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Isolating and Expanding Endothelial Progenitor Cells from Peripheral Blood on Peptide-functionalized Polystyrene Surfaces

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ABSTRACT

The expansion of human peripheral blood endothelial progenitor cells to obtain therapeutically relevant endothelial colony forming cells (ECFCs) has been commonly performed on xeno-derived extracellular matrix proteins. For cellular therapy applications, xeno-free culture conditions are desirable to improve product safety and reduce process variability. We have previously described a novel fluorophore-tagged RGD peptide (RGD-TAMRA) that enhanced the adhesion of mature endothelial cells *in vitro*. To investigate whether this peptide can replace animal-derived extracellular matrix proteins in the isolation and expansion of ECFCs, peripheral blood mononuclear cells from 22 adult healthy adult donors were seeded on RGD-TAMRA-modified polystyrene culture surfaces. Endothelial colony formation was significantly enhanced on RGD-TAMRA-modified surfaces compared to the unmodified control. No phenotypic differences were detected between ECFCs obtained on RGD-TAMRA compared to ECFCs obtained on rat tail collagen-coated surfaces. Compared to collagen-coated surfaces and unmodified surfaces, RGD-TAMRA surfaces promoted ECFC adhesion, cell spreading, and clonal expansion. This work presents a platform that allows for a comprehensive *in vitro* evaluation of peptide-based biofunctionalization as a promising avenue for *ex vivo* ECFC expansion.

KEY WORDS: endothelial progenitor cells, extracellular matrix-derived-derived peptides, bio-functionalization, *ex vivo* expansion, xeno-free, vascular regeneration

1. INTRODUCTION

In the last two decades, endothelial progenitor cells (EPCs) emerged as a promising patient-specific vascular repair agent that can be targeted to treat multiple ischemic diseases, with over 150 registered interventional clinical trials in the United States and Canada seeking to utilize their therapeutic potential (Ceradini et al., 2004; Chong, Ng, & Chan, 2016). Since the first description of putative EPCs , these bone marrow-derived progenitors have been shown to directly contribute to various vascular regeneration mechanisms due to their availability in the blood and their high proliferative capacity (Takayuki Asahara et al., 1999; T. Asahara et al., 1997). Different cell populations grouped under the term "EPC" leading to inconsistent report but can be distinguished based on phenotypic and functional attributes (Medina et al., 2017). For example, two distinct cell populations expressing endothelial cell markers can be obtained by culturing peripheral blood mononuclear cells (PBMCs) in different conditions: myeloid angiogenic cells and endothelial colony forming cells (ECFCs). While ECFCs carry the ability to directly contribute to vessel formation and repair, myeloid angiogenic cells are thought to promote vascular repair through only paracrine effects (Ingram et al., 2004; Timmermans et al., 2009).

Integrin signaling has been linked to EPC trafficking from the circulating blood resulting in the activation of biochemical pathways responsible for necessary endothelial function including proliferation, nitric oxide secretion, and mechanotransduction (Malinin, Pluskota, & Byzova, 2012; Simon & Burridge, 1994). Integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ along with intercellular adhesion molecule-1 (ICAM-1)/ β_2 interactions are thought to mediate endothelial cell adhesion to extracellular matrix (ECM) proteins such as fibronectin and collagen (Chavakis et al., 2005; De Falco et al., 2004; Robson, Pichla, Zhou, & Baldwin, 2002; Yoon et al., 2006). These integrins also play an essential role in neo-vessel formation, maturation, and repair (Malan et al., 2010). To mimic this environment *ex vivo*, blood mononuclear cells are seeded on xeno-derived collagen coated culture surfaces for EPCs to adhere, differentiate, and proliferate forming colonies of ECFCs (Hirschi, Ingram, & Yoder, 2008). However, maintaining the integrity, conformation and function of surface-grafted or surface-adsorbed collagen on culture surfaces during sterilization, or performing surface modifications in aseptic conditions remains a major hurdle for the timely processing of EPCs for clinical applications. From a

safety and regulatory perspective, eliminating xeno-derived proteins, such as rat tail collagen, can also potentially enhance the clinical progress of EPC-based cell therapeutics.

Alternatively, cell culture surfaces used for ECFC expansion can be functionalized with ECM-derived peptides such as RGD to promote cell adhesion. RGD is a common motif that contributes to the cell adhesive properties of various ECM proteins including fibronectin, vitronectin, fibrinogen, osteopontin and some collagens due to its affinity to several members of the integrin receptor family (Pierschbacher & Ruoslahti, 1984; Ruoslahti & Pierschbacher, 1987).Surface-bound cyclic RGD molecules were found to enhance the formation of ECFC colonies derived from porcine PBMCs in a study conducted by Blindt *et al.* (Blindt et al., 2006). However, the characterization of the colonies obtained was limited to morphology, as well as the expression of low-density lipoprotein and glycans with no functional analysis. Other pro-endothelialization surfaces targeting EPCs were studied using endothelial cell lines such as human umbilical vein endothelial cells (HUVECs), which may not be predictive of the interactions of EPCs with the same materials (Galiazzo et al., 2014; Jang et al., 2015; Noel, Hachem, Merhi, & De Crescenzo, 2015).

So far, most studies aiming to optimize EPC expansion have focused on the effects of soluble factors present in cell culture media (Bauman, Granja, & Barrias, 2018). To our knowledge, there have been no studies on the ability of ECM-derived peptides to promote the formation ECFC colonies from human PBMCs. We have previously found that a fluorophore-tagged peptide containing the GRGDS sequence, referred to as RGD-TAMRA, significantly enhanced human saphenous vein endothelial cell spreading (C. A. Hoesli et al., 2014) and retention under arterial levels of laminar shear stress (Corinne A. Hoesli et al., 2018). Here, we covalently grafted the RGD-TAMRA peptide on aminated polystyrene surfaces and studied the effects of these surfaces on colony formation, adhesion, and expansion of ECFCs derived from human adult PBMCs.

2. MATERIALS AND METHODS

2.1 Peptides applied to surfaces or used as soluble inhibitors

The RGD-containing fluorophore tagged peptide, referred to as RGD-TAMRA (synthesized through custom services by Anaspec Inc., Fremont, CA), consists of the following sequence: CG-K(PEG3-TAMRA)-GGRGDS-NH2 where the PEG3-TAMRA is a 3-unit long polyethylene glycol side branch conjugated to a

TAMRA fluorophore (Figure 1A). The peptide sequences CGKGGRGDS-NH_2 and CGKGGRDGS-NH_2 were synthesized by Bio Basic Inc. (Markham, ON).

2.2 Surface functionalization

Substrates utilized for surface modification as well as controls were PureCoat™ Amine culture plates (Corning®, Corning, NY), referred to as "unmodified surfaces". For covalent conjugation, the bi-functional linking arm sulfosuccinimidyl 4-(N-maleimidophenyl)butyrate (referred to as "sulfo-SMPB"; G Biosciences®, St. Louis, MO) was first grafted onto the surfaces by adding 0.1 mL/cm^2 of a 3 mg/mL suspension of sulfo-SMPB in 7.4 pH phosphate-buffered saline (PBS, Thermo Fischer Scientific™, Waltham, MA). After 2 hours of incubation at room temperature in the dark with 50 rpm agitation, the surfaces were washed with reverse osmosis water, air dried and peptides were grafted by adding 0.1 mL/cm^2 of a 20 μM RGD-TAMRA peptide solution prepared in 7.4 pH 0.1 M citrate buffer, followed by incubation for 3 hours at room temperature in the dark with 50 rpm agitation. In adhesion studies, surfaces modified with CGKGGRDGS peptide were also prepared as controls – referred to as "RDG surfaces". Surfaces were then rinsed thoroughly with 0.2 μm filtered PBS and stored in PBS at 4 °C for up to 2 days. Immediately before use, the culture plates were sterilized by submerging in 95% ethanol (Commercial Alcohols Inc., Brampton, ON) for 15 minutes, left to dry for 30 mins in a biosafety cabinet, and then rinsed thoroughly with Dulbecco's phosphate buffered saline (DPBS; Thermo Fischer Scientific™). To prepare the culture surfaces modified via adsorption, the 20 μM RGD-TAMRA solution in citric acid buffer was directly added to PureCoat™ Amine culture plates (without S-SMPB treatment) and incubated for 3 hours at room temperature in the dark with 50 rpm agitation followed by thorough rinsing with 0.2 μm filtered PBS. Collagen-coated surfaces were prepared by adding 0.15 mL/cm^2 of 50 μg/mL type 1 rat tail collagen (Thermo Fischer Scientific™) in 0.02 N acetic acid (Thermo Fischer Scientific™) to sterilized (by 95% ethanol) PureCoat™ Amine culture plates and incubated for an hour before rinsing with sterile PBS and using on the same day to seed cells.

2.3 RGD-TAMRA longevity

To study the durability of the RGD-TAMRA modification strategy, 2 cm x 3 cm aminated polystyrene surfaces were cut from PureCoat™ Amine Petri dishes using a hot wire (Hot Wire Foam Factory, Lampoc,

CA). Spots of RGD-TAMRA with ~2 mm diameter were created by pipetting 2 μL of the RGD-TAMRA solution directly onto the PureCoat™ Amine surfaces (adsorption) or PureCoat™ Amine surfaces reacted with sulfo-SMPB (conjugation) and incubating for 3 hours at room temperature. At the end of the reaction time, all samples were rinsed with PBS twice, then with reverse osmosis water twice, and dried using compressed air. The modified surfaces were then stored in the dark at room temperature either in a 9 cm diameter petri dish with 25 ml of PBS or in dry conditions for 5 minutes, 7 days, 14 days, or 28 days. Immediately before imaging, surfaces were air-dried, and fluorescence images were obtained using a Zeiss LSM 510 Meta Confocal Microscope and fluorescence intensities were quantified using the CellProfiler™ software via the "Measure Object Intensity" plug-in.

2.4 Blood donation and PBMC isolation

Blood samples (80-120 mL) were collected in 60 mL syringes coated with 2 mL of 1000 unit/ml heparin (Sandoz Canada Inc., Boucherville, QC) from 22 healthy donors (12 males and 10 females) after obtaining informed consent. All protocols were approved by the Institutional Review Board at McGill university (IRB Study No. A06-M33-15A). Blood from each donor was diluted in a 1:1 ratio with sterile DPBS before the isolation of PBMCs via density centrifugation using SepMate™ tubes (Stem Cell Technologies Inc., Vancouver, BC) according to the manufacturer's instructions. Briefly, Histopaque®-1077 solution (Sigma-Aldrich®, St. Louis, MO) was used by adding 15 mL to the bottom of each tube followed by 30 mL of the diluted blood. Tubes were centrifuged and the top PBMC-containing fraction was poured off. The PBMCs were washed twice with endothelial cell growth basal medium (EBM-2; Lonza, Basel, Switzerland) and then resuspended in complete endothelial cell growth medium-2 (complete EGM-2) at 12.5 million cells/mL. The complete medium consisted of EGM-2 BulletKit™ (Lonza), topped up to 10% fetal bovine serum (FBS) with Hyclone™ FBS (GE Healthcare Life Sciences). 4 ml of cell suspension were then seeded on each well of either RGD-TAMRA-modified 6-well plates, collagen-coated 6-well plates, or directly on the unmodified PureCoat™ Amine 6-well plates and then placed in a humidified incubator at 37 ˚C and 5% CO_2.

2.5 Cell culture

The spent cell culture medium along with non-adherent cells after seeding of PBMCs were aspirated and fresh 2 mL of complete EGM-2 medium were added to each well of the 6-well plates 24 hours after seeding.

This was repeated every day for the first 7 days, after which medium was changed every other day. After the 7th day, the wells were visually screened for colonies of single layered cells with cobblestone morphology on a trinocular inverted microscope under phase contrast (VWR International, Radnor, PA) twice per week for 3 weeks (until day 28 after seeding). Each colony was allowed to grow for a week before removing it from the surface using TrypLE (Thermo Fischer Scientific™) and re-distributing the harvested cells on 3 wells of a 6 well plate with the same surface treatment to expand the ECFCs for further experiments. ECFCs were passed at confluency using a 1:8 dilution ratio and further experiments were conducted with ECFCs at passage 3-6. Human abdominal aortic endothelial cells (HAAECs; Coriell Institute for Medical Research, Camden, NJ) were cultured on gelatin (type A from porcine skin; Sigma-Aldrich®) coated T-75 flasks (VWR International) using complete EGM-2 medium and used at confluency at passage 6.

2.6 Flow cytometry

ECFCs derived on RGD-TAMRA and collagen surfaces along with HAAECs were separately removed from the culture surface using TrypLE and re-suspended in complete EGM-2 medium for an hour for surface antigen recovery. Cells were then first incubated with recombinant Fc protein block (BD biosciences, Franklin Lakes, NJ) at 2.5 µg/10^6 cells suspended in a staining buffer (PBS with 3% FBS and 0.09% sodium azide (Sigma-Aldrich™)) for 10 minutes at room temperature to block non-specific antibody adhesion. Cells were labelled with fixed viability stain 660 (BD Biosciences) to discriminate between live and dead cells. Mouse anti-human primary fluorescent labelled antibodies (all BD biosciences unless otherwise indicated) were then used to label cell surface antigens at 4°C for 20 minutes in the absence of light. Primary antibodies included FITC-conjugated anti-human CD144, PE-conjugated anti-human CD31, FITC-conjugated anti-human CD105, PE-conjugated anti-human CD146, FITC-conjugated anti-human CD34, PE-conjugated anti-human CD309 (BioLegend®, San Diego, CA), FITC-conjugated anti-human CD14, and PE-conjugated anti-human CD45. Unstained cells, FITC and PE mouse IgG1 k isotype control (BD biosciences), and compensation beads (BD biosciences) were used as controls. A flow cytometer (BD Accuri™) was used to record a minimum of 10,000 events per sample after gating on live cells. The data was analyzed using FlowJo (FlowJo, LLC, Ashland, OR).

2.7 Matrigel network formation assay

Matrigel-coated 96-well plates were prepared by adding 50 μL per well of undiluted Matrigel® Growth Factor-reduced Membrane Matrix (Corning®), followed by incubation at 37°C for 30 minutes. ECFCs suspended in complete EGM-2 medium were then seeded onto of the gelled Matrigel® by adding 80 μL at a seeding density of 175,000 cells/mL. HAAECs were seeded at the same density as a positive control. Monocytes were isolated from human PBMCs using the CD14+ MidiMACS™ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions, resuspended in complete EGM-2 medium at the same density, and seeded on top of gelled Matrigel® as a negative control. Seeded wells were imaged 4 hours after seeding using a trinocular inverted microscope (VWR International).

2.8 Flow chamber cell alignment

A previously described custom made parallel plate flow chamber (Corinne A. Hoesli et al., 2018) was used to study cellular and cytoskeletal alignment of the ECFCs obtained from RGD-TAMRA and collagen surfaces. The flow chamber, including silane-treated glass test surfaces (Electron Microscopy Sciences, Hatfiedld, PA) coated with gelatin, and circulation loops were assembled aseptically as previously described (Corinne A. Hoesli et al., 2018). A peristaltic pump (Masterflex L/S Economy, Cole-Parmer, Vernon Hills, IL) with two mounted 2-channel 4-roller pump heads (Masterflex L/S Easy-Load® II, Cole-Palmer) was used to apply flow immediately upstream from the pulse dampener. Warm pH equilibrated complete EGM-2 media was added to the medium reservoirs and the entire assembly was placed in a 37 ˚C, 5% CO_2 incubator. The complete EGM-2 medium was circulated in the system 1 hour prior to cell seeding by turning on the peristaltic pump. Next, 2 mL a 1 x10^6 cells/mL ECFC suspension were injected per flow chamber to obtain a seeding density of 50,000 cells/cm^2. After 3 hours of adhesion under static conditions, the adhered ECFCs were maintained overnight with medium circulation at low wall shear stress (0.2 dyn/cm^2). The next day the average wall shear stress was gradually increased to 22 dyn/cm^2 and maintained at 22 dyn/cm^2 for 6 hours. After the 6 hours, flow was stopped, the chamber was disassembled, and glass test surfaces with adhered cells were immediately retrieved and fixed for immunocytochemistry.

2.9 ECFC adhesion and inhibition

Solutions containing 3 mg/mL of CGKGGRGDS or CGKGGRDGS in EBM-2 medium were prepared for ECFC adhesion studies, respectively termed "RGD solution" (used to inhibit adhesion) and "RDG solution" (used as control). ECFCs were re-suspended at 200,000 cells/mL and seeded at 50,000 cells/cm^2 onto RGD-TAMRA or RDG-modified surfaces, as well as collagen-coated and unmodified surfaces. After 3 hours of adhesion in an incubator at 37°C and 5% CO_2, media and unadhered cells were aspirated, surfaces with cells were washed with PBS, and cells were fixed for immunohistochemistry.

2.10 Colony formation frequency and clonal expansion of ECFCs

Amine PureCoat™ 96-well culture plates were processed by modifying half of the plate while leaving the other half unmodified. The modified half was either treated to covalently conjugate RGD-TAMRA or coated with rat tail collagen. The 96 well culture plates were then sterilized in 100% ethanol for 15 minutes before rinsing with sterile PBS twice. Next, 175 µL of complete EGM-2 medium were added to each well and the plates were placed inside an incubator at 5% CO_2 and 37°C and only removed from incubation immediately prior to cell sorting. ECFCs were resuspended in PBS with 3% FBS and 0.09% sodium azide at 1x10^6 cells/mL. Single cells were seeded into each of the 96 wells using a FACSAria III (BD Biosciences) operated with the FACSDiva 6 software by gating on the live cells through their expected forward and side scatter. The plates were maintained for 24 hours inside an incubator (37ºC, 5% CO_2) before the first 175 µL/well medium exchange, followed by changing the medium every other day. After 10 days of culture, colonies formed from the single ECFCs were fixed using a 4% paraformaldehyde solution, and cell nuclei were stained as described in ***Error! Reference source not found.***.

2.11 Immunocytochemistry

Cells were first washed with PBS and incubated with 4% paraformaldehyde solution (VWR International) for 10 minutes. Fixed cells were washed with PBS twice and stored in PBS at 4 ˚C for further steps. Fixed cells were permeabilized using a 0.1% Triton™ X-100 (Sigma-Aldrich®) solution incubated for 15 mins at room temperature. This was followed by 15 mins of incubation with Dako® Protein Block (Agilent Technologies Inc., Santa Clara, CA) to limit non-specific adhesion. FITC phalloidin (Sigma-Aldrich®) or

TRITC Phalloidin (Life Technologies, Carlsbad, CA) was diluted 1:200 in Dako® Anti-body Diluent (Agilent Technologies Inc.), added to cells and incubated for 2 hours at room temperature in the dark. Adhered cells were washed with PBS, and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich®) was then used at 1 ug/mL in filtered distilled water to stain the nuclei by incubating for 10 mins. Samples were washed with filtered distilled water and PBS before imaging.

2.12 Imaging and image analysis

For cell adhesion or cell alignment studies, random fluorescence images were acquired using an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a pre-programmable Prior® stage (Prior Scientific Inc., Rockland, MA). The number of images acquired was of 9 images (5.56 mm^2 of surface) per sample surface for cell adhesion studies and flow chamber experiments chosen blindly using a programmable stage. For the quantification of alignment, the "Directionality" tool on image J was used to generate a histogram of alignment of Actin filaments for the images in each condition. For cell adhesion studies, TRITC images displaying actin filaments were used to calculate the surface area per cell by adjusting the threshold of the signal, outlining the perimeter of the cells, and calculating the area occupied by atleast 500 cells per sample using the "ROI manager" tool. For colony-forming assays, entire wells were imaged by pre-programming the Prior® stage and multiple images of a single colony were combined using the "stitching" plugin in imageJ. The number of cell nuclei in each DAPI image was quantified using the threshold, mask, "watershed", and "analyze particles" tools.

2.13 Statistical methods

Unless otherwise stated, data represent the average ± the standard deviation of 3 independent experiments. Differences between sample groups were considered to be statistically significant at $p < 0.05$. For experiments with cells, each experiment was conducted with ECFCs derived from at least 3 different donors. Student's t-test was used for two-way comparisons between two samples groups unless otherwise stated. Error bars represent 95% confidence intervals. In ECFC adhesion and inhibition experiments, multiple condition means were compared using two-way ANOVA followed by the Tukey-Kramer test. Significant donor to donor variability resulting in non-normal distribution was observed when quantifying colony

formation frequency from blood, as well as when quantifying colony size after single cell sorting. For these experiments, non-parametric paired (within donor) comparisons were conducted using the non-parametric Wilcoxon Rank test. The above statistical analyses were done using JMP® (SAS Institute Inc., Cary, NC).

3. RESULTS

3.1 Durable covalent surface conjugation of RGD-TAMRA

The stability of peptide-modified surfaces is critical in interpreting *in vitro* culture results as well as for potential applications of peptide-modified surfaces for ECFC expansion. As shown in Figure 1B, the aminated culture surfaces were successfully modified with RGD-TAMRA (Figure 1A) spots both with the sulfo-SMPB linking arm via covalent conjugation, and without the linking arm, via adsorption. Fluorescence imaging of samples immediately after conjugation or adsorption with the same initial concentration of RGD-TAMRA revealed significantly higher level of fluorescence when the peptides were covalently grafted rather than adsorbed. When stored under dry conditions, which should theoretically not lead to any peptide losses, samples with conjugated RGD-TAMRA retained 52% ± 2.64% of their initial fluorescence intensity. Compared to these control samples maintained in dry conditions, a significant decrease in signal was observed after briefly submerging the surfaces with conjugated or adsorbed RGD-TAMRA in PBS, followed by drying and imaging. Over the following 28 days of incubation in PBS, a significant loss in fluorescence signal was observed (Figure 1C). However, samples with conjugated RGD-TAMRA stored in PBS retained 44.7% ± 6.2% of their initial fluorescence (at day 0) after 28 days, contrary to surfaces with adsorbed peptides which did not retain detectable signal after 7 days. Based on the stability of detectable fluorescence signal for at least 28 days in PBS, the stability of the RGD-TAMRA surface conjugation was considered sufficient for subsequent cell culture studies.

3.2 RGD-TAMRA modified surfaces promote colony outgrowth from PBMCs

To determine whether ECFC colonies could be generated on the peptide-modified surfaces, human PBMCS were seeded on aminated polystyrene surfaces with covalently grafted RGD-TAMRA, coated with collagen (positive control), or left untreated (negative control; referred to as “unmodified surfaces”). The number of colonies formed on RGD-TAMRA surfaces or on collagen surfaces was significantly higher than the unmodified controls (Figure 2A). The average colony formation frequency on RGD-TAMRA and collagen

was not significantly different (P=0.284). Donor to donor variability was observed in the number of colonies formed per 100 million PBMCs, the time taken for a colony to form, and the speed at which the colony expanded to reach more than 1 mm in diameter (i.e. readily identifiable colonies). The colonies formed on both RGD-TAMRA and collagen surfaces exhibited cobblestone morphology growing radially outwards (Figure 2B). Colonies were less frequent on the unmodified surfaces: only one colony was observed (out of cultures from 9 donors). The cells in this colony had a different morphology consisting of elongated stromal-like cell, contrary to the cobblestone morphology of colonies obtained on collagen or RGD-TAMRA.

3.3 Outgrowth colonies from RGD-TAMRA show endothelial characteristics

To complement morphological assessment, the ECFC identity of the cells obtained on the RGD-TAMRA surfaces was determined via further phenotypical and functional characterization. Flow cytometry was used to study the surface marker expression of the ECFCs from RGD-TAMRA-modified and collagen-coated surfaces. Endothelial markers, hematopoietic markers, and a progenitor marker were analyzed. Results reflect similar presence of endothelial surface markers between the ECFC populations obtained on RGD-TAMRA and collagen surfaces, as well as similar absence of hematopoietic markers as shown in Figure 3A, with no statistically significant difference identified. When compared to HAAECs, both ECFCs from collagen and RGD-TAMRA surfaces had significantly higher expression of CD34, and CD309 (vascular endothelial growth factor receptor-2).

ECFCs from both surfaces were also seeded on Matrigel® to analyze their capacity to form endothelial networks, a characteristic of ECFCs but not myeloid angiogenic cells(Hirschi et al., 2008; Madri & Pratt, 1986). Four hours after seeding, clear network-like structures were formed on both surfaces (Figure 3B). Similar behavior was observed by human abdominal aortic endothelial cells (HAAECs) which were used as a positive control while no visible networks were observed with the CD14+ fraction of PBMCs, primarily composed of monocytes. Furthermore, endothelial cells are expected to align in the direction of laminar fluid flow (Steward, Tambe, Hardin, Krishnan, & Fredberg, 2015). To further analyze the functionality of the ECFC populations obtained on both surfaces, passage 5 ECFCs were exposed to 22 dyn/cm2 of shear stress for 6 hours in a parallel plate flow chamber (Corinne A. Hoesli et al., 2018). As shown in Figure 4, ECFCs

from both surfaces exhibited evidence of cellular alignment in the direction of the flow compared to the static control.

3.4 RGD-based signaling controls ECFC surface adhesion

To analyze whether the RGD-TAMRA peptide can promote the adhesion of ECFCs expanded from PBMCs, ECFCs were seeded on RGD-TAMRA-modified surfaces, collagen-coated surfaces, or unmodified surfaces. This was done in the presence and absence of competing RGD peptides (or RDG peptides as control) in solution to assess the role of integrin-based signaling on the process. After 3 hours of adhesion without competing peptide in solution, the number of attached cells and the average surface area per cell was significantly higher on the RGD-TAMRA surfaces compared to RDG-modified surfaces and unmodified controls (Figure 5). In the presence of competing RGD peptides in solution, this effect diminished with no statistical difference observed between the 4 surface conditions. This inhibitory effect was not observed in the presence of the control RDG peptide in solution. Significantly higher average surface area per cell was also observed on the collagen-coated surface compared to the unmodified control in the absence of inhibition, and no statistical difference was observed upon comparison with the RGD-TAMRA surfaces.

3.5 Enhanced clonal expansion of ECFCs on RGD-TAMRA-modified surfaces

An effective biofunctionalization strategy for EPCs must also aim to maximize cell proliferation while considering their low initial frequency. For the proposed peptide surface modifications, the proliferation potential of pre-expanded ECFCs on the modified culture surfaces was studied by quantifying the colony formation frequency and expansion potential of ECFCs seeded as single cells onto RGD-TAMRA surfaces, collagen-coated surfaces or unmodified surfaces (Figure 6A). After 10 days of culture, no statistically significant differences in the colony formation frequency was observed between the surfaces. However, significantly higher clonal ECFC expansion was observed on the RGD-TAMRA surfaces compared to the collagen-coated or unmodified surfaces (Figure 6B & C).

4. DISCUSSION

In this work, we have utilized the RGD-TAMRA peptide to modify tissue culture plate surfaces using a durable covalent conjugation strategy that is applicable to other ECM-derived peptides with an available

thiol group. The RGD-TAMRA-modified surfaces promoted the formation of ECFC colonies from PBMCs that exhibited typical endothelial surface markers and hallmarks. The addition of competing RGD peptide to the medium led to reduced ECFC adhesion and spreading, suggesting that ECFC adhesion both on RGD-TAMRA and collagen surfaces is at least partly mediated by integrins. Interestingly, single ECFCs were also shown to form significantly larger colonies when seeded on RGD-TAMRA-modified surfaces compared to both collagen-coated and unmodified surfaces. Therefore, this works presents, for the first time, an ECM-derived peptide-based functionalization strategy that can successfully be utilized to promote EPC adhesion and ECFC expansion from human peripheral blood.

The injection of *ex vivo* expanded ECFCs from peripheral blood remains the main therapeutic strategy for a significant number of ongoing EPC-based clinical trials targeting myocardial infarction, coronary artery disease, and critical limb ischemia (Chong et al., 2016). Available *ex vivo* culture methods rely heavily on animal derived collagen coatings despite the safety risk of transmissible pathogens associated with xeno-derived reagents and the resulting regulatory challenges. While xeno-free *ex vivo* culture surfaces prepared by using of human-derived collagen or fibronectin have shown similar efficacy, scale-up remains challenging (Bauman et al., 2018). ECM-derived cell adhesion peptides such as RGD provide a chemically defined substitute to ECM proteins that is easier to study, engineer, and produce (Collier & Segura, 2011). Future directions towards 100% xeno-free techniques would potentially use the combination of peptide-based surfaces, like the RGD-TAMRA-modified surfaces, and serum-free ECFC culture media such as ECFC-specific chemically defined substitutes (Masuda et al., 2012).

During *ex vivo* EPC culture, readily identifiable ECFC colonies typically appear 14-21 days after seeding PBMCs on the surface and are then left to grow for up to a week before being transferred to a larger surface area. Therefore, the durability of an applied bio-functionalization is most important during the first 2-4 weeks. While surfaces with covalently conjugated RGD-TAMRA peptides retained fluorescence signal for at least 4 weeks, no detectable signal was observed after one week of aqueous buffer incubation for adsorbed peptides. The dry condition is not expected to have any peptide losses due to desorption. The observed significant decrease in fluorescence intensity of the dry conjugated sample over the span of the first 14 days might be linked to changes in surface water content, to time-dependent photo-bleaching, or a combination of

both. Although the fluorescence signal detection does not differentiate between adsorbed and conjugated peptides nor peptide losses versus photobleaching, these observations point towards a significantly higher stability of surfaces with covalent peptide conjugation.

Since the first isolation of EPCs from adult human peripheral blood, the *ex vivo* expansion of adult human EPCs has been done solely by seeding PBMCs or sorted PBMC subsets on protein-coated surfaces, mostly collagen or fibronectin. Replacing these ECM proteins by a defined 1400 Da peptide with reduced lot-to-lot variability and reduced susceptibility to proteolytic cleavage presents distinctive advantages. In our study, PBMCs from adult human donors were seeded on RGD-TAMRA-modified surfaces and the resulting number of colonies was not statistically different from the number of colonies obtained on collagen-coated surfaces. The frequency of ECFC colonies obtained on RGD-TAMRA was consistent with the number of colonies previously obtained from human peripheral blood on collagen (Ingram et al., 2004). The lack of effective colony formation on the unmodified surface can possibly be explained by the absence of signaling proteins or peptide ligands on the surface to mediate either EPC attachment or ECFC colony outgrowth. The presence of the peptide or the collagen could also potentially mediate this colony formation via interactions with serum proteins in the media or proteins secreted by the ECFCs.

The choice of the protein coating affects the selection of the type of adhered colony forming cells obtained from PBMCs. Fibronectin coating results in myeloid angiogenic cells appearing 5-9 days after seeding, while collagen coating results in ECFCs appearing 14-21 days after seeding. The presence of the two distinct subtypes grouped under the same EPC label, as illustrated by Yoder *et al.*(Hirschi et al., 2008; Yoder et al., 2007) and others (Cheng et al., 2013; Fadini, Losordo, & Dimmeler, 2012) highlights the need for further characterization of resulting ECFC colonies. ECFCs derived on RGD-TAMRA expressed mature endothelial markers, variable levels of CD34 and did not express hematopoietic markers including CD45 and CD14. This expression profile concords with ECFCs rather than myeloid angiogenic cells (Fadini et al., 2012; Yoder et al., 2007) or other hematopoietic populations. The significant difference in EPC-linked CD34 and CD309 expression between the two ECFC populations and HAAECs is points to the existence of a higher level of progenitors in the ECFC populations (Ferreras, Cole, Urban, Jayson, & Avizienyte, 2015). The ability of the obtained ECFC populations to form network-like structure in Matrigel® along with their

alignment in the direction of laminar flow provides more functional evidence of their endothelial behaviour in physiologically relevant conditions (Cheng et al., 2013; Yoder et al., 2007). To further demonstrate the functional potential of the ECFCs produced on RGD-TAMRA, future studies could investigate the capability of these cells to directly contribute to neo-vasculature *in vivo* (Melero-Martin et al., 2007).

The nature of cell adhesion to RGD in general is highly dependent on the recognition of the RGD motif by integrin receptors such as $\alpha_v\beta_3$ (Eble & Kühn, 1997). The significant increase in both cell spreading and number of cells on RGD-TAMRA-modified surfaces compared to RDG-modified surfaces points to a sequence specific recognition of the RGD sequence by cell surface receptors, most likely integrins. The decreased adhesion to both RGD-TAMRA-modified surfaces as well as collagen-coated surfaces in the presence of RGD in the medium points to a general dependence of ECFC adhesion on an RGD-dependent pathway, most likely integrins.

In addition to cell adhesion, endothelial cell growth – and hence likely ECFC expansion – is believed to be an anchorage-dependant process where integrin signaling stimulates cell proliferation via promoting cell cycle progression (Danen & Yamada, 2001). After 10 days of clonal expansion of the expanded ECFCs, significant differences between the size of colonies on RGD-TAMRA-modified surfaces and the unmodified surfaces were observed, highlighting the effect of integrin signaling on their proliferation capacity (Figure 6). The significant difference in the size of the colonies observed on the RGD-TAMRA surface compared to those observed on the collagen surfaces can be attributed to the difference in the concentration of the available active biological sites on the surface whether for direct cell interaction or interaction with serum proteins in cell medium. Inducing integrin signalling is likely critical in maximizing ECFC expansion for therapeutic delivery.

The techniques developed in this work to study the ability of the RGD-TAMRA peptide to promote the expansion of peripheral blood-derived EPCs into ECFCs with phenotypical and functional endothelial hallmarks provides a platform for studying an array of ECM-derived peptides. While surface-bound RGD-TAMRA appeared to enhance colony formation, ECFC adhesion and proliferation, the RGD peptide sequence can bind a range of integrin receptors. This lack of specificity can potentially enhance the adhesion and survival of other undesirable hematopoietic and progenitor populations in the blood stream (Wang et al.,

2013; Wendel, Avci-Adali, & Ziemer, 2010). However, using the platform presented in this work, other ECM-derived peptides that have been identified for more specific endothelial reaction like YIGSR and REDV can be tested for their potential on enhancing peripheral blood-derived ECFC expansion (Noel et al., 2015). Other studies have also used *in vitro* ECFC adhesion and phage display technologies to identify and synthesize longer chain peptide sequences than are specifically tailored for ECFC affinity (Anka N. Veleva, Cooper, & Patterson, 2007; A. N. Veleva, Heath, Cooper, & Patterson, 2008). The potential of such bio-functionalization strategies can be further explored using the techniques presented.

5. CONCLUSION

ECM-derived peptide-based biofunctionalization of culturesurfaces could enhance the efficiency of EPC adhesion, colony formation, and proliferation. Covalent conjugation of the peptides could ensure a higher surface concentration that is available for the duration of the application compared to adsorption-based techniques. RGD-TAMRA-modified surfaces promoted endothelial colony formation from human PBMCs. ECFCs from the resulting colonies displayed uniform endothelial surface marker expression, showed network formation ability in Matrigel®, and aligned in the direction of laminar flow. The presence of surface bound RGD-TAMRA promoted higher ECFC adhesion through an RGD-dependent integrin pathway. ECFC expansion from single cells into multicellular colonies was also significantly enhanced upon seeding on RGD-TAMRA-modified surfaces. The results and techniques presented in this work therefore highlight the potential of peptide-based surface biofunctionalization strategies to enhance the *ex vivo* expansion of peripheral blood-derived EPCs.

Conflicts of interest

The authors declare no competing financial interest.

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Supporting Information

Brightfield images of resulting colonies from PBMC ex vivo culture on different surface modifications (Figure S.1)

Average number of junctions and average length of branch of networks formed upon seeding of collagen ECFCs, RGD-TAMRA ECFCs, and HAAECs on Matrigel® (Figure S.2)

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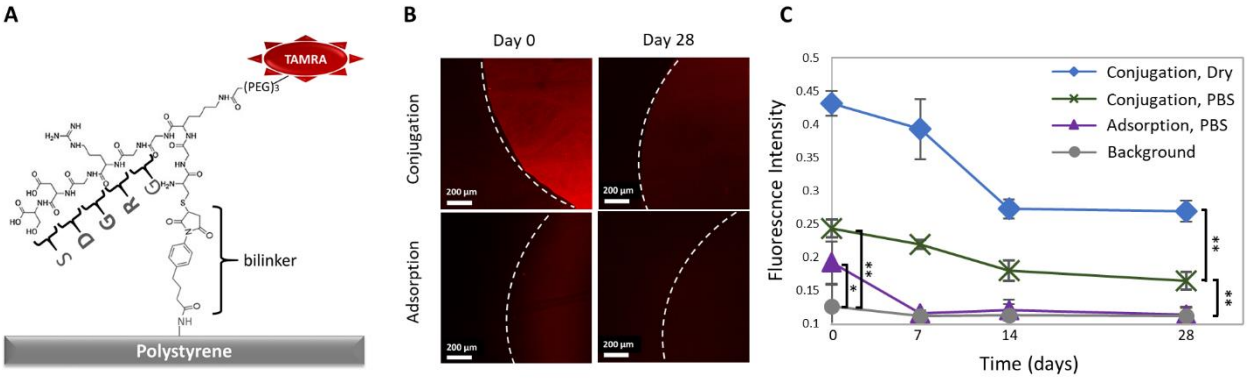

Figure 1. Surface functionalization and durability of RGD-TAMRA surface modification via adsorption vs covalent conjugation. (A) Structure of RGD-TAMRA-modified polystyrene surfaces using sulfo-SMPB as a bilinker for covalent conjugation. (B) Fluorescence imaging of spots of RGD-TAMRA (shown in red) coatings that were prepared via either covalent conjugation (with sulfo-SMPB) or adsorption (without sulfo-SMPB and imaged immediately after modification or 28 days after. (C) Quantification of the average fluorescence intensity of RGD-TAMRA spots after up to 28 days. *P < 0.05; **P < 0.01 with N=3.

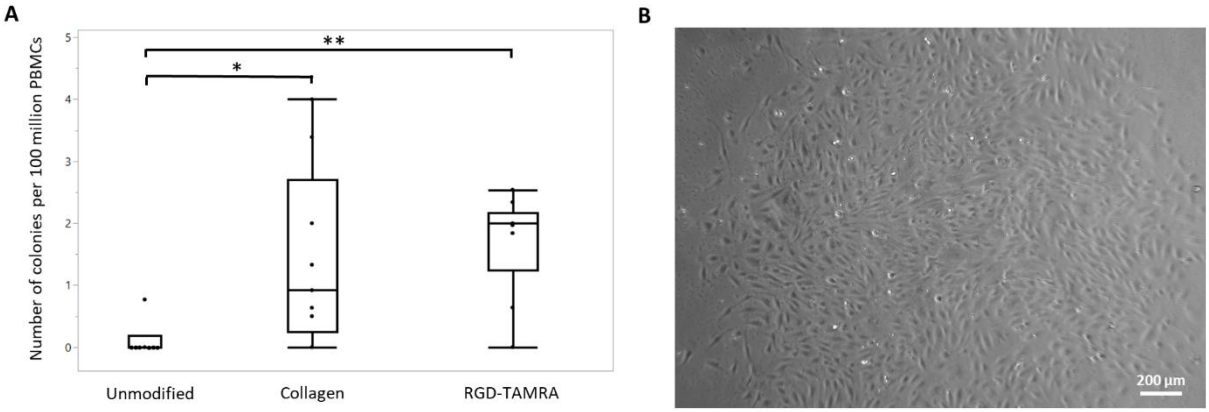

Figure 2. Formation of colonies with cobblestone morphology from human PBMCs after 14-21 days of *ex vivo* culture. (A) Number of colonies formed on RGD-TAMRA, collagen, and unmodified surfaces per 100 million seeded PBMCs. The box plots represent quartiles. *P < 0.05; **P < 0.01 with N=9-12. (B) Resulting colony with a typical cobblestone morphology and radial outgrowth formed on an RGD-TAMRA modified surface 20 days after seeding.

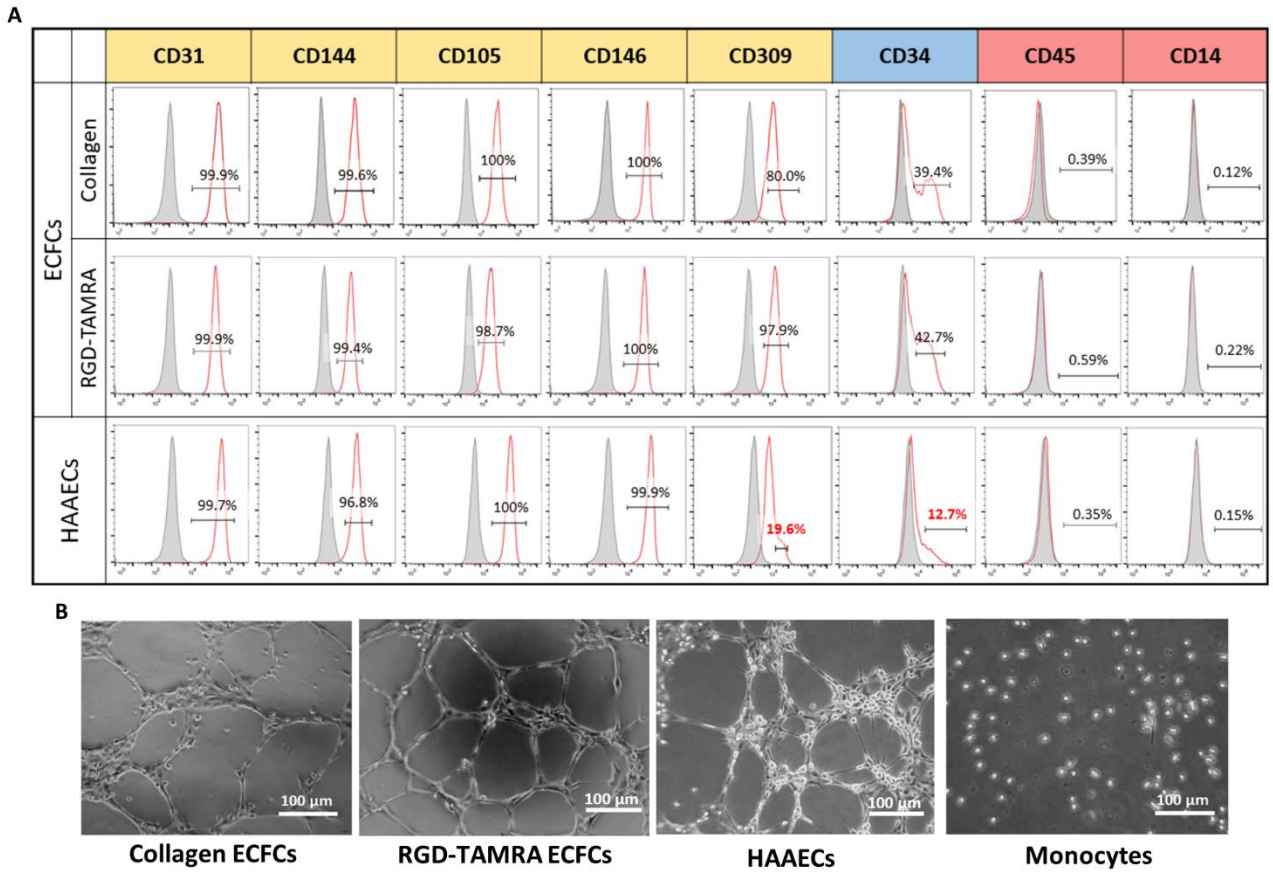

Figure 3. Phenotypical and functional characterization of ECFCs isolated and expanded on RGD-TAMRA and collagen surfaces. (A) Surface marker expression of ECFCs from RGD-TAMRA and collagen surfaces compared to mature HAAECs grown on gelatin. Grey histograms represent isotype controls. Red percentages represent statistically significant differences between the fraction of positive cells obtained for HAAECs compared to ECFCs ($p < 0.05$). (B) Network formation on Matrigel by ECFCs from RGD-TAMRA and collagen surfaces compared to HAAECs (positive control), and CD14+ PBMCs (monocytes, negative control).

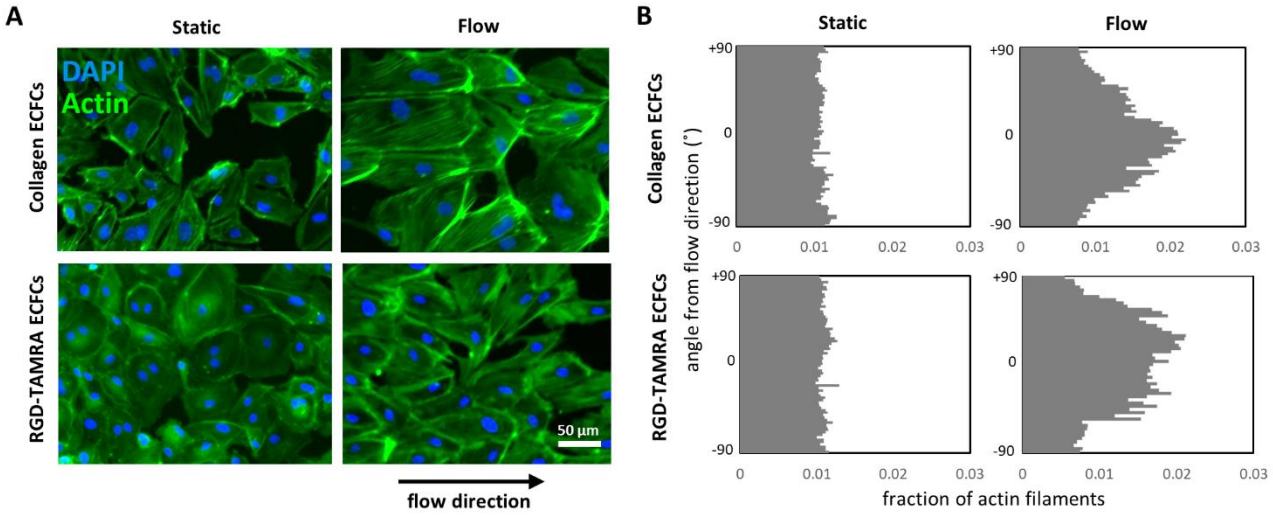

Figure 4. ECFC alignment in the direction of laminar shear stress. (A) Fluorescence imaging of ECFCs after 6 hours of exposure to 22 dyn/cm2 laminar flow (horizontal flow to the right) compared to static controls. ECFCs were stained for phalloidin (F-actin, green) and DAPI (nuclei, blue). (B) Histograms of the angle of actin filament alignment relative to the direction of the flow (0°).

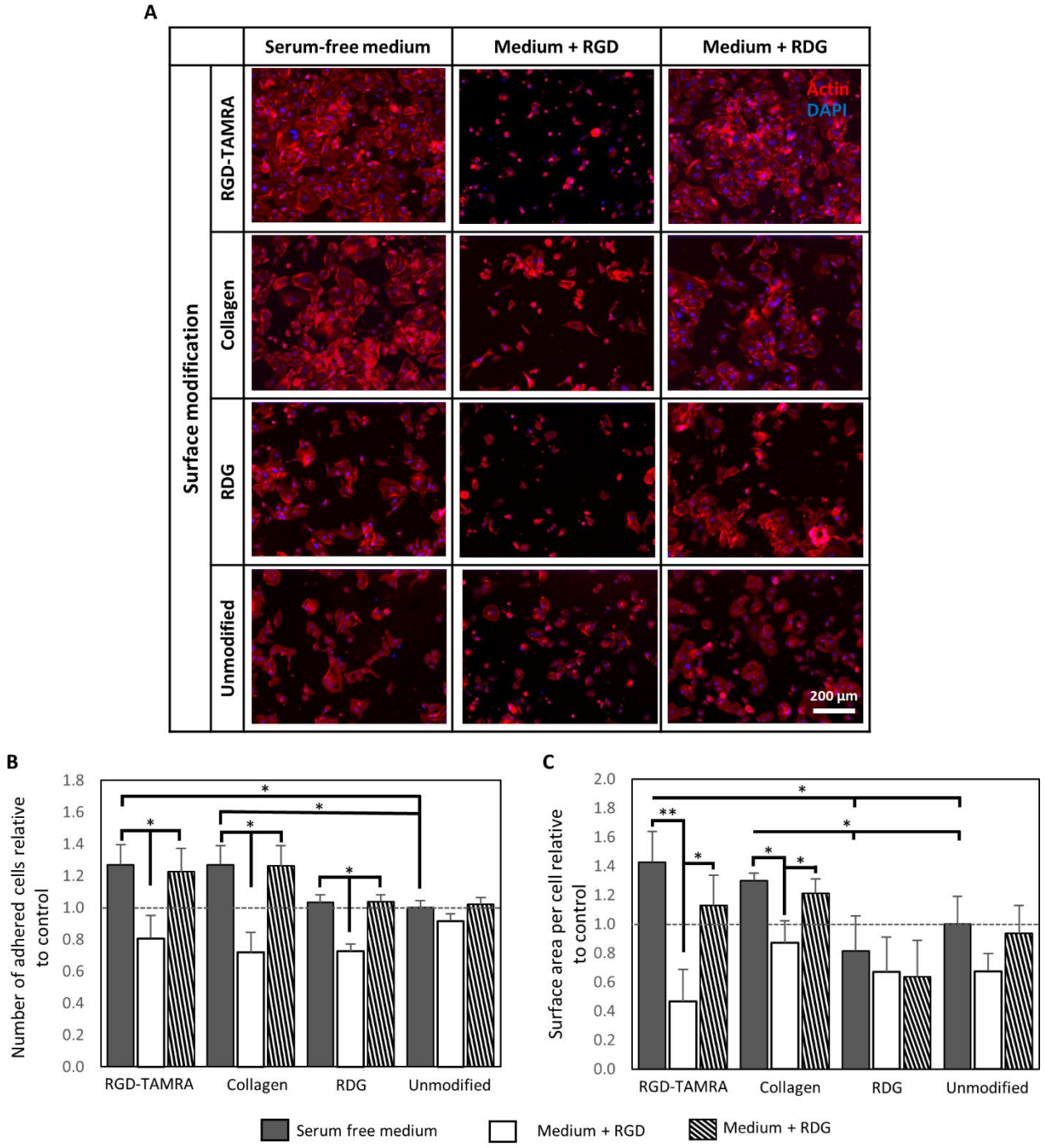

Figure 5. ECFC adhesion after 3 hours of seeding on RGD TAMRA, collagen, RDG, and unmodified surfaces in serum-free medium with or without RGD (inhibitor) or RDG (control) peptides. (A) ECFCs were stained for filamentous actin (red) and nuclei (blue). (B) Number of adhered cells per mm^2 relative to unmodified surfaces with serum free medium without peptides added in solution. (C) Average surface area per cell (measured in μm^2) relative to unmodified surfaces with serum-free medium without peptides added in solution. *P < 0.05; **P < 0.01 with N=3.

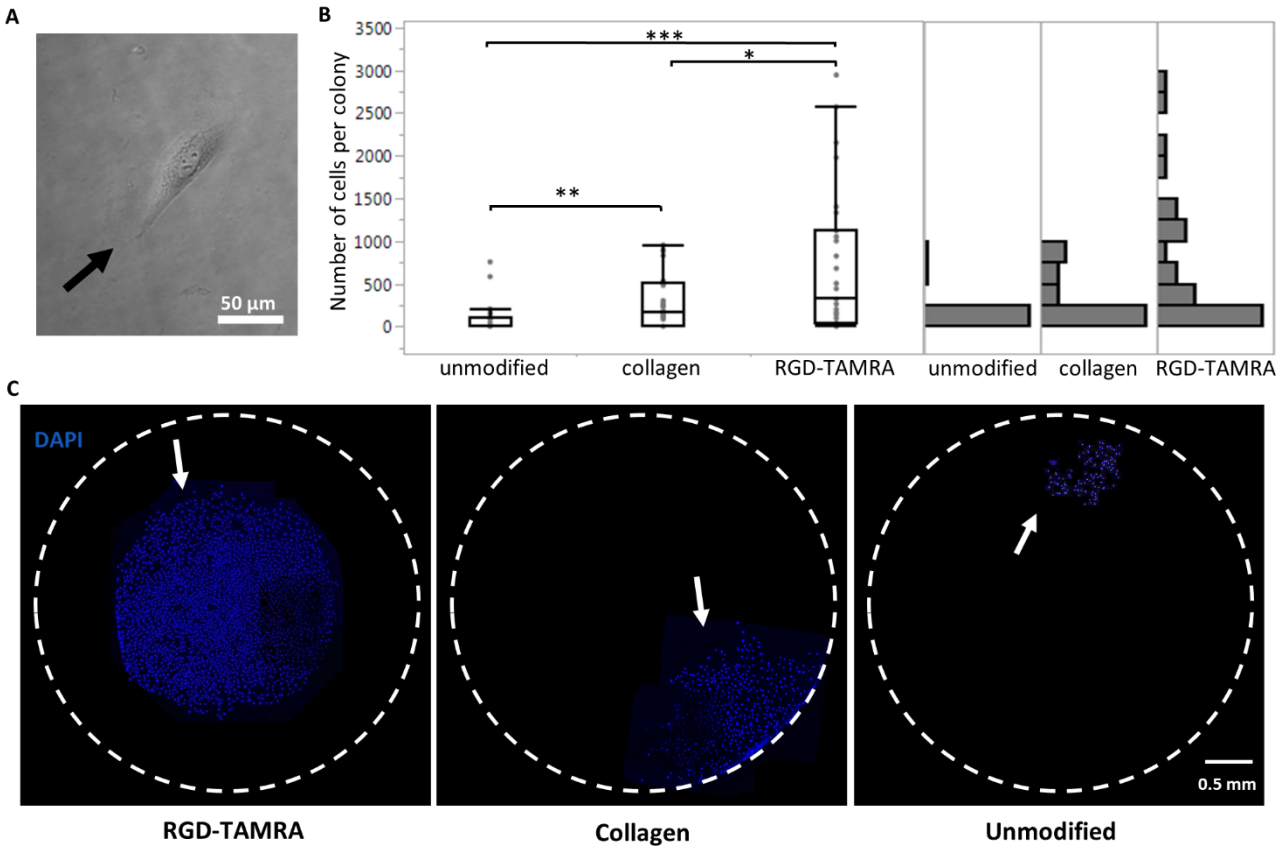

Figure 6. Single cell sorting of ECFCs and formation of colonies with varying sizes on RGD-TAMRA, collagen, and unmodified surfaces. (A) Phase contrast image of a passage 5 ECFC immediately after single cell sorting on an RGD-TAMRA-modified surface. (B) Number of cells per colony formed 10 days after single cell sorting with a box plot and sample distribution for each condition. *P < 0.05; **P < 0.01; ***P < 0.001 with N=3. (C) Fluorescence images of sample colonies from each surface 10 days after single cell sorting in 96 well plates. Nuclei were stained with DAPI (blue).