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## The influence of tethered epidermal growth factor on connective tissue progenitor colony formation

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### Abstract

Strategies to combine aspirated marrow cells with scaffolds to treat connective tissue defects are gaining increasing clinical attention and use. In situations such as large defects where initial survival and proliferation of transplanted connective tissue progenitors (CTPs) is limiting, therapeutic outcomes might be improved by using the scaffold to deliver growth factors that promote the early stages of cell function in the graft. Signaling by the epidermal growth factor receptor (EGFR) plays a role in cell survival and has been implicated in bone development and homeostasis. Providing epidermal growth factor (EGF) in a scaffold-tethered format may sustain local delivery and shift EGFR signaling to pro-survival modes compared to soluble ligand. We therefore examined the effect of tethered EGF on osteogenic colony formation from human bone marrow aspirates in the context of three different adhesion environments using a total of 39 donors. We found that tethered EGF, but not soluble EGF, increased the numbers of colonies formed regardless of adhesion background, and that tethered EGF did not impair early stages of osteogenic differentiation.

### Keywords

Connective tissue; Mesenchymal stem cell; Stem cell; Bone tissue engineering; Growth factors; Adhesion molecule

### Introduction

Bone marrow contains a diverse population of connective tissue stem and progenitor cells that contribute to the formation of new tissues after injury. When marrow aspirate is plated

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in culture, these cells adhere, exhibit fibroblastic morphology, and proliferate to form colonies with multilineage differentiation potential [1]. Such colony-forming cells -- about one out of 20,000 nucleated marrow aspirate cells [2, 3] -- can form bone, cartilage and fat when transplanted in vivo [4]. Colonies formed from marrow aspirates are heterogeneous in size and appearance. This may reflect a spectrum of stem-to-early progenitor properties in the population giving rise to colonies or the presence of progenitor populations from multiple niches in native tissue. Various terminologies have been employed to describe the culture-expanded and selected progeny of tissue derived progenitors from various sources, including mesenchymal stem cells (MSCs) [5], skeletal stem cells [6], multipotential adult progenitor cells [7, 8], and multipotent mesenchymal stromal cells [9]. The present work characterizes properties of the mixed population of connective tissue progenitors (CTPs) extracted from native bone marrow whose progeny can differentiate into one or more connective tissues [10, 11]. Addition of autologous bone marrow aspirate containing CTPs to bone grafts (including ceramics and demineralized bone matrix) is already clinically practiced [12-14]. Recently, it has been shown in canine models of spinal fusion and segmental defect repair that the success of bone grafts can be enhanced using selective retention of CTPs over other nucleated cells based on the preferential adhesion of CTPs over other non-CTP populations [15-17]. These results motivate additional studies aimed at understanding and manipulating the CTP population in marrow-supplemented grafts to improve healing.

Survival and function of CTPs following implantation are influenced by local environmental cues. The scaffold or matrix used in the graft provides mechanical support and is a source of adhesive molecules and soluble factors that regulate CTP behavior. While natural scaffolds such as demineralized bone provide such cues, such scaffolds also have inherent limitations in size, structure, mechanical properties, biological activity and other features from lot-to-lot, thus motivating design of synthetic scaffolds with precise properties for particular indications.

Providing signals that act on transplanted CTPs to enhance survival and proliferation, upstream of differentiation programs, may enhance overall healing by increasing the number of cells that are capable of forming bone. Ligands for the epidermal growth factor receptor (EGFR) are candidates for such cues, as the EGFR plays important roles in bone development and homeostasis [18-21], and is an important regulator of CTP behavior [22-27]. Most importantly, EGFR signaling can expand a CTP pool without either committing it to differentiation or interfering with subsequent differentiation cues [28].

Although in vitro studies typically employ soluble EGF, there are several potential advantages in presenting EGF as a matrix-tethered molecule for applications in tissue engineering [29] so that it is competent to bind and activate the EGFR, but not be internalized and degraded. This mode mimics features of the physiological presentation of EGFR ligands that act in juxtacrine fashion or are matrix-bound. The potential advantages include a better control of local EGF concentration, reduction in receptor downregulation, and prolonging overall signaling [29]. Tethered EGF (tEGF) may also alter the balance of downstream signaling pathways activated by the EGFR compared to activation by soluble EGF. tEGF, but not soluble EGF, enhances cell spreading and protects culture-expanded human CTPs from pro-death inflammatory cues [30].

We therefore hypothesized that tEGF might also act beneficially on CTPs in fresh human bone marrow aspirates. We tested the ability of tEGF to increase the number of osteogenic CTP colonies generated from freshly aspirated human bone marrow plated in serum-containing medium, and whether the effects of tEGF were influenced by variation of the adhesive properties of the substrate.

## Materials and Methods

### Ligand-Modified Culture Substrates

Substrates were prepared to present tEGF against an adhesion background of pre-adsorbed fibronectin (FN), adsorbed serum proteins, or a branched minimal FN-derived adhesion peptide designed to mimic the adhesion sites in the 9<sup>th</sup>-10<sup>th</sup> domains of FN. This peptide, which we designate “PHSRN-(K)-RGD” contains two lysine-linked branches, one containing a RGD-adhesion domain and the other a PHSRN-synergy sequence, and a GGC stem attached to the lysine side chain for covalent linkage to the polymer [31]. Polymer-coated glass slides modified with ligands of interest were prepared as described earlier [30] with minor modifications to expand the range of adhesive ligands presented with tEGF. All substrates were prepared from blends of two different poly(methyl methacrylate)-graft-poly(ethylene oxide) (PMMA-g-PEO) amphiphilic comb copolymers [32]: (i) CC1 (32 wt% PEO) resists cell adhesion unless modified with adhesion peptides and fosters high-density ligand clustering [31-33]; and (ii) CC2 (20 wt% PEO) allows cell attachment mediated by serum protein adsorption [30]. CC1 was activated with 4-nitrophenyl chloroformate (NPC) (Alpha Aesar, Ward Hill, MA) [30, 33] to target the N-terminal amine of murine EGF, and with N-[p-Maleimidophenyl]isocyanate (PMPI) (Pierce Biochemical, Rockford, IL) to target thiol-terminated adhesion peptides, as described previously [31, 34]. Unmodified CC1, unmodified CC2, PMPI-activated CC1, and NPC-activated CC1 were blended in specific proportions to give a 20 mg/mL solution in toluene, spin-coated onto 18 mm square glass coverslips pre-treated with Siliclad (Gelest Inc., Morrisville, PA) to a thickness of ~75 nm, vacuum-dried, then modified by adhesion ligands and/or EGF (see below) to create 4 unique polymer surfaces (Table 1), some of which were further treated by adsorption of FN or serum.

Covalent linkage of the PHSRN-(K)-RGD peptide was accomplished by reacting substrates with a 25  $\mu$ M solution of the peptide in phosphate buffered saline (PBS) containing 10 mM Tris(2-Carboxyethyl) phosphine hydrochloride (TCEP; Sigma, St. Louis, MO) at pH 7.5 for 2 hr at room temperature [31]. The peptide density was about 20,000 RGD/ $\mu$ m<sup>2</sup> based on previous measurements [31]. Covalent linkage of murine EGF (PeproTech, Rocky Hill, NJ) to substrates, through the N-terminal amine of the EGF, was accomplished by incubating substrates for 20-24 hr with a 25  $\mu$ g/mL solution of EGF in 100 mM phosphate buffer (pH 8.5-8.7) at room temperature, followed by rinsing 3X and blocking unreacted sites with 100 mM Tris buffer (pH 9.0), to achieve a surface density of approximately 5000-7000 tethered EGF/ $\mu$ m<sup>2</sup> [30]. Substrates presenting tEGF against the PHSRN-(K)-RGD adhesion background were produced by sequential reaction of each ligand, because the PMPI reaction with thiols and the NPC reaction with amines proceed at different values of pH [30, 31]. Where indicated, FN (Sigma, St. Louis, MO) was adsorbed to substrates 1 and 2 (Table 1) by incubating substrates with a 10  $\mu$ g/mL solution in PBS for 2 hr at room temperature, rinsing 3X with PBS, then blocking for 1 hour at room temperature with 1% bovine serum albumin (Sigma, St. Louis, MO), and rinsing 3X. FN coating was performed within a day of use.

### Bone Marrow Aspiration

Human bone marrow was obtained from 39 normal donors and patients presenting to Dr. G.F. Muschler prior to an elective orthopedic procedure. All subjects were enrolled with full informed consent under a protocol approved by the Institutional Review Board of the Cleveland Clinic. 2 mL of bone marrow was isolated from the iliac crest of volunteers as described previously [3, 33]. Briefly, bone marrow was aspirated into a 10-ml plastic syringe containing 1 ml of saline containing 1000 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- $\alpha$  containing 2 unit/ml Na-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.

The aspirated bone marrow was centrifuged for 10 minutes at 1500 RPM. The buffy coat was isolated and re-suspended in complete medium, MEM- $\alpha$  (Invitrogen, Chicago, IL) containing, 50 mg/ml sodium ascorbate (Sigma, St. Louis, MO), antibiotic-antimycotic (Invitrogen, Chicago, IL), and 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA), using a lot that was selected on the basis of enhancing osteogenic differentiation.

### CTP/CFU Assay

Treated coverslips were placed in the wells of 2-chamber Lab-Tek culture slides (Nunc, Rochester, 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 cells (approximately  $1.5 \times 10^5$  nucleated cells/cm<sup>2</sup>) in complete medium.

For each donor, 4 coverslips for each surface condition being examined were seeded with bone marrow cells. In addition to comb copolymer surfaces, untreated glass surfaces were also seeded, and served as the positive control for each donor.

Cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The culture medium was changed 48 h after plating and the non-adherent cells from each chamber were replated on Lab-Tek slide surfaces. Where indicated, soluble EGF was added at 10 ng/mL at plating and with each subsequent medium change.

The initial and replated cultures were maintained for 4 and 6 additional days, respectively. Cultures were then washed twice with PBS, fixed, and stained with DAPI and for alkaline phosphatase (AP) with VectorRed (Vector Labs, Burlingame, CA). Clusters of 8 or more cells staining positive for AP were scored as an osteogenic colony. Colonies on original substrates were defined as “early adherent colonies,” as they originated from cells that adhered to the test surfaces within 48 h of plating. Colonies formed on Lab-Tek slides from the replating of cells that were non-adherent at 48 h were defined as “late adherent colonies.” Fig. 1A shows the schematic of the colony-forming unit (CFU) assay for CTP colony formation.

Nine different combinations of adhesion and growth factor environments were studied (Fig. 1B), in 3 different subsets of combinations so that multiple conditions could be compared using marrow from the same donor to account for patient-to-patient variability in CTP prevalence. For each experimental condition, we measured the prevalence of osteogenic colonies that formed per 1 million seeded cells and calculated relative colony forming efficiency (CFE) on each surface by normalizing to the prevalence on the glass control surfaces for that donor. The same procedure was applied with the late adherent colonies to determine the normalized number of late adherent colonies that formed on glass after being removed from a given experimental surface.

### Image Analysis

Quantitative image analysis was performed on data from 8 donors where indicated, using a procedure described previously [35]. Briefly, experimental and glass surfaces of fixed and stained cells were imaged at 10X. 100 individual images were combined into a single image such that colonies could be outlined and analyzed based on fluorescence, using software developed by Powell et al. [35]. Following rendering of outlines by the software, images were examined and colonies that were incorrectly outlined due to debris were adjusted

manually by the reviewer. The following metrics were analyzed for each colony: the area of the colony expressing AP above a threshold value (set by the reviewer) normalized to the number of cells in the colony; the number of cells per colony; and the cell density per colony. For each donor, the median value for each metric was assessed for all colonies and normalized to the median value for colonies formed on glass controls.

### Statistical Analysis

For each data set, every normalized data point was log base-2 transformed and an average value for each experimental condition was calculated across all patients. 95% confidence levels were calculated in log base-2 space. Three points for CFE had a measured value of 0, and these values were converted to 1/2 the smallest non-zero CFE for the rest of the data set for that condition, prior to the log transformation. Additionally, for two donors, the glass control surfaces had 0 late adherent colonies, precluding normalization. These data were therefore not included in the calculation of mean and confidence levels.

A Shapiro-Wilk test was used to assess the normality of each data set, and data sets were assumed to be normal if  $p > 0.05$ . To test for statistical differences between surface conditions, a two-way matched pairs t-test was performed in log base-2 space for each pair of conditions. In the event that a data set for a surface condition was non-normally distributed, a Wilcoxon signed-rank test was also used to test for significant differences between surface conditions. Using  $p = 0.05$  as the cutoff for significance, no differences in statistical conclusions were observed for any statistical comparison with non-normally distributed data sets using either the matched pair t-test or the Wilcoxon signed-rank test with one exception described below. For all plots, the anti-log of all data, as well as the mean and confidence intervals, are plotted on log base-2 axes. For plots containing data from 12 donors or fewer, data points from a single donor are connected by a line.

## Results

### Colony Formation on Adhesion Peptides with tEGF

In the CFU assay, marrow aspirates are plated at a density of  $1.5 \times 10^5$  nucleated cells/cm<sup>2</sup>, non-adherent cells are removed after 48 hr, and after a total culture time of 6 days, the number of colonies (8 or more cells in a cluster) is enumerated and the differentiation status of each colony is analyzed. Differences in prevalence (i.e., number of colonies per 2 million cells) may arise from differences in the number of CTPs present and adhering initially, entry of CTPs into the cell cycle (activation), or differences in proliferation. These processes are influenced by cues from the culture substrate and soluble medium components.

We first evaluated whether tEGF could enhance colony formation under conditions where adhesion was expected to be the limiting process in colony formation, as tEGF has been shown to enhance attachment and spreading of culture-expanded CTPs [30]. We therefore used substrates that foster attachment through the minimal peptide adhesion moiety RGD (using the PHSRN-(K)-RGD peptide) presented in a clustered format. While substrates presenting minimal RGD peptides in such a clustered format support proliferation of culture-expanded CTPs, the formation of colonies from human marrow aspirates on these substrates is significantly diminished compared to control adhesion environments [33] even though CTPs express integrins known to interact with RGD-containing matrix proteins [33, 36, 37].

We observed here an average CFE of 0.27 on PHSRN-(K)-RGD substrates, confirming that colony formation on clustered RGD is significantly diminished compared to glass controls (Fig. 2). tEGF increased colony formation on PHSRN-(K)-RGD almost 3-fold, resulting in an average CFE of 0.73 ( $p < 0.00002$ ). The minimal RGD adhesion sequence, even when

presented in nanoclusters, is not sufficient to engender full adhesion and spreading by all integrins that recognize the RGD sequence [38-41]. tEGF may be acting locally to enhance actin polymerization and adhesion [42], allowing adhesion to an otherwise poorly adherent substrate, or may be initiating inside-out activation of integrins [43] to enhance the fraction of CTPs that adhere. We thus sought to determine whether tEGF would increase colony formation on substrates expected to provide a greater level of intrinsic adhesion.

### Colony Formation on Adsorbed Serum and FN

Osteogenic colony formation is typically assessed by plating cells onto glass or plastic substrates in the presence of serum, so that adhesion is mediated by adsorbed serum proteins. Plating marrow in serum on control comb co-polymer substrates that allow protein adsorption (Blend 1 in Table 1; i.e. an “adsorbed serum” adhesion environment) we observed a significant increase in colony formation (CFE = 0.41), compared to tethered PHSRN-(K)-RGD peptide (0.27,  $p < 0.02$ ) substrates alone (Fig. 2). Even on this more favorable adhesion background, tEGF nearly doubled colony formation (CFE = 0.74,  $p < 0.00004$ ). Because average CFE was  $< 1.0$  (suggesting that CTP availability was not depleted), we would expect the magnitude of the increase in colony formation to be similar on adsorbed serum and PHSRN-(K)-RGD, if tEGF was increasing colony formation primarily by stimulating proliferation post-adhesion and activation. However, in the presence of tEGF, CFE was statistically similar on PHSRN-(K)-RGD and adsorbed serum ( $p > 0.9$ ).

Among the adhesion molecules in serum, FN has been shown to foster colony formation in serum-free medium in a manner comparable to serum-supplemented medium. In addition to the PHSRN and RGD sites in the 9<sup>th</sup>-10<sup>th</sup> domains, FN contains binding sites for integrin  $\alpha 4\beta 1$ , syndecans, and other receptors reported to be expressed by CTPs [36, 37, 44]. Hence, FN may offer an adhesive environment more conducive to colony formation than the minimal PHSRN-(K)-RGD sequence. Using a different cohort of donors than those studied in Fig. 2, we found that colony formation on FN (CFE = 0.46) was statistically similar to that for cells plated on adsorbed serum (CFE = 0.37,  $p > 0.09$ ), and greater than on PHSRN-(K)-RGD (CFE = 0.23,  $p < 0.004$ ) (Fig. 3). Notably, tEGF increased colony formation for cells on FN (CFE = 0.59,  $p < 0.005$ ) (Fig. 3), behavior consistent with the effect of tEGF on cells from this cohort plated on adsorbed serum or PHSRN-(K)-RGD (Fig. 3). Even though CFE was much greater on FN than on RGD in the absence of tEGF, when EGF was tethered to the substrate, CFE was similar on FN and on PHSRN-(K)-RGD, lending support to our hypothesis that tEGF is increasing the adhesion, survival, and/or activation of CTPs, rather than increasing proliferation.

### Colony formation with soluble EGF

In culture-expanded human CTPs and in an immortalized human MSC cell line, soluble EGF is far less effective than tEGF in promoting cell spreading and in protecting cells from FasL-mediated cell death [33] and decreases colony formation from rabbit marrow cells plated in the presence of serum [26]. In contrast, soluble EGF promotes colony formation from human bone marrow cells on FN in serum-free medium to a degree comparable to serum-supplemented medium [25]. When we add soluble EGF (10 ng/mL) to cells plated on FN in serum-containing medium, colony formation is significantly decreased compared to the control no EGF case (CFE = 0.32,  $p < 0.003$ ) as shown in Fig. 3. Thus, soluble EGF elicits a different response than tEGF, which significantly increased colony formation on FN in serum-containing media (Fig. 3). Phenotypic differences between soluble EGF and tEGF are also observed in the context of our two other adhesion environments, adsorbed serum and PHSRN-(K)-RGD peptide (Fig. 4): tEGF increased colony formation on both substrates ( $p < 0.04$ ), whereas soluble EGF did not ( $p > 0.1$ ).

## Influence of Donor Population Characteristics

The prevalence and biological performance of CTPs from bone marrow is affected by age, gender, and health of the donor [3], and it is possible that CTPs from different donor pools may respond differently to extracellular cues such as tEGF. We therefore compared the colony-forming responses of marrow aspirates from donors with a disease state, osteoarthritis, to those of normal healthy donors. We found that tEGF significantly increased CFE in both donor populations (Figs. 5A and 5B), on both PHSRN-(K)-RGD and adsorbed serum ( $p < 0.006$ ), demonstrating that the effects of tEGF are not limited to marrow from healthy donors.

Indeed, the effects of tEGF were consistent across all 39 donors (Fig. 5C). Without tEGF, colony formation was slightly increased on adsorbed serum (CFE = 0.33) compared to PHSRN-(K)-RGD (CFE = 0.24,  $p < 0.006$ ). However, with tEGF, CFE increased ~2.6X on PHSRN-(K)-RGD (CFE = 0.62,  $p < 0.0001$ ) and ~1.7X on serum (CFE = 0.57,  $p < 0.0001$ ). In the presence of tEGF, CFE was similar on serum and PHSRN-(K)-RGD ( $p > 0.2$ ), supporting our hypothesis that tEGF was enhancing the adhesion, survival, and/or activation of CTPs.

## Colony size on tEGF substrates

In diverse cell types, EGFR signaling influences downstream migration and proliferation responses in addition to survival and adhesion. In the CFU assay employed here, where colonies are examined visually following 6 days of culture, changes in cell proliferation and migration rates would likely influence two measurable properties: the number of cells per colony, and the density of cells in each colony (cells per unit area). We used a quantitative image analysis algorithm (see Methods) to measure these properties for marrow-derived CTPs plated on either PHSRN-(K)-RGD peptide or on adsorbed serum proteins, in the presence and absence of tEGF, with the data normalized to those obtained on glass for each donor. On the PHSRN-(K)-RGD peptide, the number of cells per colony (Fig. 6A) was similar with or without tEGF ( $p > 0.7$ ). However, on adsorbed serum, tEGF promoted an increase in colony size ( $p < 0.002$ ), suggestive of cell proliferation. Unlike changes in CFE, increased cell proliferation with tEGF appears to be unique to serum-adsorbed surfaces, suggesting that this effect is distinct from mechanisms underlying tEGF-enhanced CFE.

Cell density within the colonies also appeared to be differentially affected by the adhesive substrate (Fig. 6B). Cell density was greater on PHSRN-(K)-RGD than on adsorbed serum in the presence ( $p < 0.0008$ ) or absence ( $p < 0.04$ ) of tEGF, suggesting that the more diverse components present in adsorbed serum elicited a greater cell migration response than the simple PHSRN-(K)-RGD peptide. The adhesive environment was the dominant effect in determining cell density, as for a particular adhesion environment, no significant differences were observed in the presence of tEGF compared to its absence (Fig. 6B).

## Osteogenic differentiation on tEGF substrates

Because EGFR-mediated signaling has been shown to influence the differentiation of culture expanded CTPs [22, 28, 45], we assessed the relative values of AP expression by dividing the area of a colony expressing AP above a threshold value by the number of cells within that colony (see Methods for details of image analysis). Values for test substrates were normalized to values for glass for the same donor. No significant differences were observed in any of the test substrates compared to control class ( $p > 0.1$  for all comparisons), suggesting that tEGF neither enhances nor inhibits this early step along the osteogenic pathway. In 6 of 8 donors however, on PHSRN-(K)-RGD, the percent of AP positive cells was decreased in the presence of tEGF, suggesting that the differentiation of CTPs derived from some donors may be affected by tEGF under some adhesion conditions. More sensitive

measures of osteogenic differentiation, including AP activity per cell and expression levels of other osteogenic markers, may reveal functional differences between these conditions.

## Discussion

Many envisioned applications of marrow-derived connective tissue stem and progenitor cells in regenerative medicine require biomaterials that can direct the attachment, survival, activation, proliferation, migration, and differentiation of these cells. To this end, we investigated how an unconventional biophysical presentation of a ligand for EGFR influences behavior of CTPs in fresh human bone marrow aspirates, with two major findings of importance for CTP biology and use. First, we found that tethered tEGF, enhances colony formation by primary CTPs independent of adhesion background (Figs. 2-5), and does so in a way that does not appear to impair the early stages of osteogenic differentiation (Fig. 6C). Second, we found that the effects of EGF on colony formation depend on its mode of presentation. In contrast to tEGF, soluble EGF at the physiological concentration of 10 ng/mL does not enhance colony formation, and slightly inhibits colony formation on FN (Figs. 3 and 4).

Although many facets of how culture-expanded human CTPs respond to EGFR ligands have been described [22, 27, 28, 45], relatively little is known about how freshly-aspirated human marrow-derived CTPs respond in this regard. In an elegant study, Gronthos and Simmons showed that 90% of the colony-forming cells in fresh human marrow aspirates co-express the EGFR and the marker Stro-1, and that when Stro-1 positive cells are plated on FN, soluble EGF at concentrations as low as 1 ng/mL fosters colony formation to an equivalent degree as plating in serum on plastic [25]. While we found soluble EGF to be slightly inhibitory to colony formation on FN, these differences may be attributed to our mixed population of cells and other complexities associated with adhesion and growth factor signaling, as well as the inclusion of serum in our experimental protocol.

EGFR has several natural ligands and activates multiple divergent signaling pathways. The duration and relative strengths of different signaling pathways activated by ligand-bound EGFR are strongly influenced by receptor internalization and trafficking [46-48]. Ligands that have low cell surface binding affinities or dissociate in the endosome, such as transforming growth factor alpha and tenascin cytoactin-encoded EGF-like repeats, preferentially signal through pathways localized at the cell surface, including PLC $\gamma$  [49]. In contrast, ligands that remain receptor-bound following internalization, such as EGF, drive sustained signaling through Ras/ERK pathways during intracellular trafficking [47, 50, 51].

Pathways activated by the EGFR stimulate lamellipod extension, stabilization of focal contacts, cell contraction, and multiple other events involved in adhesion and spreading processes that are essential early events in colony formation. Under conditions where the number or strength of adhesion receptor-extracellular matrix bonds is low -- e.g., where the number of matrix adhesion sites is limiting, the adhesion ligand has low affinity for the receptor, or the matrix proteins are only weakly adsorbed to the substrate -- stimulation by EGF (or other growth factors) can cause cell rounding and detachment by creating an unfavorable ratio of cell contractile force to adhesion force [52]. The polymer substrates we used for analyzing colony formation in response to tethered and soluble EGF inhibit protein binding either partially (adsorbed serum and FN conditions) or completely (RGD condition) [33]. Although we have previously observed that several cell lines are highly adhesive on similar RGD substrates [33, 53], we observed here that the number of colonies formed from human marrow aspirates plated on the three test adhesion environments (RGD, FN, adsorbed serum proteins) was reduced compared to glass for the control condition (no EGF), suggesting these substrates are inherently less adhesive for CTPs than glass in the presence



of serum. In this setting, soluble EGF may further impair colony formation on these substrates by pushing cell contraction to overwhelm adhesion forces. However, because tEGF preferentially stimulates pathways localized to the cell surface, including very rapid local actin polymerization and lamellipod extrusion [42], the overall balance of signaling pathways on tEGF may favor the adhesion, and thus survival and activation, of attached cells, conditions permissive for proliferation and colony formation.

We have focused on the potential role of EGFR stimulation to affect events that occur in the first few hours to days following transplantation of freshly-aspirated human CTPs, reasoning that increasing attachment, survival, activation, and proliferation of CTPs at this stage may facilitate more robust bone formation in large defects. Although our results suggest that tEGF does not impair osteogenic differentiation at least at the early stage examined here (Fig. 6C), it is difficult to predict how tEGF may influence downstream events in bone wound healing *in vivo* due to the complexity of factors at play and the fact that its direct effects would be limited to the scaffold surface. Studies in mice with genetically reduced EGFR levels, and of osteoblastic cells stimulated with EGFR ligands *in vitro*, suggest that activation of EGFR simulates proliferation of progenitor cells and inhibits osteoblastic differentiation [20, 27, 53], while other studies support EGF promotion of proliferation *in vitro* without inhibiting osteogenic differentiation [28, 45] or enhancement of differentiation of culture-expanded CTPs [22, 54]. In addition to the context-dependent effects on osteogenic cells expressing EGFR, EGFR signaling promotes angiogenesis [55] and influences osteoclast activity [56]. Hence, *in vivo* studies are required to determine if the integrated effects elicited by tEGF offer clinical advantages in tissue regeneration. Our preliminary studies here and elsewhere [30] motivate such *in vivo* studies, in both 2D and 3D, as they demonstrate that tEGF exerts positive influences on CTP behaviors important in the early post-transplant period.

## Conclusion

In conclusion, this study has shown that tEGF enhances human CTP osteogenic colony formation across multiple adhesion conditions via a mechanism most likely related to improved survival and activation of CTPs as well as proliferation. The effects of tEGF stand in contrast to those of soluble EGF. Hence, tEGF may be a useful molecular signal in transplantation of human marrow-derived CTPs for regenerative medicine.

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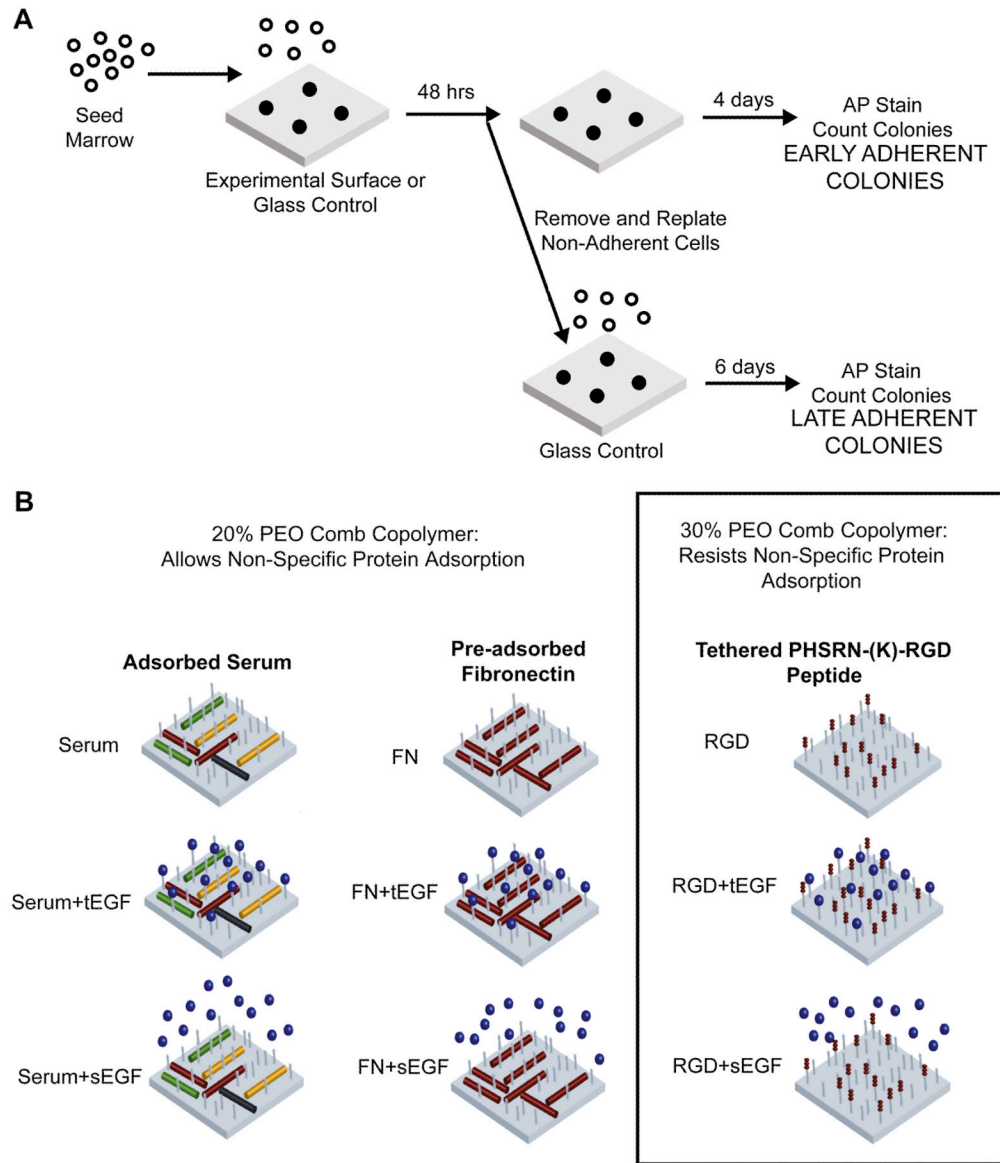
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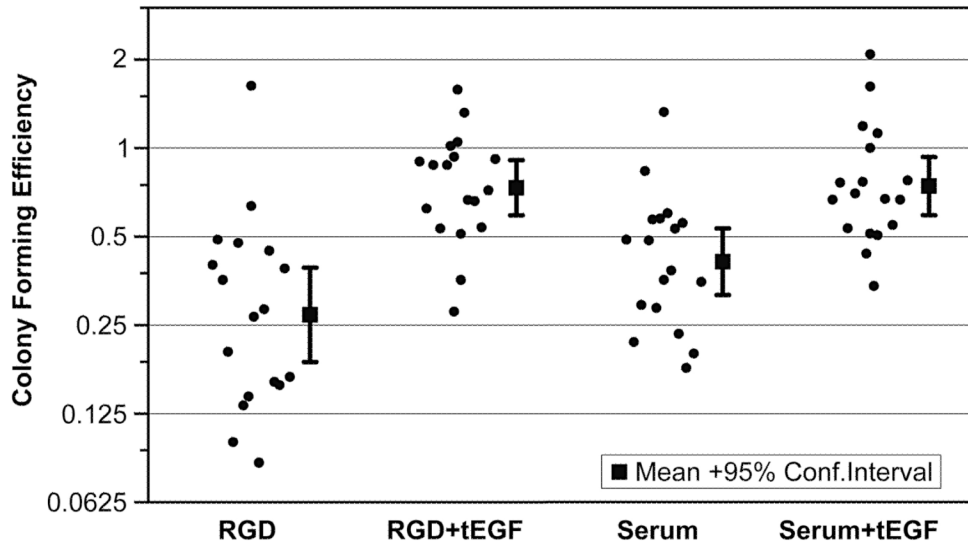
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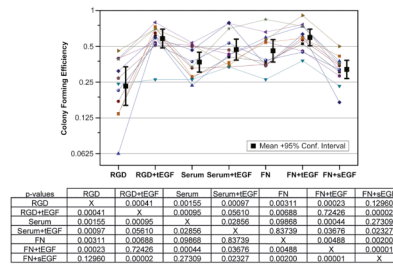


**Figure 1.** Experimental setup used to examine the effect of tethered epidermal growth factor (tEGF) on osteogenic connective tissue progenitor (CTP) colony formation from freshly aspirated bone marrow. **(A)** Protocol for bone marrow aspirate colony forming unit assay. Open circles: non-adherent bone marrow cells. Closed circles: colonies of adherent cells derived from adherent connective tissue progenitors. **(B)** Osteogenic CTP colony formation was studied without epidermal growth factor, with tEGF, or with soluble epidermal growth factor at 10  $\mu\text{g}/\text{mL}$  on three adhesive conditions: adsorbed serum, pre-adsorbed fibronectin, or tethered PHSRN-(K)-RGD peptide, as depicted schematically. Abbreviations: PEO, poly(ethylene oxide); serum, adsorbed serum; FN, pre-adsorbed fibronectin; RGD, tethered PHSRN-(K)-RGD peptide; tEGF, tethered epidermal growth factor; sEGF, soluble epidermal growth factor.

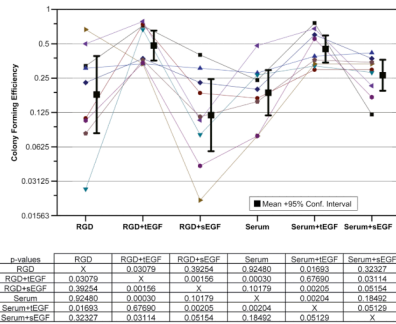


| p-values   | RGD     | RGD+tEGF | Serum   | Serum+tEGF |
|------------|---------|----------|---------|------------|
| RGD        | X       | 0.00001  | 0.01164 | 0.00007    |
| RGD+tEGF   | 0.00001 | X        | 0.00038 | 0.90039    |
| Serum      | 0.01164 | 0.00038  | X       | 0.00003    |
| Serum+tEGF | 0.00007 | 0.90039  | 0.00003 | X          |

**Figure 2.** Tethered epidermal growth factor (tEGF) enhances connective tissue progenitor (CTP) adherent colony formation. The relative colony forming efficiency (CFE) of adherent CTPs was measured for a total of 18 donors with an average age of 62. Each point represents data from an individual donor on a given experimental surface condition. Error bars indicate mean values and 95% confidence levels for each experimental condition. p-values are calculated using a matched-pairs t-test (matched by donor) and are shown in the tables. The osteogenic colony formation, indicated by CFE, of adherent CTPs from freshly aspirated bone marrow on tethered PHSRN-(K)-RGD peptide and adsorbed serum is increased in the presence of tEGF ( $p < 0.00004$ ). Without tEGF, CFE is greater on adsorbed serum than on PHSRN-(K)-RGD ( $p < 0.02$ ), while with tEGF, CFE is similar on PHSRN-(K)-RGD and adsorbed serum ( $p > 0.9$ ). Abbreviations: RGD, tethered PHSRN-(K)-RGD peptide; tEGF, tethered epidermal growth factor; serum, adsorbed serum.

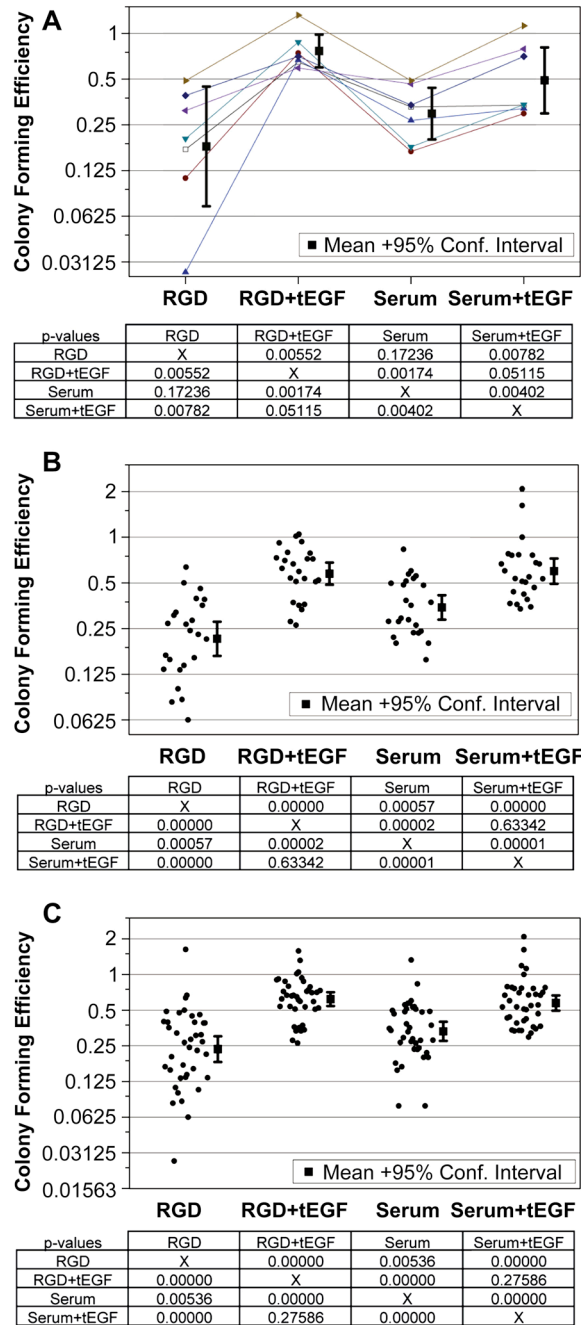


**Figure 3.** Tethered epidermal growth factor (tEGF) enhances connective tissue progenitor (CTP) colony formation on both minimally adhesive and highly adhesive substrates. The osteogenic colony formation, indicated by relative colony forming efficiency (CFE), of CTPs from freshly aspirated bone marrow was increased on tethered PHSRN-(K)-RGD peptide, adsorbed serum and pre-adsorbed fibronectin in the presence of tEGF ( $p < 0.03$ ). CFE is decreased on fibronectin in the presence of soluble epidermal growth factor at 10 ng/mL ( $p < 0.003$ ). Each point represents CFE for an individual donor on a given experimental surface condition for a total of 12 donors with an average age of 51. Lines connect data for a specific donor. Error bars indicate mean CFE and 95% confidence levels for each experimental condition. p-values are calculated using a matched-pairs t-test (matched by donor) and are shown in the table. Abbreviations: RGD, tethered PHSRN-(K)-RGD peptide; tEGF, tethered epidermal growth factor; serum, adsorbed serum; FN, pre-adsorbed fibronectin; sEGF, soluble epidermal growth factor.



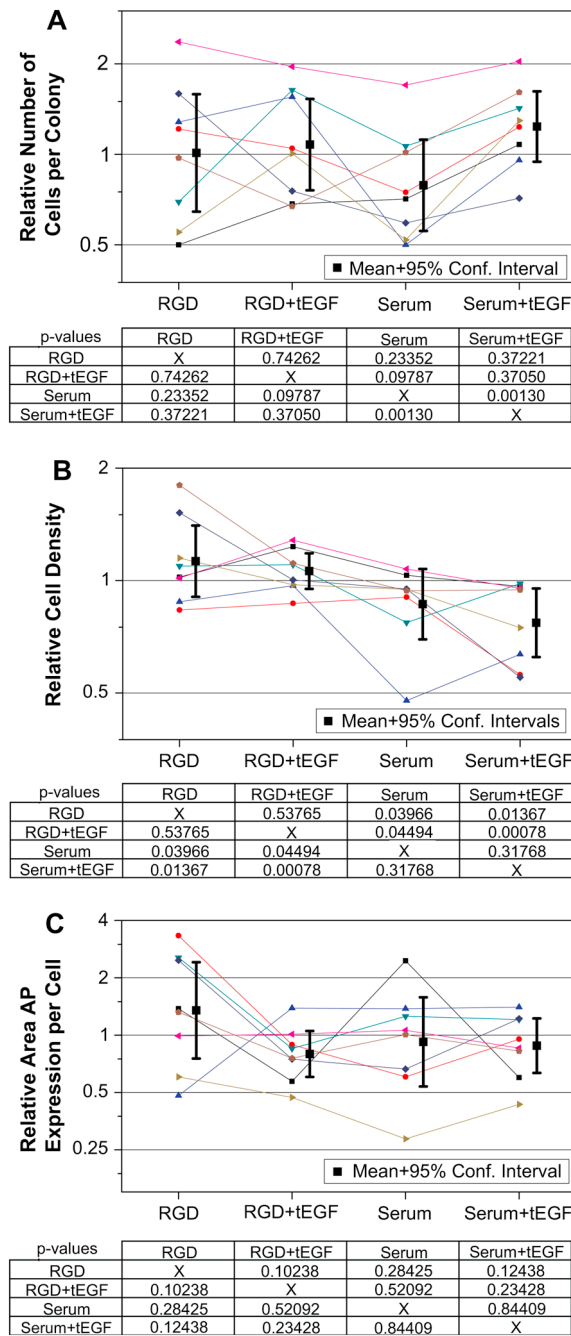
**Figure 4.** Tethered epidermal growth factor (tEGF), but not soluble epidermal growth factor, enhances connective tissue progenitor (CTP) colony formation. The osteogenic colony formation, indicated by relative colony forming efficiency (CFE), of CTPs from freshly aspirated bone marrow was increased on tethered PHSRN-(K)-RGD peptide and adsorbed serum in the presence of tEGF ( $p < 0.04$ ). Soluble epidermal growth factor at 10 ng/mL does not significantly affect CFE for either adhesive condition ( $p > 0.05$ ). Each point represents CFE for an individual donor on a given experimental surface condition for a total of 9 donors with an average age of 58. Lines connect data for a specific donor. Error bars indicate mean CFE and 95% confidence levels for each experimental condition. p-values are calculated using a matched-pairs t-test (matched by donor) and are shown in the table. Abbreviations: RGD, tethered PHSRN-(K)-RGD peptide; tEGF, tethered epidermal growth factor; serum, adsorbed serum; sEGF, soluble epidermal growth factor.





**Figure 5.** Tethered epidermal growth factor (tEGF) enhances connective tissue progenitor (CTP) colony formation in multiple donor populations. Colony forming efficiency (CFE) data was pooled from all three donor cohorts to assess the effect of tEGF on osteogenic colony formation in both healthy volunteers as well as donors with osteoarthritis. Each point represents CFE for an individual donor on a given experimental surface condition. Error bars indicate mean CFE and 95% confidence levels for each experimental condition. p-values are calculated using a matched-pairs t-test (matched by donor). (A) CFE as measured in 7 healthy donors with average age of 39, with lines connecting data for each specific donor. tEGF enhances CFE on both tethered PHSRN-(K)-RGD peptide and adsorbed serum

( $p < 0.008$ ). **(B)** CFE as measured in 24 donors with osteoarthritis with average age of 63. As observed with healthy donors, tEGF enhances CFE on both tethered PHSRN-(K)-RGD peptide and adsorbed serum ( $p < 0.00002$ ). **(C)** Data pooled from all 39 donors, with average age of 58 (8 donors presented with indications other than or in addition to osteoarthritis). tEGF enhances CFE on both tethered PHSRN-(K)-RGD peptide and adsorbed serum ( $p < 0.00001$ ). In the absence of tEGF, CFE is enhanced on adsorbed serum compared to tethered PHSRN-(K)-RGD peptide ( $p < 0.006$ ). Abbreviations: RGD, tethered PHSRN-(K)-RGD peptide; tEGF, tethered epidermal growth factor; serum, adsorbed serum.



**Figure 6.** Connective tissue progenitor colony size, cell density, and differentiation status as measured with quantitative image analysis. Data was compiled from 8 donors with an average age of 65. Individual points represent median values for an experimental substrate normalized to median values on control surfaces for each donor. Lines connect data points from a specific donor and error bars indicate mean colony values and 95% confidence levels for each experimental condition. p-values are calculated using a matched-pairs t-test (matched by donor). **(A)** Tethered epidermal growth factor (tEGF) increases the number of cells per colony on adsorbed serum ( $p < 0.002$ ), but not on tethered PHSRN-(K)-RGD ( $p > 0.7$ ). **(B)** tEGF did not affect cell density on either tethered PHSRN-(K)-RGD peptide or adsorbed

serum ( $p > 0.3$ ). However, cell density was greater on tethered PHSRN-(K)-RGD peptide compared to adsorbed serum, both with and without tEGF ( $p < 0.05$ ). (C) To assess differentiation, the area of a colony expressing alkaline-phosphatase (AP) above a given threshold was normalized to the number of cells in the colony. Using this metric, tEGF was not observed to promote a statistically significant increase in the number of cells within a colony expressing AP ( $p > 0.1$ ). However, on PHSRN-(K)-RGD, tEGF decreased the number of cells expressing AP in 6 of 8 donors. Abbreviations: RGD, tethered PHSRN-(K)-RGD peptide; tEGF, tethered epidermal growth factor; serum, adsorbed serum; AP, alkaline phosphatase.

Polymer blends used for experimental substrates presenting adhesion peptides and/or tethered epidermal growth factor.

**Table 1**

| Blend | Adhesion-Mediating Component | %    | Tethered Growth Factor Component | %   | Bulk          | %   |
|-------|------------------------------|------|----------------------------------|-----|---------------|-----|
| 1     | Non-Resistant CC2            | 100% | None                             | -   | -             | -   |
| 2     | Non-Resistant CC2            | 60%  | EGF-modified CC1                 | 40% | -             | -   |
| 3     | PHSRN-K-RGD-modified CC1     | 25%  | None                             | -   | Resistant CC1 | 75% |
| 4     | PHSRN-K-RGD-modified CC1     | 25%  | EGF-modified CC1                 | 40% | Resistant CC1 | 35% |

Abbreviations: CC1, poly(methyl methacrylate)-graft-poly(ethylene oxide) with 32% poly(ethylene oxide) by weight; CC2, poly(methyl methacrylate)-graft-poly(ethylene oxide) with 20% 20% poly(ethylene oxide) by weight; EGF, epidermal growth factor.