required in any bone healing process. When human marrow aspirates are plated *in vitro* on glass or plastic, fewer than 0.01% of the nucleated cells adhere, but over 90% of the adherent colony-forming cells can differentiate along the osteoblast lineage [4-8]. Bone grafts supplemented with marrow-derived osteogenic CTPs show enhanced efficacy of bone formation [3,9]. Mesenchymal stem cells (MSC) are a subset of CTPs that are self-renewing and pluripotent; i.e., a single MSC can give rise to progeny that differentiate into bone, cartilage, fat, tendon, or other connective tissues. Demonstration of the presence of MSC within culture-expanded cell populations from marrow is accomplished by culture expansion of a single clone, subsequent parallel culture of the progeny in a panel of different culture media (each containing additives that foster development of a particular lineage), and finally assays showing that the different phenotypes are achieved [10]. While MSC are likely present in most cultures of adherent cells from marrow at the early stages of culture, the adherent cell population is heterogeneous and also contains more committed progenitor cells which may be useful clinically [11-14]. Depending on the specific methods used to isolate and expand cells in the CTP population, the resulting cells may be referred to as MSC, marrow stromal cells, multipotential adult progenitor cells, or given specific lineage designations.

Bone marrow can be aspirated intraoperatively to provide a practical CTP source for bone grafting, and thus effective methods for using marrow in bone grafts are being defined [3]. One strategy to enhance the efficacy of grafts is to increase the concentration of osteogenic CTPs within the graft over that in the marrow aspirate using selective adhesion of osteogenic CTPs to the graft surface. The potential of this approach has been demonstrated with cancellous bone grafts and porous hydroxyapatite grafts [12-15]. When marrow aspirates are passed through these grafts using a simple intraoperative protocol, the total nucleated cell concentration in the grafts is increased by approximately 2-fold (i.e., all cells are more concentrated), and the grafts show an additional modest (~2-fold) selectivity toward CTPs as assessed by analysis of excess cells washed out of the grafts during seeding. Grafts containing CTPs concentrated in this way show enhanced healing [12-15].

However, the graft materials used to date are not ideal in terms of mechanical properties, availability, and risk of disease transmission. If specific molecular interactions that foster interactions of CTPs with graft surfaces can be defined, grafts made from any underlying bulk material can potentially be modified to retain CTPs within grafts during intraoperative seeding with fresh marrow. Integrin ligands are an attractive target for surface modification, as integrins are the major class of cell-matrix adhesion receptors and the more than 20 known integrins are differentially expressed on different cell types [16]. Integrin ligands offer functionally relevant adhesive interactions, and thus may allow for the initial CTP attachment as well as subsequent survival, proliferation and differentiation into bone.

The integrin profile of freshly-isolated human connective tissue progenitors (i.e., cells contained in marrow aspirates) is obscure, and the relative paucity of functional information about this cell population prompted the current study. Integrin profiles of several types of culture-expanded osteogenic cell types have been described, and these profiles offer a starting point for speculation of the integrin profiles of freshly isolated CTPs. Mesenchymal stem cell

populations obtained by *in vitro* subculturing and expansion of adhesive cells from marrow, as well as several permanent transformed cell lines with characteristics of early osteoblastic progenitors, have been reported to express integrins, α_1 , α_2 , α_3 , α_4 , α_5 , α_v , α_6 , β_1 , β_3 , and β_6 as well as other non-integrin receptors [10,17-27]. Further, these cells respond functionally by proliferating and differentiating when they adhere to cognate extracellular matrix molecules or small peptide ligands derived from them. Several studies have reported that culture-expanded mesenchymal stem cells or osteoblastic cell lines attach to small peptide ligands containing the prototypical fibronectin-derived RGD adhesion sequence, and that attached cells can differentiate down an osteoblastic lineage [28-35]. However, the response of fresh marrow aspirates (i.e., cells that have not been previously cultured) to these ligands is not known. Integrin profiles can change quickly in culture, and thus the response of freshly-aspirated marrow cells may differ from that previously reported for various kinds of culture-expanded cells.

In order to assess cell attachment via specific molecular interactions, the ligand must be presented against an otherwise non-adhesive background. Polyethylene oxide (PEO) brushes, comprising close-packed PEO chains tethered to the substrate at one end, with a free hydroxyl available for modification, can be created by myriad methods and are resistant to cell and protein adsorption [36]. Such brushes are used widely for presentation of biological ligands, especially when some ligand mobility is required against an otherwise inert background, although not all approaches are adaptable to coating complex devices such as bone scaffolds.

Comb copolymers that present relatively short, closely-spaced PEO chains emanating from a hydrophobic backbone, such as poly(methyl methacrylate) (PMMA), offer a facile means for creating PEO brushes. PMMA-PEO comb copolymers with side chains of 6-9 ethylene oxide units and >35% PEO content are highly resistant to cell attachment even in the presence of serum [37]. These polymer molecules form 2-dimensional random-walk discs at the polymer film/water interface, and thus can be used to present local clusters of ligand when activated and inert comb polymer are mixed [38]. Clustered ligands improve cell adhesion over comparable ligand surface densities presented in a random format [38].

Here, we assess the potential for peptide adhesion ligands presented against an inert background to foster attachment, proliferation, and differentiation of CTPs from fresh human marrow aspirates. We first validated that the substrates foster attachment and expansion of osteoblastic cell lines and primary culture-expanded pig CTPs. Behavior of fresh human marrow on the same substrates was assessed following a protocol where marrow is seeded directly onto peptide substrates before any culture or expansion on glass or plastic.

Materials and methods

Comb copolymer synthesis and surface preparations

Comb copolymers were synthesized by free radical polymerization of methyl methacrylate, polyethylene glycol methacrylate (HPOEM) and polyethylene glycol methyl ether methacrylate (MPOEM) in toluene using azo(bis)isobutyronitrile (AIBN) as an initiator following the general protocols described previously [37,39] and adapted for the specific compositions described here. A panel of polymers comprising systematic variations in the ratios of each monomer, and in the number of PEO units in the HPOEM and MPOEM monomers was synthesized. All reagents were obtained from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise specified. HPOEM of molecular weight $M_n \sim 360$ g/mol (6-mer PEO units) and $M_n \sim 526$ g/mol (9.7-mer PEO units) and MPOEM with $M_n \sim 475$ g/mol (9-mer PEO units) and $M_n \sim 2,000$ g/mol (45-mer PEO units) were obtained from Aldrich. HPOEM (22-mer and 33-mer) were synthesized as follows: polyethylene glycol (PEG) was dissolved in dichloromethane with two molar equivalents of triethylamine and cooled on ice. 0.75 molar

equivalents of methacryloyl-chloride were then added dropwise with vigorous stirring and the reaction was allowed to proceed for four hours. The crude product was washed once with 0.01 N hydrochloric acid, twice with brine and then once with deionized water. The product was dried over magnesium sulfate and rotary-evaporated to dryness. Composition and purity were verified by thin layer chromatography and proton NMR (Bruker Avance DPX-400 proton NMR running at 400 MHz).

The molecular weight and polydispersity index (PDI) of the resulting comb polymers were determined by gel permeation chromatography with in-line light scattering (GPC-LS, Wyatt MiniDawn) based on polystyrene standards. The weight ratio of the three monomers in the resulting polymers was determined by proton NMR using 1% copolymer solution in deuterated chloroform. The PDI of all comb polymers was less than 3.5 and other specifications of the polymers are given in Table 1.

Polymer substrates were prepared by spincoating 0.2 μ m-filtered polymer solution on glass coverslips previously silanized with methacryloxypropyltrimethoxysilane (MPTS, Gelest Inc, PA) to render the glass surfaces more hydrophobic. Coverslips were sonicated in 100% ethanol for 30 minutes prior to silanization to remove debris. Coverslips were then rinsed and placed in a 4% solution of MPTS in 95% ethanol and 5% water (pH 4-5) with constant agitation for 30 minutes. The coverslips were then rinsed five times in 100% ethanol and cured at room temperature overnight or at 130 °C for one hour. Polymer solutions were prepared by dissolving comb copolymer in 3:1 methyl ethyl ketone to toluene solution at 20 mg/mL. Residual solvent was removed by incubating the spincoated coverslips *in vacuo* at room temperature overnight. Ellipsometry measurements indicated that the comb polymer thin films produced by this method were between 800-1000 Å.

Preparation and characterization of peptide-modified polymer substrates

Hydroxyl side chains were activated with 4-nitrophenyl chloroformate (NPC) as follows: The H10M0 comb copolymer was lyophilized from benzene. The polymer was dissolved in dichloromethane with 4.5 times excess (relative to the number of hydroxyls) of triethylamine (TEA). The reaction mixture was cooled in an ice bath and purged with nitrogen. 2.5 molar excess of NPC dissolved in dichloromethane was then added dropwise. The mixture was allowed to react for 2 hours on ice and then overnight at room temperature. NPC-activated polymer was concentrated by rotary evaporation and redissolved in ethyl acetate. TEA salts were removed by filtration. The product was purified by repeated precipitation in petroleum ether and finally dried *in vacuo*. NPC comb polymer was aliquoted and stored at -20°C until use.

NPC-activated comb copolymer was analyzed by gel permeation chromatography with in-line light scattering. No detectable differences in molecular weight and polydispersity index before and after NPC activation were found. The percentage conversion of side chain hydroxyl terminal groups to NPC was determined by NMR. ¹H NMR spectra were obtained for 1% copolymer solutions in deuterated chloroform. The characteristic phenyl peaks of 4-nitrophenyl carbonate at $\delta \sim 7.4$ -8.4 ppm along with the appearance of a new peak at $\delta \sim 4.4$ ppm (ethylene protons adjacent to 4-nitrophenyl carbonate, **d** in Figure 1) indicated the successful activation by NPC (Figure 1) and were consistent with previously-reported NMR spectra of NPC-activated polymer [40]. The ratio of peak intensity at $\delta \sim 4.4$ ppm to the peak at $\delta \sim 4.1$ ppm (ethylene protons adjacent to the backbone ester, **c** in Figure 1) was used to determine the percentage of activated PEO side chains, which was over 80%.

To prepare surfaces with various peptide densities while retaining a local clustering of peptides, a series of comb copolymer solutions was made by mixing NPC-activated comb copolymer with its non-activated counterpart in varying defined proportions. These solutions were then

spincoated on glass coverslips, as described above, to give rise to surfaces with various densities of NPC-activated chains. We found that peptide surface densities did not increase monotonically with increasing percentage of NPC-activated comb in the film, presumably due to inhibition by the reaction products at high surface concentrations of NPC, as addition of products to the initial coupling mixture reduced the degree of coupling (data not shown). Peptide surface densities increased linearly with increasing NPC-activated comb content up to a value of about 40-50% activated comb, then declined at values above ~75% activated comb. We thus used a maximum of 25% activated comb in films in this study and to achieve the highest local peptide density (number per activated chain).

Peptides (GRGDSPY, GRGESPY, GRAASPY and YGGFHRRIKA) were obtained from the MIT Biopolymers Laboratory and Tufts University Core Facility. All peptides were purified by high performance liquid chromatography to greater than 95 % purity and analyzed by MALDI mass spectroscopy. For reaction with NPC-activated polymer substrates, peptides were dissolved in 0.1 M sodium bicarbonate (NaHCO_3) buffer (pH 8.3) at ~ 1 mM, unless otherwise specified and added to substrates in 1000-fold stoichiometric excess of peptide to NPC groups (1.1 μL of peptide solution was added to each mm^2 of polymer surface). The reaction was allowed to proceed for 4-5 hours. Substrates were then rinsed twice with NaHCO_3 buffer, incubated in ethanolamine solution (0.025M ethanamine in 0.25M NaHCO_3 buffer) at 37°C overnight to deactivate remaining unreacted NPC groups, and rinsed three times with NaHCO_3 buffer and finally with milliQ water to remove residual bicarbonate salts. The arginine (pKa 12.5) and lysine (pKa 10.5) residues of the peptide YGGFHRRIKA are largely protonated in the pH 8-8.5 buffer used for peptide coupling, so that the majority of peptides should couple to the polymer surface through their amine terminus.

Peptide surface density was quantified with peptides radiolabelled with iodine-125 using the iodobead method (Pierce, Rockford, IL). After the reaction of peptides and ^{125}I , and subsequent quenching with sodium metabisulfite (12 mg/mL in PBS) and cold potassium iodide solution, peptides were purified with a C18 Sep-Pak Reverse phase cartridge (Waters, MA). A series of water-based solutions containing 1% trifluoroacetic acid (TFA) and an increasing amount of methanol (10% to 80%) was used to elute the ^{125}I -bounded peptides from the cartridge and separate them from free ^{125}I . Only the fractions containing the highest concentration of ^{125}I -peptides were pooled. An excess but known amount of cold peptides were then added to the ^{125}I -peptide fraction. The peptide solution was then titrated to approximately pH 8.3 and diluted to the appropriate concentration for coupling, which proceeded as described above. Resulting activity of the ^{125}I -peptide-coupled coverslips was measured with a Packard Cobra II Gamma Counter and densities calculated using a standard calibration curve of gamma activity versus number of ^{125}I -peptides. A dependence of apparent peptide density on film thickness was observed for coupling cysteine-terminated RGD peptides to films prepared with combs activated with N-[*p*-Maleimidophenyl]isocyanate (PMPI), an effect attributed to reaction of peptide in the bulk of water-swollen films and resulting in overestimation of surface densities by a factor of 3 for films of thickness ~900 Å (W. Kuhlman, personal communication). This phenomenon would likely be less pronounced in films prepared with NPC-activated comb, as we found that the reaction product (4-nitrophenol) inhibited coupling and these products would accumulate in the bulk during reaction. Reported peptide densities represent total substrate-associated peptide (i.e., include both bulk and surface peptide) and are thus referred to as nominal peptide densities.

To study the effect of peptide-clustering on cell adhesion, RGD surfaces with different degrees of RGD clustering were prepared by mixing various ratios of NPC-activated comb and its non-activated counterpart. Further, the average overall ligand density was stoichiometrically varied by mixing the active peptide GRGDSPY with the inactive peptide GRGESPY. Peptide surfaces with the maximum number of active ligands (GRGDSPY) per cluster were prepared by

spincoating on coverslips polymer solutions of 0/100, 5/95, 15/85 and 25/75 of NPC-activated comb/non-activated comb and subsequently reacted with 1mM GRGDSPY solution to yield surfaces with peptide surface densities of (0 ± 500) peptides/ μm^2 , (4150 ± 450) peptides / μm^2 , (9300 ± 900) peptides/ μm^2 , and (14000 ± 450) peptides/ μm^2 respectively. Since a fixed concentration of GRGDSPY was used, the local peptide density within a cluster was invariant. The number of active ligands per cluster (N_{ligand}) for all these surfaces was 18, the maximum achievable. Alternatively, surfaces with comparable average GRGDSPY densities can be created by varying the number of active ligands per cluster using a fixed ratio of active: inactive polymer (1:3) and coupling with mixture of inactive ligand (GRGESPY) with active ligand (GRGDSPY) in the coupling solution (GRGDSPY: GRGESPY = 0:1, 1:4, 3:2 and 1:0). This reduces the number of active peptides/cluster while keeping the overall peptide density constant. Mixture compositions were chosen so that the average peptide density in clusters where $N_{\text{ligand}} < 18$ would correspond to the set of average peptide densities for $N_{\text{ligand}} = 18$. Peptide densities were verified by ^{125}I -labelled peptide quantification. All peptide-modified substrates were used within 3 weeks of preparation and sterilized by exposure to UV light for 30-45 min prior to cell culture.

Protein resistance experiments

Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) was radiolabelled with ^{125}I using iodobeads. After purification, ^{125}I -BSA was diluted to concentrations ranging from 0.01 to 1 mg/mL in phosphate-buffered saline (PBS, pH 7-7.5) and incubated with polymer substrates for 2 hours at 20°C. 1 μL of BSA solution was used per cm^2 of polymer surface. The substrates were then washed four times with PBS and the amount of BSA adsorbed was calculated by measuring gamma activity, as described in the previous section, and compared to the ^{125}I -BSA standard calibration curve.

Cell isolation and culture

Wild type NR6 fibroblasts (wtNR6) were cultured in minimum essential medium- α (MEM- α) supplement with 7.5% fetal bovine serum (FBS) as previously described [38]. All materials were obtained from Gibco, Grand Island, NY, USA. The mouse embryonic fetal calvarial osteoblastic cell line (MC3T3-E1), obtained from the American Type Culture Collection (ATCC), was cultured in MEM- α supplemented with 10% FBS and 100% i.u/mL penicillin, 200 $\mu\text{g/mL}$ streptomycin, 1mM sodium pyruvate and 50 $\mu\text{g/mL}$ ascorbic acid. Both cell lines were maintained at 37°C with 5% CO_2 and split when they were near 80% confluency.

Pig culture-expanded mesenchymal stem cells (pMSCs) were a gift from J. P. Vacanti and H. Abukawa (Massachusetts General Hospital). The pMSC-enriched fraction obtained by centrifugation of aspirated marrow in 70% Percoll followed by suspension in culture medium and seeding at a density of 1.6×10^6 cells per cm^2 using published protocols [41]. Culture medium was changed every 3 days. Cells were subcultured via trypsinization when ~80% confluent (~10 days after initial seeding) and used within the third passage.

Human bone marrow was obtained from normal donors and from patients presenting to Dr. G.F. Muschler prior to an elective orthopedic procedure. All subjects were enrolled with full informed consent under a protocol which was approved by the Institutional Review Board of Cleveland Clinic. Bone marrow aspirates were obtained from the iliac crest using a procedure described previously [8]. A 2-ml sample of bone marrow was aspirated into a 10-ml plastic syringe containing 1 ml of saline containing 1,000 units of heparin. Subsequent aspirates were taken using identical technique through separate cortical perforations separated by at least 1 cm, moving posteriorly along the iliac crest. Four aspirates were harvested from each side. The heparinized marrow sample from each site was suspended into 20 ml of MEM- α -heparin and

sealed in a 50-ml test tube for transportation to the cell culture laboratory. All samples were harvested by Dr G.F. Muschler.

Cell attachment to unmodified polymers

The ability of unmodified substrates to resist non-specific cell attachment was assessed using wtNR6 fibroblasts and human marrow aspirates. For wtNR6 experiments, 12 mm glass coverslips spincoated with a test polymer were fixed in 24 well-plates with silicone rings (cut from medical grade silicone tubing). Surfaces were sterilized under UV light for 30-45 min, incubated with wtNR6 growth medium (7.5% serum) overnight, then seeded with 14,000 wtNR6 cells/cm² in wtNR6 growth medium. Cell adhesion was assessed on day 7. After two gentle rinses of the surfaces with divalent-ion-containing PBS, quantitative analysis of three images was performed per coverslip per time point with triplicate coverslips per condition, using Openlab software (Improvision, Lexington, MA) for image processing and manual counting of cells. Three separate experiments were performed for each comb polymer substrate with triplicate samples in each experiment.

For testing attachment of marrow, test polymers were spincoated on 18 × 18 mm² square glass coverslips as described above. Polymer-coated coverslips were placed in the wells of 2-chamber Lab-tek culture slides (Nunc, NY), with one coverslip covering the bottom of each chamber on the slide, sterilized under UV for 30 min, and seeded with 0.5 million fresh marrow aspirate cells in DMEM with 10% FBS, using a lot of FBS selected on the basis of enhancing osteogenic differentiation. Glass coverslips were used as the positive control. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. The culture medium was changed 48 hours after plating and the non-adherent cells from each chamber were replated on Lab-tek slide surfaces. The initial and replated cultures were maintained for 4 and 6 additional days respectively. Cultures were then washed twice with PBS and fixed [42]. Colonies (clusters of 8 or more cells), and sub-colony clusters were counted and single cell adhesion was scored as + (0-10 cells per slip/chamber), ++ (10-100 cells per slip/chamber) and +++ (>100 cells per slip/chamber). Colonies on original substrates were defined as “early adherent colonies”, as they originated from cells that adhered to the test surfaces within 48 hours of plating. Colonies formed on Lab-tek slides from the re-plating of cells that were non-adherent at 48 hr were defined as “late adherent colonies”. Figure 2 shows the schematic of the colony forming unit (CFU) assay for CTP colony formation. Four slips/chambers were used for each type of comb copolymer surface per marrow sample and three patient samples were used in this study.

Cell attachment to peptide-modified substrates

Peptide-coupled substrates were placed in 24-well plates and fixed with silicone rings as described above. wtNR6, MC3T3-E1 or primary pMSCs (10,000 cells per cm²) were seeded on peptide surfaces in the presence of serum-containing media free of phenol red and incubated for 16-18 hours. Cell attachment was then assessed by microscopy as described above. In experiments to determine the effect of peptide-clustering on adhesion, the software Scion Image (Scion Corp., Fredrick, MD) was used, in addition, to trace cell boundaries and calculate cell spread areas. Colony formation by pMSC was assessed by culturing attached cells for 5 days after seeding. Three experiments were carried out for each cell type, and for each experiment, triplicates of each type of surface were used.

Assay for human marrow-derived osteogenic colony forming units (CFUs) on peptide-modified substrates

The protocol for assessing bone marrow cell adhesion and colony formation on peptide-modified substrates was similar to that described above for unmodified substrates, except 0.5 × 10⁶ cells were seeded per cm² (2 million cells per coverslip). Further, cells and colonies on substrates in these experiments were stained for alkaline phosphatase (AP) expression.

Substrates were first rinsed with Hanks balanced salt solution and then incubating with 120 mM Tris buffer, pH 8.4, containing 0.9 mM Naphthol AS-MX Phosphate and 1.8 mM Fast Red TR. Naphthol AS-MX Phosphate was solubilized with N,N-dimethylformamide (Curtis Matheson Scientific, Houston, TX, USA) prior to dilution with Tris buffer. After 30 min at 37°C, the cultures were washed with deionized water. Any CFU that was AP-positive was denoted as CFU-AP.

Statistical analysis

Results from cell resistance experiments were analyzed by single-factor analysis of variance (ANOVA) with MSeExcel. Pairwise comparisons were performed by using Fisher LSD post hoc test. Confidence level was as indicated in the text and figure captions. In wtNR6 adhesion experiments on peptide surfaces with different degree of clustering, student t-tests were employed to determine whether the difference in cell spreading area between surfaces with different cluster size were significant. 99% confidence levels were reported, unless otherwise noted.

Results

Protein and cell resistance of unmodified comb polymers

We screened a panel of unmodified comb polymers with systematic variation in properties for their ability to resist adsorption of a model protein (BSA), resist adhesion of a model cell line (wtNR6 fibroblast) and resist attachment and colony formation by fresh primary human bone marrow cells. Comb copolymers with > 40 wt% PEO are water soluble, and below 30 wt% PEO, those with 6-9 unit PEO side chains support non-specific fibroblast adhesion [37]. Thus polymers in the 30-40 wt% PEO range were tested. Previous work in our lab showed that certain comb copolymers with more methoxy (-OCH₃) side chain ends are more resistant than those with more hydroxyl (-OH) chain ends (unpublished data) although hydroxy-terminated short PEG chains are more resistant in self-assembled monolayers [43]. Only hydroxyl side chain ends can be activated for peptide modification, but the side chain functional groups can be mixed within a single polymer to maximize cell resistance while providing an adequate number of active end-groups. We thus investigated the properties of both types of end groups.

Adsorption isotherms for BSA on each of the comb polymers listed in Table 1 indicate that all polymers are substantially more resistant than glass (Figure 3), with the least resistant comb showing < 13% adsorption compared to glass at saturation. We measured a saturation density of 500 ng/cm² on glass, where the theoretical close-packed density of an adsorbed BSA monolayer under our experimental conditions is estimated to be about 225 ng/cm² of BSA [44,45]. Isotherms of H6M0, H10M0, H6M9_3, H6M9_1 all reached an asymptotic adsorption value of less than 30 ng/cm², comparable to surfaces considered highly non-fouling [46]. There was no apparent trend of BSA adsorption in correlation to the attributes of the comb polymer (side chain end-groups, side chain lengths, or percent PEO). Indeed, the polymer with the greatest % PEO (H0M45, 50% PEO) was least resistant to BSA adsorption. This same polymer (H0M45) was among the most resistant to wtNR6 adhesion assessed following a pre-incubation in serum and 7 day culture period (Figure 4).

All comb copolymers exhibited wtNR6 fibroblast attachment of less than 25% of that seen on TCPS at 7 days as assessed by quantitative analysis of microscopy images (p < 0.001, Fisher LSD post hoc test). Cells were observed only in occasional defects (scratches and edges) on substrates that exhibited < 3% adhesion, and only a small number of rounded cells were observed by microscopy on even the most adhesive of these surfaces. Comb copolymers H6M9_1 and H22M9, which were of intermediate resistance in the series, were used as reference points for pair-wise statistical comparison with all other polymers. Comb polymers

with only methoxy-terminated POEM sidechains, the 9-mer H0M9 and the 45-mer H0M45, as well as the 10-mer HPOEM sidechain comb H10M0 were the most cell resistant (Figure 4) and exhibited a statistically lower relative adhesion than H6M9_1 with $p < 0.05$ and H22M9 with $p < 0.01$. Comb polymers with 6-mer HPOEM side chains, H6M0 and H6M9_3 (a copolymer with a mixture of 6-mer HPOEM and 9-mer MPOEM side chains), also exhibited very good wtNR6 fibroblast cell resistance, $2.1 \pm 1.2 \%$ and $2.0 \pm 1.2 \%$, respectively ($p < 0.01$ vs. H22M9).

A different ranking of the cell attachment resistance of this polymer series was observed in experiments with human bone marrow. Performance was assessed by measuring the number of colonies (clusters of 8 or more cells), sub-colony clusters, and single cells adherent on the substrates after 2 millions of marrow cells were seeded (for a surface area of 16 cm^2) and cultured for a total of 6 days (according to the protocol in Figure 2). Relative colony-forming efficiency (CFE) is defined as the ratio of early adherent colonies formed on a test surface to the number of early adherent colonies formed on glass or Lab-tek control substrates. With the exception of H6M9_2 (CFE = $38 \pm 15 \%$), all comb copolymers showed significant reduction of CFE when compared to glass control ($p < 0.001$) (striped bars, Figure 5). The total number of colonies formed on glass was typically 60-100, and thus total numbers on comb polymers (typically 10 or fewer) were too low for discerning statistically-significant trends. Only H6M9_1 and H6M9_3 showed significantly less colony formation (striped bars) than H6M9_2 ($p < 0.05$, Fisher LSD post hoc test). When subcolony-clusters were taken into consideration, and the ratio of the sum of colonies and subcolony-clusters on comb surfaces to that on glass controls was plotted for each comb copolymer (solid bars), we found that combs with longer side chains (H22M9, H33M0 and H33M9) were generally less resistant than those with shorter side chains (Figure 5). H6M0, H10M0, H22M0 were the most resistant to non-specific colony and cluster formation ($p < 0.05$ vs. H22M9. H22M9 was used as the reference point for calculating statistical significance), followed by combs with only methoxy-terminated POEM side chains ($p < 0.1$ vs. H22M9). The degree of single cell adhesion was similar for all surfaces (Table 2) with fewer than 100 cells total adherent on any surface.

From the marrow cell adhesion resistance studies, H6M0 and H10M0 emerged as the best candidates for peptide modification in terms of good cell adhesion resistance and sufficient hydroxyl side chain ends. GPC and NMR results showed that the H6M0 polymer had a number average molecular weight (M_n) of 75,000 with an average of 86 side chains per molecule, while H10M0 molecule had a M_n of 142,000 and an average of 107 side chains per molecule. The H10M0 comb copolymer was selected based on its greater number of hydroxyl groups available for peptide modification.

Peptide clustering enhances wtNR6 spreading

Adhesion and migration of wtNR6 fibroblasts on substrates presenting RGD-containing peptides were previously shown to be enhanced when the peptides are presented at the same overall average surface density in a clustered compared to a random format [38,47]. In previous studies using PMMA-PEO comb copolymers, peptides were covalently linked to carboxylated combs by N-hydroxysuccinimide and dicyclohexocarbodiimide prior to film casting to achieve a maximum cluster size of ~ 5 peptides/cluster [38].

In the current protocol, PMMA-PEO comb copolymers were activated with NPC, cast into films, and then reacted with adhesion peptides to form N-terminus linkages. Because a different coupling chemistry was used compared to our previous work, and because the characteristics of the polymer used were slightly different, we established the range of cell responses as a function of ligand density and clustering using a circumscribed set of conditions compared to those previously investigated for fibroblasts [38]. A panel of substrates in which the average nominal surface peptide density and average number of peptides in a cluster were

systematically and independently varied was created by mixing different proportions of activated and inert comb. Surfaces modified with only the control peptides GRGESPY or GRAASPY were resistant to wtNR6 adhesion at all peptide densities (data not shown). Attachment of wtNR6 to the active peptide GRGDSPY was comparable to TCPS when the highest cluster size and peptide density was employed—the ratio of cell adhesion on peptide surface to that on TCPS was 1.2 ± 0.3 ; however, this ratio decreased to 0.2 ± 0.04 at the lowest peptide density (nominally 4140 ± 430 GRGDSPY/ μm^2) even though the degree of peptide clustering remained maximal (nominally 18 peptides/cluster). Notably, fibroblast spreading was enhanced with increased cluster size, when the average total peptide density was kept constant (Figure 6). Conditions that engendered a comparable response to TCPS were a nominal density of 14,000 GRGDSPY/ μm^2 and a nominal cluster size of 18 peptides per cluster.

Attachment of pig MSCs and MC3T3-E1 cells on peptide-modified surfaces

Primary pMSCs were used as an initial screen for the ability of the peptide-modified substrates to support the attachment and proliferation of osteoblastic cells. Relative to TCPS, few cells attached to control peptides GRGESPY and GRAASPY even at nominal peptide densities greater than 15,000 peptides/ μm^2 (Figure 7a). In contrast, the number of pMSC attached to GRGDSPY-modified substrates was comparable to the positive control (TCPS) at a nominal peptide density of 15,000 peptides/ μm^2 (Figure 7a), and decreased at lower peptide densities to a value just above the negative control values at low peptide density (4140 peptides/ μm^2). The ~20% background adhesion at 0 density for all peptides in Figure 7 was due to cells accumulated at the edge of the wall of each culture well. Visual observation of cells (via microscopy) on the control peptide surfaces confirmed that cell attachment was similar to control unmodified comb surfaces and was negligible. The GRGDSPY peptide-modified substrates supported not only adhesion but also colony formation of pMSCs (Figure 7b).

The bone sialoprotein-derived peptide FHRRIKA has been reported to foster attachment of primary rat calvarial osteoblasts via proteoglycan receptors [48,49], particularly when used in mixtures with RGD [48]. Primary pMSCs did not attach to this peptide (Figure 7c), even at a nominal peptide density three times greater than that which induced maximal attachment of pMSC to GRGDSPY. The murine pre-osteoblastic line MC3T3-E1 cells exhibited modest adhesion to FHRRIKA at peptide densities above 150,000 peptides/ μm^2 (Figure 7c).

GRGDSPY and FHRRIKA peptide-modified substrates support attachment and colony formation by human CTPs

Marrow aspirate samples from seven human subjects were plated on substrates presenting GRGDSPY (14000 ± 440 peptides/ μm^2) or GRGESPY (15330 ± 1040 peptides/ μm^2). Marrow from five of these same subjects was also plated on substrates presenting GRAASPY (27440 ± 3540 peptides/ μm^2), or FHRRIKA (327000 ± 9000 peptides/ μm^2) and formation of colonies was assessed using the protocol in Figure 2 in comparison to controls. We found that colony formation was enhanced on GRGDSPY surfaces compared to its inactive peptide analog and non-activated comb control in 5 of the 7 patients, as assessed by relative colony forming efficiency (CFE, Figure 8). All colonies on GRGDSPY, GRGESPY and GRAASPY substrates and comb surfaces were stained positive for AP, except for 30% of the colonies on GRGESPY surfaces with subject #4. Even though GRGDSPY enhanced CFE relative to controls in five out of seven marrow samples tested, the degree of enhancement varied widely among subjects. The bone sialoprotein peptide FHRRIKA also showed a significantly higher CFE in comparison with the negative control GRAASPY and unmodified comb control (Figure 8).

The number of colonies formed on a given peptide or control surface by different patient samples varied greatly on all substrates including glass controls, reflecting expected variation

in CTP prevalence among different donor subjects [8,42]. The number of early adherent CTPs forming colonies on glass from the 7 marrow aspirate samples ranged from 10 to 70 and the total number of colonies (sum of the early-adherent and late-adherent CTP colonies) formed from the 8 million nucleated marrow cells originally seeded (total of 4 substrates with 2 million cells each) also showed a 4-fold variation among the 7 patients (Figure 10). However, the ratio of colonies that express alkaline phosphatase to all CTP-derived colonies on glass control showed a much smaller variation ($91.3 \pm 4.8\%$). Few subcolony clusters were found on the peptide surfaces and no condition had more than 100 single adherent cells. In many cases, fewer than 10 adherent single cells were observed.

Discussion

Marrow-derived CTPs offer great potential for regenerating defects in bone, by direct addition of marrow (or CTPs that are concentrated or selected from marrow) to bone grafts, or by expanding the progeny of CTPs in culture and adding them to the graft. Intraoperative addition of marrow to grafts is particularly appealing as the risk and expense of *ex vivo* cell manipulations are avoided. CTPs are prevalent in aspirated human marrow at a frequency of about 1 in 20,000 nucleated cells [8] and about 1,000 CTPs per mL of aspirate under optimal aspiration conditions [42].

While the cells other than CTPs that are present in marrow may contribute to bone formation in as yet unknown ways, it is likely that in large defects, the vast majority of these additional cells will impede the survival and function of CTPs by competing for nutrients and growth factors within the graft following implantation. Models of nutrient diffusion and consumption within avascular cell-seeded grafts predict that the interior region of grafts more than a cm thick and seeded with even moderate cell densities ($\sim 10^6$ cells/cm³) will be severely hypoxic [50]. Although certain behaviors of mesenchymal stem cells appear to be enhanced under moderately hypoxic culture conditions in both 2 and 3 dimensional culture, in keeping with a hypothesis that these cells are normally in a relatively hypoxic niche *in vivo* or are activated under hypoxic conditions during wound healing [51-54], extreme hypoxia inhibits colony formation and growth *in vitro* (G. Muschler, unpublished data). Some experimental evidence supports the concept that diffusion of nutrients limits survival of bone-forming cells in grafts just a few mm thick [55,56], although bone can form *in vitro* throughout cell-seeded grafts when dimensions are small enough [57].

Further, *in vitro*, hematopoietic cells present in whole bone marrow aspirate cultures appear to be suppressed or die off in 3D scaffolds in the absence of hematopoietic stimuli, while the progeny of osteogenic CTPs proliferate and retain differentiation potential [58]. *In vivo*, death of non-osteogenic cells (including the less adhesive lineage-committed cells) will be accompanied by an inflammatory response resulting in recruitment of additional cells to clear the debris, further contributing to a hostile environment for survival and function of CTPs and other cells that may be essential for the bone formation process. These effects will likely be exacerbated in large grafts, and may not be as apparent in studies done with small implants. Approaches that enrich for CTPs and limit or reduce the concentration of non-osteogenic cells within the graft may improve performance of grafts seeded intraoperatively with marrow aspirates [3,6,12,13]. We thus sought to define specific molecular interactions that would foster attachment, survival and colony formation by CTPs derived from human marrow aspirates, as a potential tool for improving bone graft substitute materials.

Peptides containing the RGD adhesion motif elicit bone-forming behaviors from a wide variety of culture-expanded progenitor and mature cell types *in vitro* and enhance bone formation or in growth *in vivo* in many experimental models [19,20,28,29,31,32,40,48,59-65]. Although the interactions of freshly-isolated human marrow with RGD peptides has not previously been

studied, linear RGD-containing peptides presented against a minimally-adhesive background induce the attachment, survival and osteogenic differentiation of the culture-expanded progeny of CTPs from human [31,64], goat [65] and rat [32]. (Depending on culture conditions and processing methods, the derived culture-expanded cells have been called bone marrow stromal cells, mesenchymal stem cells, multipotential adult progenitor cells, or mesenchymal progenitor cells; all such designations apply to cells that have been expanded and subcultured, and thus likely have adhesion receptor profiles that reflect adaptation to culture conditions.) Similar influences of linear RGD peptides are seen with the murine osteoblast progenitor line MC3T3-E1 [28,29] and primary rat calvarial osteoblast-like cells [48,66].

In agreement with these previous reports, we find that culture-expanded pMSC, when used at low passage number, attach to and proliferate on a surface of linear RGD peptide presented against an otherwise adhesion-resistant background. These same cells do not attach to the same surfaces modified with comparable densities of control peptides, confirming the general adhesion-resistance of these substrates in the presence of medium containing serum. At an average nominal peptide surface density of 15,000 peptides/ μm^2 , pMSC attachment to RGD-modified comb polymer is comparable to attachment to TCPS and cells are well-spread (Figure 7a and b). Robust differentiation of osteoblastic cells (from bone explants) on a linear RGD-containing peptides has been reported to occur at peptide densities of $\sim 4,000$ RGD peptides/ μm^2 [66].

Because the polymer substrates comprised a blend of peptide-modified comb copolymer with unmodified comb copolymer (in a ratio 1:3 or less), the peptide surface distribution was not random and the local peptide density at points of cell adhesion is higher than the average. Simulations of the interfacial configuration of the comb copolymer chains, combined with experimental measurements of chain conformation using transmission electron microscopy, show that the comb copolymer chains are confined to quasi-2D semi-interpenetrating discs at the interface, with the PEO chains extending into the aqueous phase [37,67].

For the comb copolymer used in these studies, H10M0, the number average radius of gyration of the polymer chain at the interface is estimated to be $R_{g,2D} = 9.2$ nm, taking into account the polydispersity measured by GPC [67]. Based on the measured average peptide densities for various blend ratios, the local peptide surface density is nominally about 64,000 peptides/ μm^2 . Thus, the average distance between adjacent peptides locally within a cluster is estimated to be less than 7 nm even under the conservative assumption that up to two-thirds of the nominal peptide density is buried in the bulk.

The integrin profile of primary CTPs derived from native tissue can change in culture [17, 27] and thus experiments with culture-expanded cells and cell lines cannot be expected to predict the behavior of fresh marrow-derived CTPs. A challenge in assessing response of fresh CTPs is that the number of cells in fresh marrow that attach to control substrates (such as glass and plastic) and form colonies is typically very low. Our finding that the number of colonies formed by early adherent CTPs on glass from the marrow aspirate derived from 7 donors ranged from 10 to 70 (from 8×10^6 total cells seeded) is consistent with previous results in comparable donor groups [8,42,68]. This presents significant challenges in assessing the properties of cells in early stages of culture, and we thus used a standard osteogenic colony assay to judge both proliferation and differentiation (by alkaline phosphatase staining) by colony formation.

We found that when data from all patients are included, CTP-derived colonies formed to some degree on all types of polymer substrates including controls, with colony numbers on polymer controls in all but one patient below 20% of colonies on glass. All colonies (except for 30% of the colonies on control peptide GRGESPY surfaces with patient #4) stained positive for AP (Figure 9), indicating their progression down the osteoblast lineage once adherent. Because

colony formation was enhanced on GRGDSPY surfaces compared to its inactive peptide analog and non-activated comb control in 5 of the 7 patients (Figure 8), it appears that some of the CTPs express sufficient levels of RGD-specific integrins to attach to and proliferate on the RGD adhesion ligand. However, CTPs from only one patient sample showed colony formation on RGD comparable to that on glass, with efficiencies in the range 0-30% for the other 6 patients tested. Thus, the linear RGD motif tested here is generally not sufficient to capture and foster proliferation of the entire CTP population in the average donor sample.

In an elegant study relating initial adhesion of osteoblast cells to subsequent differentiation in response to a panel of RGD-containing linear and cyclic peptides presented against a non-adherent background, Healy and co-workers recently demonstrated that a tyrosine residue immediately following the RGDS motif was essential to obtain robust cell proliferation and mineralization in osteoblast-like cells from rat calvaria [48], and it is thus possible that RGD-containing peptides with a slightly different set of flanking peptides may enhance colony formation beyond that observed. The peptide we used, GRGDSPY, contains a tyrosine residue following the RGD (with an additional intervening proline) and enabled robust proliferation of primary culture-expanded pMSCs. The linear GRGDSPY peptide is known to have relatively low affinity and thus one interpretation of our results is that only cells that express high levels of integrins bind to this low-affinity peptide and gain sufficient integrin occupancy to attach firmly (and then undergo subsequent attachment-dependent behaviors); use of higher affinity ligands, e.g. cyclic peptides or peptide fragments containing the synergy site in the FN 9-10 domain, may engender greater colony formation. A corollary hypothesis is that cells may require engagement of adhesion receptors other than those that recognize RGD-either in addition to or instead of those that engage RGD- and that co-tethering of multiple ligands will be required to achieve colony formation by such cells. Alternatively, certain integrins, including $\alpha_5\beta_1$, can adopt an inactive configuration on the cell surface and require activation to switch to a conformation that can bind to external ligand [16,69], and the activation state of integrins in these studies is not known. Finally, our assays require that cells attach and survive 6 days until enumerated, and it is possible that some CTPs that are initially adherent to the substrates are unable to survive on just the peptide ligand with the culture medium employed in these studies. Although nearly all the colonies formed from fresh human marrow were osteogenic *in vitro* in the assay used, it cannot be concluded that the peptides used would be selective for CTPs in a clinically-relevant situation-*in vivo*, other cell types may also interact with this peptide.

At least some subpopulation of cells in fresh marrow appears to have proteoglycan receptors that can bind the peptide FHRRIKA, as this peptide enhanced the number of colonies above controls in 4 out of the 5 patients tested and was at least as effective as RGD in all patients where both peptides were tested. This peptide has been reported as substantially more effective in fostering adhesion-mediated events when used in combination with RGD-containing peptides rather than alone, when tested against osteoblast-like cells from rat calvaria [48], thus future studies with these substrates may be directed toward the role of peptide combinations on CTPs. In accordance, we found that the FHRRIKA peptide did not induce attachment of primary pMSCs above control substrates, but had a modest effect on the attachment of MC3T3-E1 cells (Figure 7c).

A few (10-100) single cells and subcolony clusters were also found on both active peptide and control substrates, although no particular trends were observed on control vs. peptide substrates. Hematopoietic progenitor cells, which are more prevalent in marrow than osteogenic CTPs, express fibronectin receptors that recognize the RGD motif [70], and might therefore also be expected to attach. Indeed, hematopoietic CD34+ cells from human cord blood, which attach and proliferate on both TCPS on carboxylated polyethylene terephthalate (PET) when cultured with hematopoietic growth factors, show enhanced expansion on

carboxylated PET when it is modified with GRGDSPC at peptides densities of $\sim 600,000$ peptides/ μm^2 ; further, cells with mesenchymal properties also appear to be expanded on these substrates [71]. The conditions used here differed from those of Jiang et al in that we presented peptides against a very poorly adhesive background, thus minimizing the additive effects (to those of RGD) of cell-secreted attachment factors, and hematopoietic growth factors were not included in the culture medium. We observed far less substantial initial cell attachment and found that subcolony clusters had morphology indicative of CTPs. Thus, our conditions do not appear to favor the attachment, survival, and proliferation of hematopoietic cells.

Conclusions

In conclusion, we have identified a polymer substrate that significantly reduces attachment and proliferation of adherent cells in fresh human marrow and can thus be used to investigate fundamental adhesion-mediated behaviors of these cells. The challenges in predicting behavior of fresh marrow cells by extrapolating from behaviors of culture expanded cells or cell lines is underscored by the substantial differences we observed between fresh primary human CTPs in bone marrow samples and culture-expanded pig MSCs and the MC3T3 cell line in their attachment and proliferation properties on peptide-modified surfaces, differences that may include effects of both culture time and species. The CTP population in marrow is heterogeneous and we did not identify a single peptide that just by itself is capable of robust selection and expansion of these cells from primary marrow. We are currently evaluating the roles of integrin activation on human CTP colony formation on defined peptide substrates.

Acknowledgements

The authors would like to acknowledge William Kuhlman for preparing some of the comb copolymers used in this work and for insightful discussions. This research was funded by NIH R01-AR42997 and R01-GM59870. We also acknowledge partial support from the National Institute of General Medical Sciences (NIGMS) Cell Migration Consortium for synthesis and characterization of polymers and the Natural Sciences and Engineering Research Council of Canada for a Postgraduate Scholarship to A.A.

References

1. Hester JF, Banerjee P, Mayes AM. Preparation of protein-resistant surfaces on poly(vinylidene fluoride) membranes via surface segregation. *Macromolecules* 1999;32(5):1643–1650.
2. Muschler GF, Midura RJ, Nakamoto C. Practical Modeling Concepts for Connective Tissue Stem Cell and Progenitor Compartment Kinetics. *J Biomed Biotechnol* 2003;2003(3):170–193. [PubMed: 12975533]
3. Muschler GF, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. *J Bone Joint Surg Am* 2004;86-A(7):1541–58. [PubMed: 15252108]
4. Bellows CG, Aubin JE, Heersche JN. M.E. A. Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. *Calcif Tissue Int* 1986;38:143–154. [PubMed: 3085892]
5. Friedenstein AJ, Chailakhyan RK, Gerasimov UV. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet* 1987;20:263–272. [PubMed: 3690622]
6. Owen M. Marrow stromal stem cells. *Journal of Cell Science* 1988;10(Suppl):63–76.
7. Aubin J. Bone stem cells. *Journal of Cellular Biochemistry Supplements* 1998;30/31:73–82.
8. Muschler GF, Nitto H, Boehm CA, Easley KA. Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors. *Journal of Orthopaedic Research* 2001;19(1):117–125. [PubMed: 11332607]
9. Tuan RS, Boland G, Tuli R. Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther* 2003;5(1):32–45. [PubMed: 12716446]

10. Pittenger MF, Mackay AM, Beck SC, Jaiwal RK, Douglas R, Mosca JD, et al. Multilineage Potential of Adult Human Mesenchymal Stem Cells. *Science* 1999;284(5411):143–147. [PubMed: 10102814]
11. Fleming JE, Cornell CN, Muschler GE. Bone cells and matrices in orthopedic tissue engineering. *Orthopedic Clinics of North America* 2000;31(3):357–374. [PubMed: 10882463]
12. Matsukura Y, Boehm C, Valdevit A, Kambic H, Davros W, Easley K, et al. Concentration of bone marrow derived osteoprogenitors for spinal fusion. *Journal of Bone and Mineral Research* 2000;15 (M199 Suppl 1):S504–S504.
13. Muschler GF, Matsukura Y, Nitto H, Boehm CA, Valdevit AD, Kambic HE, et al. Selective retention of bone marrow-derived cells to enhance spinal fusion. *Clin Orthop Relat Res* 2005;432:242–51. [PubMed: 15738828]
14. Muschler GF, Nitto H, Matsukura Y, Boehm C, Valdevit A, Kambic H, et al. Spine fusion using cell matrix composites enriched in bone marrow-derived cells. *Clinical orthopaedics and related research* 2003;407:102–118. [PubMed: 12567137]
15. Brodke D, Pedrozo HA, Kapur TA, Attawia M, Kraus KH, Holy CE, et al. Bone grafts prepared with selective cell retention technology heal canine segmental defects as effectively as autograft. *J Orthop Res* 2006;24(5):857–866. [PubMed: 16602110]
16. Hynes R. Integrins. Bidirectional, allosteric signaling machines. *Cell* 2002;110:673–687. [PubMed: 12297042]
17. Chastain SR, Kundu AK, Dhar S, Calvert JW, Putnam AJ. Adhesion of mesenchymal stem cells to polymer scaffolds occurs via distinct ECM ligands and controls their osteogenic differentiation. *J Biomed Mater Res A* 2006;78(1):73–85. [PubMed: 16602124]
18. Reyes CD, Garcia AJ. Alpha2beta1 integrin-specific collagen-mimetic surfaces supporting osteoblastic differentiation. *J Biomed Mater Res A* 2004;69(4):591–600. [PubMed: 15162400]
19. Schaffner P, Dard MM. Structure and function of RGD peptides involved in bone biology. *Cell Mol Life Sci* 2003;60(1):119–32. [PubMed: 12613662]
20. Kilpadi KL, Sawyer AA, Prince CW, Chang PL, Bellis SL. Primary human marrow stromal cells and Saos-2 osteosarcoma cells use different mechanisms to adhere to hydroxylapatite. *J Biomed Mater Res A* 2004;68(2):273–85. [PubMed: 14704969]
21. Klees RF, Salaszyk RM, Kingsley K, Williams WA, Boskey A, Plopper GE. Laminin-5 induces osteogenic gene expression in human mesenchymal stem cells through an ERK-dependent pathway. *Mol Biol Cell* 2005;16(2):881–90. [PubMed: 15574877]
22. Clover J, Dodds RA, Gowen M. Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. *Journal of Cell Science* 1992;103:267–271. [PubMed: 1429908]
23. Moursi AM, Globus RK, Damsky CH. Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro. *J Cell Sci* 1997;110(Pt 18):2187–96. [PubMed: 9378768]
24. Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol* 1999;181(1):67–73. [PubMed: 10457354]
25. Roche P, Rousselle P, Lissitzky JC, Delmas PD, Malaval L. Isoform-specific attachment of osteoprogenitors to laminins: mapping to the short arms of laminin-1. *Exp Cell Res* 1999;250(2):465–74. [PubMed: 10413600]
26. 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(2):207–13. [PubMed: 10867645]
27. Deschaseaux F, Charbord P. Human marrow stromal precursors are alpha 1 integrin subunit-positive. *J Cell Physiol* 2000;184(3):319–25. [PubMed: 10911362]
28. Alsberg E, Anderson KW, Albeiruti A, Franceschi RT, Mooney DJ. Cell-interactive alginate hydrogels for bone tissue engineering. *J Dent Res* 2001;80(11):2025–9. [PubMed: 11759015]
29. Cavalcant-Adam EA, Shapiro IM, Composto RJ, Macarak EJ, Adams CS. RGD peptides immobilized on a mechanically deformable surface promote osteoblast differentiation. *J Bone Miner Res* 2002;17 (12):2130–40. [PubMed: 12469907]
30. Grigoriou V, Shapiro IM, Cavalcanti-Adam EA, Composto RJ, Ducheyne P, Adams CS. Apoptosis and survival of osteoblast-like cells are regulated by surface attachment. *J Biol Chem* 2005;280(3):1733–9. [PubMed: 15522882]

31. Meinel L, Karageorgiou V, Hofmann S, Fajardo R, Snyder B, Li C, et al. Engineering bone-like tissue in vitro using human bone marrow stem cells and silk scaffolds. *J Biomed Mater Res A* 2004;71(1):25–34. [PubMed: 15316936]
32. Shin H, Temenoff JS, Bowden GC, Zygourakis K, Farach-Carson MC, Yaszemski MJ, et al. Osteogenic differentiation of rat bone marrow stromal cells cultured on Arg-Gly-Asp modified hydrogels without dexamethasone and beta-glycerol phosphate. *Biomaterials* 2005;26(17):3645–54. [PubMed: 15621255]
33. Yang XB, Roach HI, Clarke NM, Howdle SM, Quirk R, Shakesheff KM, et al. Human osteoprogenitor growth and differentiation on synthetic biodegradable structures after surface modification. *Bone* 2001;29(6):523–31. [PubMed: 11728922]
34. Cutler SM, Garcia AJ. Engineering cell adhesive surfaces that direct integrin alpha5beta1 binding using a recombinant fragment of fibronectin. *Biomaterials* 2003;24(10):1759–70. [PubMed: 12593958]
35. Verrier S, Pallu S, Bareille R, Jonczyk A, Meyer J, Dard M, et al. Function of linear and cyclic RGD-containing peptides in osteoprogenitor cells adhesion process. *Biomaterials* 2002;23(2):585–96. [PubMed: 11761179]
36. Harris, JM.; Zapilsky, S., editors. Poly(ethylene glycol): Chemistry and Biological Applications. American Chemical Society; Washington, DC: 1997.
37. Irvine DJ, Mayes AM, Griffith LG. Nanoscale clustering of RGD peptides at surfaces using comb polymers. 1 Synthesis and characterization of comb thin films. *Biomacromolecules* 2001a;2(1):85–94. [PubMed: 11749159]
38. Koo LY, Irvine DJ, Mayes AM, Lauffenburger DA, Griffith LG. Co-regulation of cell adhesion by nanoscale RGD organization and mechanical stimulus. *Journal of Cell Science* 2002;115(7):1423–1433. [PubMed: 11896190]
39. Irvine DJ, Ruzette AVG, Mayes AM, Griffith LG. Nanoscale clustering of RGD peptides at surfaces using comb polymers. 2 Surface segregation of comb polymers in polylactide. *Biomacromolecules* 2001b;2(2):545–556. [PubMed: 11749219]
40. Jo S, Shin H, Mikos AG. Modification of oligo(poly(ethylene glycol) fumarate) macromer with a GRGD peptide for the preparation of functionalized polymer networks. *Biomacromolecules* 2001;2(1):255–261. [PubMed: 11749181]
41. Abukawa H, Shin M, Williams WB, Vacanti JP, Kaban LB, Troulis MJ. Reconstruction of mandibular defects with autologous tissue-engineered bone. *Journal of Oral Maxillofacial Surgery* 2004;62:601–606. [PubMed: 15122567]
42. Muschler GF, Boehm C, Easley K. Aspiration to obtain osteoblastic progenitor cells from human bone marrow: the influence of aspiration volume. *Journal of Bone and Joint Surgery (Am)* 1997;79:1699–1709.
43. Prime KL, Whitesides GM. Self-assembled organic monolayers: model systems for studying adsorption of proteins at surfaces. *Science* 1991;252(5010):1164–7.
44. Peters T. Serum-Albumin. *Advances in Protein Chemistry* 1985;37:161–245. [PubMed: 3904348]
45. Giacomelli CE, Avena MJ, DePauli CP. Adsorption of bovine serum albumin onto TiO₂ particles. *Journal of Colloid and Interface Science* 1997;188(2):387–395.
46. Lopez GP, Ratner BD, Tidwell CD, Haycox CL, Rapoza RJ, Horbett TA. Glow discharge plasma deposition of tetraethylene glycol dimethyl ether for fouling-resistant biomaterial surfaces. *Journal of Biomedical Materials Research* 1992;26:415–439. [PubMed: 1601898]
47. Maheshwari G, Brown G, Lauffenburger DA, Wells A, Griffith LG. Cell adhesion and motility depend on nanoscale RGD clustering. *Journal of Cell Science* 2000;113:1677–1686. [PubMed: 10769199]
48. Harbers GM, Healy KE. The effect of ligand type and density on osteoblast adhesion, proliferation, and matrix mineralization. *J Biomed Mater Res A* 2005;75(4):855–69. [PubMed: 16121356]
49. Rezaia A, Healy KE. Biomimetic peptide surfaces that regulate adhesion, spreading, cytoskeletal organization, and mineralization of the matrix deposited by osteoblast-like cells. *Biotechnol Prog* 1999;15(1):19–32. [PubMed: 9933510]
50. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. *Nat Rev Mol Cell Biol* 2006;7(3):211–24. [PubMed: 16496023]

51. Grayson WL, Zhao F, Izadpanah R, Bunnell B, Ma T. Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. *J Cell Physiol* 2006;207(2):331–9. [PubMed: 16331674]
52. Schooley JC, Kullgren B, Fletcher BL. Growth of murine bone marrow adherent stromal cells in culture without hydrocortisone in a low oxygen environment. *Int J Cell Cloning* 1985;3(1):2–9. [PubMed: 3882859]
53. Gupta V, Rajaraman S, Costanzi JJ. Effect of oxygen on the clonal growth of adherent cells (CFU-F) from different compartments of mouse bone marrow. *Exp Hematol* 1987;15(11):1153–7. [PubMed: 3678412]
54. Lennon DP, Edmison JM, Caplan AI. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on *in vitro* and *in vivo* osteochondrogenesis. *J Cell Physiol* 2001;187(3):345–55. [PubMed: 11319758]
55. Ishaug-Riley SL, Crane-Kruger GM, Yaszemski MJ, Mikos AG. Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* 1998;19(15):1405–12. [PubMed: 9758040]
56. Glowacki J, Mizuno S, Greenberger JS. Perfusion enhances functions of bone marrow stromal cells in three-dimensional culture. *Cell Transplant* 1998;7(3):319–26. [PubMed: 9647441]
57. Holy CE, Shoichet MS, Davies JE. Engineering three-dimensional bone tissue *in vitro* using biodegradable scaffolds: investigating initial cell-seeding density and culture period. *J Biomed Mater Res* 2000;51(3):376–82. [PubMed: 10880079]
58. Scaglione S, Braccini A, Wendt D, Jaquiere C, Beltrame F, Quarto R, et al. Engineering of osteoinductive grafts by isolation and expansion of ovine bone marrow stromal cells directly on 3D ceramic scaffolds. *Biotechnol Bioeng* 2006;93(1):181–7. [PubMed: 16245346]
59. Chen J, Altman GH, Karageorgiou V, Horan R, Collette A, Volloch V, et al. Human bone marrow stromal cell and ligament fibroblast responses on RGD-modified silk fibers. *J Biomed Mater Res A* 2003;67(2):559–70. [PubMed: 14566798]
60. Eid K, Chen E, Griffith L, Glowacki J. Effect of RGD coating on osteocompatibility of PLGA-polymer disks in a rat tibial wound. *J Biomed Mater Res* 2001;57(2):224–31. [PubMed: 11484185]
61. 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* 2005;75(2):249–55. [PubMed: 16106438]
62. Kroese-Deutman HC, van den Dolder J, Spauwen PH, Jansen JA. Influence of RGD-loaded titanium implants on bone formation *in vivo*. *Tissue Eng* 2005;11(11):1867–75. [PubMed: 16411833]
63. Sawyer AA, Hennessy KM, Bellis SL. Regulation of mesenchymal stem cell attachment and spreading on hydroxyapatite by RGD peptides and adsorbed serum proteins. *Biomaterials* 2005;26(13):1467–75. [PubMed: 15522748]
64. Nuttelman CR, Tripodi MC, Anseth KS. Synthetic hydrogel niches that promote hMSC viability. *Matrix Biol* 2005;24(3):208–18. [PubMed: 15896949]
65. Yang F, Williams CG, Wang DA, Lee H, Manson PN, Elisseeff J. The effect of incorporating RGD adhesive peptide in polyethylene glycol diacrylate hydrogel on osteogenesis of bone marrow stromal cells. *Biomaterials* 2005;26(30):5991–8. [PubMed: 15878198]
66. Rezaia A, Healy KE. The effect of peptide surface density on mineralization of a matrix deposited by osteogenic cells. *J Biomed Mater Res* 2000;52(4):595–600. [PubMed: 11033541]
67. Kuhlman W, Olivetti E, Mayes AM, Griffith LG. Chain conformations at the surface of a polydisperse comb co-polymer film. *Macromolecules* 2006;39:5122–5126. [PubMed: 19079731]
68. Majors AK, Boehm C, Nitto H, Midura RJ, Muschler GF. Characterization of human bone marrow stromal cells with respect to osteoblastic differentiation. *Journal of Orthopaedic Research* 1997;15:546–557. [PubMed: 9379264]
69. Takagi J, Springer TA. Integrin activation and structural rearrangement. *Immunol Rev* 2002;186:141–63. [PubMed: 12234369]
70. Roy V, Verfaillie CM. Expression and function of cell adhesion molecules on fetal liver, cord blood and bone marrow hematopoietic progenitors: implications for anatomical localization and developmental stage specific regulation of hematopoiesis. *Exp Hematol* 1999;27(2):302–12. [PubMed: 10029170]

71. Jiang XS, Chai C, Zhang Y, Zhuo RX, Mao HQ, Leong KW. Surface-immobilization of adhesion peptides on substrate for ex vivo expansion of cryopreserved umbilical cord blood CD34+ cells. *Biomaterials* 2006;27(13):2723–32. [PubMed: 16376984]

Figure 1.

¹H NMR spectra of NPC-activated comb copolymer. **e** refers to the two hydrogens immediate to -COO- in all POEM sidechains. The ratio of **c** to **e** gives the percentage of -OH groups to -NPC.

Figure 2.

Sequence of events in the marrow aspirate CFU assay for CTP-derived colonies. Non-adherent bone marrow cells (●); colonies of adherent cells derived from adherent CTPs (▲).

Figure 3. Relative percentage adsorption of BSA on comb copolymers with various POEM content, sidechain lengths and endgroups compared to glass control.

Figure 4.

Relative attachment of wtNR6 fibroblasts to comb copolymer surfaces compared to TCPS control after 7 days of culture. Cell adhesion was assessed by counting the number of adherent cells in microscopic images of surfaces. Results are mean $\pm$ SE of three biological replicates. (Error of the 3 technical replicates within each biological replicate is smaller than that between biological replicates.) The differences observed among the comb copolymers were significant, $p < 0.0001$ (ANOVA). Comb surfaces were compared with H6M9_1 and H22M9 using the Fisher LSD post hoc test. * $p < 0.05$ vs. H22M9, ** $p < 0.01$ vs. H22M9, and # $p < 0.05$ vs. H6M9_1.

Figure 5.

Colony and subcolony cluster formation on comb copolymer surfaces. Striped bar shows colony forming efficiency (CFE) (ratio of early adherent colonies on test surfaces to that on glass). Solid bar represents the ratio of the sum of early adherent colonies and clusters on comb copolymer surfaces to the sum of early adherent colonies and clusters on glass control. Results are mean $\pm$ SE. Statistical significance, $p < 0.0001$ and $p < 0.001$, ANOVA, was observed in the differences in CFE and colony-cluster efficiency respectively. Relative colony and cluster formation of all comb copolymers were lower than that on glass control ($p < 0.02$, Fisher LSD post hoc). Solid bars: * $p < 0.1$ vs. H22M9, ** $p < 0.05$ vs. H22M9; # $p < 0.1$ vs. H33M0 and H33M9. ($n = 5$). For CFE (striped bars), other than a significance level of $p < 0.001$ vs. glass was observed for all comb copolymers and $^{\dagger} p < 0.05$ for H6M9_1 and H6M9_3 vs. H6M9_2, the difference in CFE of the other comb copolymers was not statistically significant. ($n = 12$ for H6M9 comb copolymers and glass; $n = 3$ for all others).

Figure 6.

wtNR6 fibroblast spreading was enhanced on clustered-peptide surfaces and increased with increasing average peptide density. Cell spreading area was quantified by cell-area tracing of microscopy images. * $p < 0.001$ for cell spreading area on surfaces with the maximum number of active ligands per cluster, $N_{\text{ligand}} = 18$ (●) and $N_{\text{ligand}} = 3.5$ (△) or $N_{\text{ligand}} = 10.6$ (○). $N_{\text{ligand}} = 0$ for (□), on which only the inactive ligand, GRGESPYPY was present. Substrates of different ligand and cluster densities were prepared as outlined in the Materials and Methods section.

Figure 7.

Primary pMSCs attach (a) and form colonies (b) on substrates presenting GRGDSPY, but pMSC do not attach to control peptides GRGESPY or GRAASPY. Primary pMSC do not attach to FHRRIKA surfaces of various peptide surface densities while MC3T3-E1 cells show modest attachment at high FHRRIKA densities. In (a) and (c) percent attachment is relative to TCPS 16-18 hr after seeding is shown. By day 5, pMSC formed colonies on GRGDSPY substrates of even the lowest peptide density tested (4140 peptides/μm²). Error bars show standard errors associated with 3 technical triplicates of 3 separate experiments.

Figure 8.

Early adherent CFU-AP efficiency of GRGDSPY, inactive peptide control, YGGFHRRKA peptide and non-activated comb surfaces. Marrow aspirates from 7 individuals were used for GRGDSPY, GRGESPY and comb surfaces while FHRRKA and GRAASPY were tested with aspirates from 5 individuals. Patient classifiers are denoted on the x axes. Number in parentheses indicates total number of early adherent CFU on glass for that patient.

Figure 9.

Microscopy images of colony formation from human bone marrow aspirates on (a) RGD, (c) glass control and (d) FHRRIKA surfaces. Cells on (b) unmodified comb polymer substrates were predominantly single cells. Marrow cells were seeded and the non-adherent fraction was removed at 48 hours according to Figure 2. Colonies shown here are the early adherent CFUs. Substrates were fixed and stained for alkaline phosphatase 6 days after the initial seeding.

Figure 10.

Prevalence of early-adherent a total CTPs is highly variable among donors. No trend was observed in the prevalence of early-adherent CTP (expressed as percent of total adherent CTP) as a function of total CTP prevalence per 2×10^6 cells seeded for each of the seven marrow aspirate samples in Figure 8.

Table 1

Specifications of comb copolymers tested

Comb	<i>m-mer</i> HPOEM <i>m</i> =	<i>n-mer</i> MPOEM <i>n</i> =	wt% HPOEM	wt% MPOEM	Total wt% PEO
H6M9_1	6	9	18 %	16 %	34 %
H6M9_2	6	9	26 %	11 %	37 %
H6M9_3	6	9	20 %	16 %	36 %
H6M0	6	----	40 %	----	40 %
H10M0	10	----	40 %	----	40 %
H22M0	22	----	40 %	----	40 %
H33M0	33	----	40 %	----	40 %
H22M9	22	9	35 %	5 %	40 %
H33M9	33	9	35 %	5 %	40 %
H0M9	----	9	----	39 %	39 %
H0M45	----	45	----	50 %	50 %

Table 2

Single marrow cell adhesion on comb copolymers. + is assigned to surfaces with 1 - 10 adherent single cells, ++ to 11-99 cells and +++ to > 100 cells. (n ≥ 5)

	Single Cell Adhesion Score
H6M9_1	+-
H6M9_2	++
H6M9_3	+-
H6M0	+-
H10M0	++
H22M0	++
H22M9	++
H33M0	++
H33M9	++
H0M9	+
H0M45	+
Glass	++