

The use of vascular endothelial growth factor functionalized agarose to guide pluripotent stem cell aggregates toward blood progenitor cells

Nafees Rahman^{a,b}, Kelly A. Purpura^{a,b}, Ryan G. Wylie^c, Peter W. Zandstra^{a,b,e,f,g,**}, Molly S. Shoichet^{a,b,c,d,e,g,*}

^a Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ontario M5S 3E5, Canada

^b Institute of Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, Room 407, Toronto, Ontario M5S 3G9, Canada

^c Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario M5S 3H6, Canada

^d Institute of Medical Science, University of Toronto, 1 King's College Circle, Toronto, Ontario M5S 1A8, Canada

^e Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, 160 College Street, Room 230, Toronto, Ontario M5S 3E1, Canada

^f Heart and Stroke Richard Lewar Centre of Excellence, University of Toronto, 150 College Street, Toronto, Ontario M5S 3E2, Canada

^g McEwen Centre for Regenerative Medicine, University Health Network, 101 College Street, MaRS Centre, Toronto Medical Discovery Tower, 8th Floor, Room 701, Toronto, Ontario M5G 1L7, Canada

ARTICLE INFO

Article history:

Received 14 May 2010

Accepted 7 July 2010

Available online 3 August 2010

Keywords:

Blood
Cell encapsulation
Hydrogel
Stem cell
Thiol
Flow cytometry

ABSTRACT

The developmental potential of pluripotent stem cells is influenced by their local cellular microenvironment. To better understand the role of vascular endothelial growth factor (VEGFA) in the embryonic cellular microenvironment, we synthesized an artificial stem cell niche wherein VEGFA was immobilized in an agarose hydrogel. Agarose was first modified with coumarin-protected thiols. Upon exposure to ultra-violet excitation, the coumarin groups were cleaved leaving reactive thiols to couple with maleimide-activated VEGFA. Mouse embryonic stem cells (ESC) aggregates were encapsulated in VEGFA immobilized agarose and cultured for 7 days as free-floating aggregates under serum-free conditions. Encapsulated aggregates were assessed for their capacity to give rise to blood progenitor cells. In the presence of bone morphogenetic protein-4 (BMP-4), cells exposed to immobilized VEGFA upregulated mesodermal markers, brachyury and VEGF receptor 2 (T+VEGFR2+) by day 4, and expressed CD34 and CD41 (CD34+CD41+) on day 7. It was found that immobilized VEGFA treatment was more efficient at inducing blood progenitors (including colony forming cells) on a per molecule basis than soluble VEGFA. This work demonstrates the use of functionalized hydrogels to guide encapsulated ESCs toward blood progenitor cells and introduces a tool capable of recapitulating aspects of the embryonic microenvironment.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the embryo and have the ability to differentiate into cells from all three germ layers. By manipulating ESC differentiation toward the mesoderm, genetic and malignant blood disorders may be treated with ESC-derived blood cells [1]. However, current cell culture techniques are limited in their capacity to replicate aspects

of the embryonic microenvironment and lack the ability to scale-up and generate differentiated cells. ESCs are commonly differentiated with soluble factors added to the media; however, niche engineering such as growth factor immobilization is becoming increasingly popular to better mimic the cellular microenvironment. In vivo, cytokines and growth factors are often presented to cells immobilized through their interactions with specific heparin-binding domains [2], extracellular matrix (ECM) molecules such as fibronectin or by directly binding to the cell membrane [3]. To study specific cellular responses, biomimetic matrices have been synthesized with immobilized growth factors that increase local concentration in proximity to cell surfaces. Studies have also shown that growth factors immobilized on biomaterials remain bioactive and effectively target signalling receptors that control stem cell fate [4–7]. We hypothesized that the incorporation of immobilized vascular endothelial growth factor (VEGFA) and soluble bone morphogenetic protein (BMP-4) in an in vitro culture system would

* Corresponding author. Terrence Donnelly Centre for Cellular and Biomolecular Research Donnelly (CCBR), University of Toronto, 160 College Street, Room #514, Toronto, Ontario M5S 3E1, Canada. Tel.: +1 416 978 1460; fax: +1 416 978 4317.

** Corresponding author. Terrence Donnelly Centre for Cellular and Biomolecular Research Donnelly (CCBR), University of Toronto, 160 College Street, Office #1116, Toronto, Ontario M5S 3E1, Canada. Tel.: +1 416 978 8888; fax: +1 416 978 2666.

E-mail addresses: peter.zandstra@utoronto.ca (P.W. Zandstra), molly.shoichet@utoronto.ca (M.S. Shoichet).

enable better control and efficiency of ESC differentiation toward blood progenitor cells.

VEGFA, one of five members of the VEGF family, is a signalling growth factor that regulates angiogenesis by promoting proliferation, migration and sprouting of endothelial cells [8]. VEGFA primarily interacts with two receptor tyrosine kinases known as VEGF receptor-1 and -2 (VEGFR1/VEGFR2). VEGFR1 is mainly found on endothelial cells whereas VEGFR2 (also known as Flk-1 in mouse cells and KDR in human cells) is expressed on endothelial cells, hematopoietic stem cells, megakaryocytes, and osteoblasts [9]. Both VEGFR1 and VEGFR2 are important in normal and pathological vascular endothelial cell biology. We previously demonstrated that temporal control of VEGFA is important to achieve a hematopoietic stem cell fate [10]. In an attempt to drive ESC differentiation using an engineering construct, Ferreira et al. reported that a dextran-based hydrogel with VEGFA-loaded microparticles enhanced vascular differentiation of human ESC-derived cell aggregates termed embryoid bodies (EBs) in comparison to soluble VEGFA [11]. However, the construct used was not amenable for scale-up culture to attain large numbers of differentiated cells.

To create a biomimetic microenvironment, we chose to use agarose as a scaffold for VEGFA immobilization to direct ESC differentiation during the early stages of development toward blood progenitor cells [10]. Agarose is a compelling material because it is transparent, allowing for photochemical modification, and can be mechanically tuned by varying its weight concentration. It is a neutral polysaccharide that gels in water at reduced temperatures, non-adhesive to cells, non-adsorptive to proteins and resistant to swelling [45]; thereby providing a blank canvas on which specific biological molecules can be immobilized. Moreover, the use of agarose for *in vivo* applications has been evaluated by investigators because it does not induce specific xenogeneic responses [12]. Agarose has been used as a carrier to deliver cell secreting factors via encapsulation [13–16]. Ito et al. have shown that hamster islet cells encapsulated in agarose beads exhibited prolonged viability and established normoglycemia in streptozotocin-induced diabetic mice compared to free injected hamster islets [16]. Similarly, Lahooti and Sefton have shown that intraperitoneally injected mouse fibroblasts co-encapsulated with poly(hydroxyethyl methacrylate-co-methyl methacrylate) [HEMA-MMA] and agarose prevented aggregation of cells and expressed alkaline phosphatase 21 days postimplantation. Fibrous tissue accumulation was negligible on the surface compared to cells within HEMA-MMA microcapsules injected without agarose [17]. To promote cell attachment and differentiation, the primary hydroxyls present on the backbone of agarose can be functionalized with adhesion molecules [18] and bioactive ligands [4]. Subsequently, agarose has been used to encapsulate human and mouse ESCs, thereby allowing cell proliferation while preventing agglomeration, in a mechanically stable environment [19].

In this study, we were interested in functionalizing agarose with moieties that facilitate VEGFA immobilization and testing these matrices for their capacity to influence ESC differentiation. The initial immobilization chemistry was optimized with α -chymotrypsinogen, a model protein for VEGFA. Cells were encapsulated within VEGFA immobilized agarose in serum-free conditions and blood progenitor cell output was assessed relative to soluble VEGFA controls in the presence of BMP-4.

2. Materials and methods

All chemicals were obtained from Sigma Aldrich (St. Louis, MO, www.sigmaaldrich.com) and used as received unless otherwise stated.

2.1. Synthesis and characterization of aminated, carboxylated and coumarin-sulphide functionalized agarose

400 mg of ultra-low gelling temperature agarose (SeaPrep Agarose, Lonza, Switzerland, www.lonza.com) was dissolved in 25 ml of anhydrous dimethyl sulphoxide (DMSO) at 80 °C, and then cooled to room temperature. 70 mg of 1,1-carbonyldiimidazole (CDI) dissolved in 2 ml of DMSO was added to the agarose solution and then stirred for 2 h. Next, small molecules containing the functional groups of interest were added to CDI-activated agarose: 2 ml of ethylenediamine for aminated agarose; 1 ml of 2-mercaptoacetic acid for carboxylated agarose; or 60 mg of coumarin-sulphide [42] in 1 ml of DMSO for coumarin-sulphide agarose (C-SH). Agarose mixtures were protected from light and stirred overnight. Thereafter, 30 ml of warm water (to maintain agarose solubility) was added to each mixture prior to dialyzing (MW cut off 3500, Spectrum laboratories, CA, www.spectrapor.com) against distilled water for 3 days with daily water changes. After dialysis, during which DMSO was extracted, agarose mixtures were lyophilized and stored at –20 °C.

The degree of substitution (DS) of the modified agarose formulations was then tested. For aminated agarose, a trinitrobenzene sulphonic acid assay (TNBSA, Thermo Scientific, Rockford, IL, www.piercenet.com) was used as outlined by the manufacturer. Fourier transform infrared spectroscopy (FTIR) was used to detect carboxylic acids on carboxylated agarose. Briefly, lyophilized samples were placed securely under a Harrick SplitPea on a Nicolet Avatar E.S.P spectrometer (Thermo Electron Co., Waltham) and spectra were acquired using OMNIC software (Thermo Electron Co., Waltham). FTIR spectra values (SplitPea, in cm^{-1}): 1050 ($\lambda_{\text{primary alcohol C-O}}$); 1150 ($\lambda_{\text{secondary alcohol C-O}}$); 1730–1700 ($\lambda_{\text{carboxylic acid C=O}}$). Ultra-violet (UV) absorbance was used to determine the DS of coumarin on agarose based on published values: λ_{max} bromohydroxy coumarin-sulphide = 373 nm; $\epsilon = 14\,500\ \text{M}^{-1}\ \text{cm}^{-1}$ [42].

2.2. Fluorescent tagging and activation of α -chymotrypsinogen

170 μl of α -chymotrypsinogen (1.5 mg/ml PBS, pH = 7.4) was added to 30 μl 5,6 carboxyfluorescein, succinimidyl ester (FAMSE; 10 mg in 1 ml DMSO; Biotium Inc., Hayward, CA, www.biotium.com). The mixture was added to 250 μl solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; 2.4 mg/ml in PBS, pH = 7.4)/N-hydroxysulphosuccinimide (Sulpho-NHS; 2 mg/ml, Thermo Scientific) or sulphosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulpho-SMCC, 1.5 mg/ml in PBS, pH = 7.4, Thermo Scientific) and allowed to react for 15 min. EDC/Sulpho-NHS and Sulpho-SMCC crosslinkers were used to conjugate proteins onto aminated agarose and coumarin-sulphide agarose respectively. Unreacted fluorescein and crosslinker molecules were removed by purifying the mixture using a Sephadex G-25 packed column and fast protein liquid chromatography (AKTA-FPLC, Amersham Pharmacia). Activated α -chymotrypsinogen was eluted in sterile PBS, pH = 7.4 and monitored at 280 nm using a UV–vis monitor (UPC-900 Pharmacia Biotech) equipped on the FPLC machine. Protein concentration was determined by using a Micro-BCA assay kit (Pierce Biotechnology) as outlined by the manufacturer.

2.3. Conjugation of activated α -chymotrypsinogen onto functionalized agarose

For each type of functionalized agarose, 1 $\mu\text{g}/\text{ml}$ of activated α -chymotrypsinogen was added to agarose gels to obtain a final concentration of 2 w/v% (PBS, pH = 7.4). For carboxylated agarose, 250 μl EDC/Sulpho-NHS (2.4/2 mg/ml) was added for 15 min to activate the hydrogel before introducing the activated protein. Activated protein in aminated and carboxylated agarose mixtures was vortexed and allowed to react for 90 min on a shaker at 37 °C. Activated protein in C-SH agarose mixtures was vortexed, UV irradiated for 5 min and incubated for 30 min at room temperature in the dark. To remove unconjugated protein, 100 μl agarose mixture aliquots were placed into a 96 well plate and gelled for 1 h at 4 °C; gels were then washed for 7 d in PBS buffer pH = 7.4 with daily buffer changes at 4 °C. After washing, the plate was placed in an oven at 50 °C for 5 min after which the re-melted gels were recovered from the wells and subsequently stored at –80 °C. Immobilized protein gels were quantified relative to a standard curve comprised of known concentrations of fluorescently tagged protein mixed with functionalized agarose. An ‘adsorbed’ control was generated with unactivated protein to assess the immobilization chemistry.

2.4. Conjugation of VEGFA onto C-SH

Sulpho-SMCC chemistry was used to immobilize VEGFA to C-SH agarose. Briefly, 0.4 mg/ml VEGFA (100 μg in 250 ml PBS pH = 7.4, Shenandoah Biotechnology, Warwick, www.shenandoah-bt.com) was added to 10 μl of fluorescent dye (Alexa-fluor 532, Invitrogen, Eugene, OR, www.invitrogen.com) and 1.5 mg/ml Sulpho-SMCC. The mixture was allowed to react for 15 min at 4 °C before it was purified using an AKTA-FPLC as previously described. 8 $\mu\text{g}/\text{ml}$ of maleimide-activated VEGFA was added to a final concentration of 2 w/v% C-SH agarose (PBS, pH = 7.4) wherein the mixture was vortexed and UV irradiated for 5 min to react with the thiols present on the agarose. The mixture was protected from light and incubated for 30 min at room temperature before unconjugated protein was washed as previously described and stored at –80 °C.

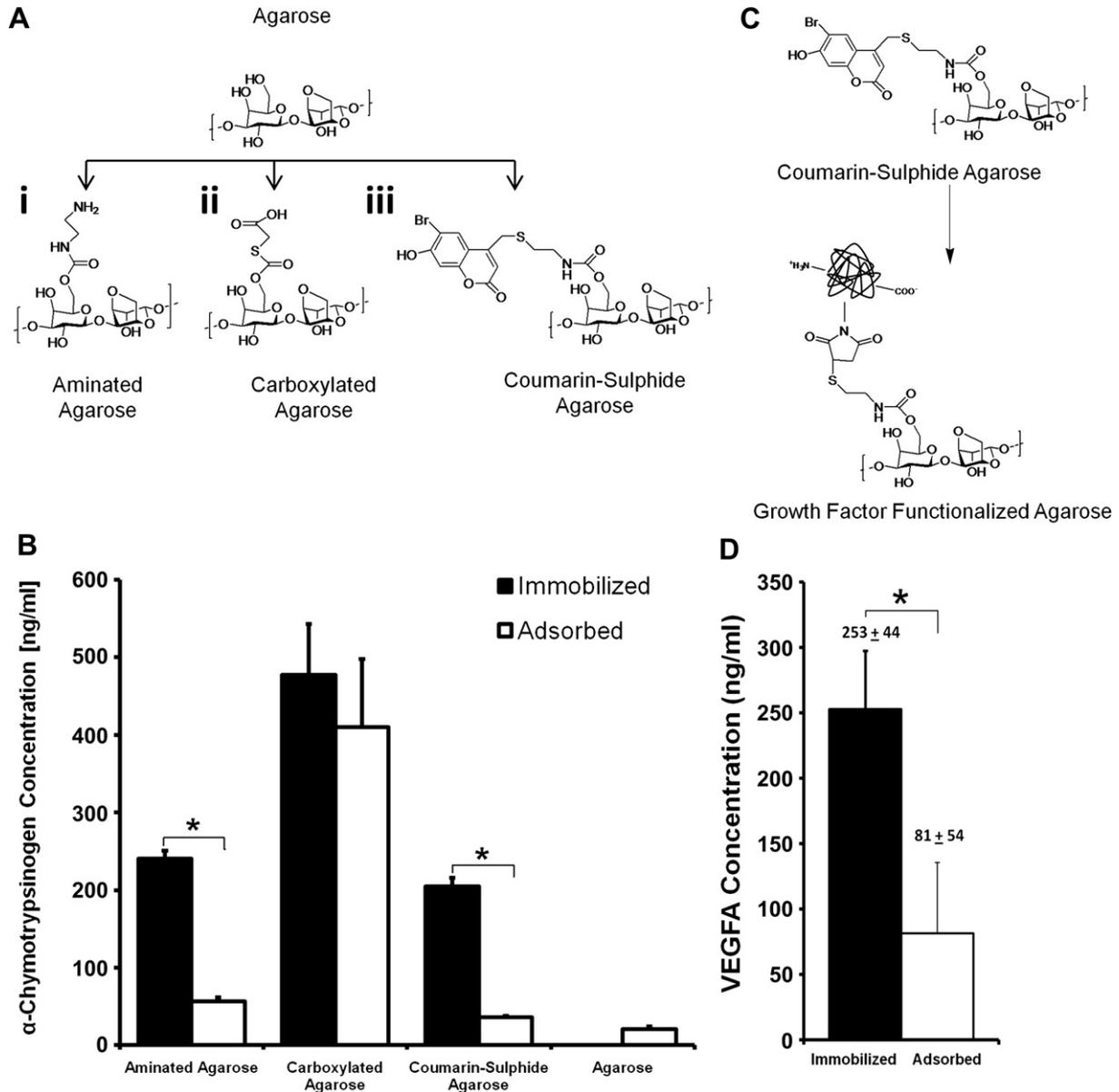


Fig. 1. Synthesis and immobilization of α -chymotrypsinogen on functionalized agarose and the reaction scheme for VEGFA immobilization on coumarin-sulphide agarose. (A) Agarose was modified using carbonyldiimidazole chemistry to synthesize (i) aminated agarose, (ii) carboxylated agarose and (iii) coumarin-sulphide agarose. A model protein, α -chymotrypsinogen, was used to test immobilization chemistries on synthesized hydrogels. (B) Gels of aminated agarose, carboxylated agarose, coumarin-sulphide agarose and native agarose were each mixed with activated fluorescently tagged α -chymotrypsinogen. Samples were gelled at 4 °C in a 96 well plate for 1 h and subsequently washed for 7 days in PBS buffer (pH = 7.4). Immobilized and adsorbed gels were collected by heating samples in an oven at 50 °C and protein concentration was quantified using fluorescence spectroscopy. (C) Sulpho-SMCC chemistry was used to conjugate VEGFA onto coumarin-sulphide agarose. Maleimide groups on VEGFA were activated using Sulfo-SMCC in PBS (pH = 7.4) and purified using FPLC. Activated VEGFA was then mixed with coumarin-sulphide agarose to form a 2 wt% gel in PBS and UV irradiated for 5 min to deprotect the thiol groups on agarose for protein conjugation. After conjugation, samples were gelled at 4 °C for 1 h, washed for 7 days in PBS buffer and quantified as previously described. (D) VEGFA immobilization was successful on coumarin-sulphide agarose ($p \leq 0.05$) at a concentration of 253 ± 44 ng/ml. Data are presented as mean \pm SD, $n = 3$. Groups with asterisks are significantly different ($p \leq 0.05$).

2.5. Maintenance of ESCs

The Brachyury/T–green fluorescent protein (GFP) [20] mouse embryonic stem cell line was maintained in 37 °C humidified air with 5% CO₂ on 0.5% gelatinized culture coated flasks (Sarstedt, Montreal, Quebec, www.sarstedt.com). Cells were kept in serum-free maintenance media comprised of Neurobasal media (48% v/v, Invitrogen), Dulbecco's Minimum Essential Media (DMEM)/F12 (48% v/v, Invitrogen), N2-supplement (0.5% v/v, Invitrogen), B27 with retinoic acid (1% v/v, Invitrogen), bovine serum albumin (BSA, 0.5 mg/ml, Sigma Aldrich), L-glutamax (1% v/v, Invitrogen), penicillin/streptomycin (1% v/v, Invitrogen), monothioglycerol (MTG, 4.5×10^{-4} M), leukemia inhibitory factor (LIF, 1000 μ l/ml, Chemicon, Billerica, MA, www.millipore.com) and BMP-4 (10 ng/ml, R&D systems, Minneapolis, MN, www.rndsystems.com). ESCs were passaged every second day and used between passage 19 and 26.

2.6. ESC encapsulation and differentiation

ESC aggregates were formed by seeding 4.5×10^6 single cells in 15 ml maintenance media in 15 cm Petri dishes for 12 h before being encapsulated by emulsification and transferred into differentiation media. Settled aggregates suspended in 100 μ l Dulbecco's Phosphate Buffered Saline (DPBS, Invitrogen) were quickly mixed with 25 μ l F-68 cell culture treated pluronic (Invitrogen) and 400 μ l of VEGFA immobilized agarose or C-SH. The cell mixture was added to 5 ml warm dimethylpolysiloxane (DMPS) in a conical tube using a micropipette tip (p1000) while being vigorously stirred. The solution was then vortexed for 1 min (Vortex Genie at setting 8, Fisher Scientific) before being cooled on ice for 10 min to allow for the formation of agarose capsules. Cell encapsulated agarose capsules were obtained by centrifugal phase separation through a wash procedure as previously described [19]. Briefly, 10 ml of cold HBSS was added to the agarose-oil mixture and

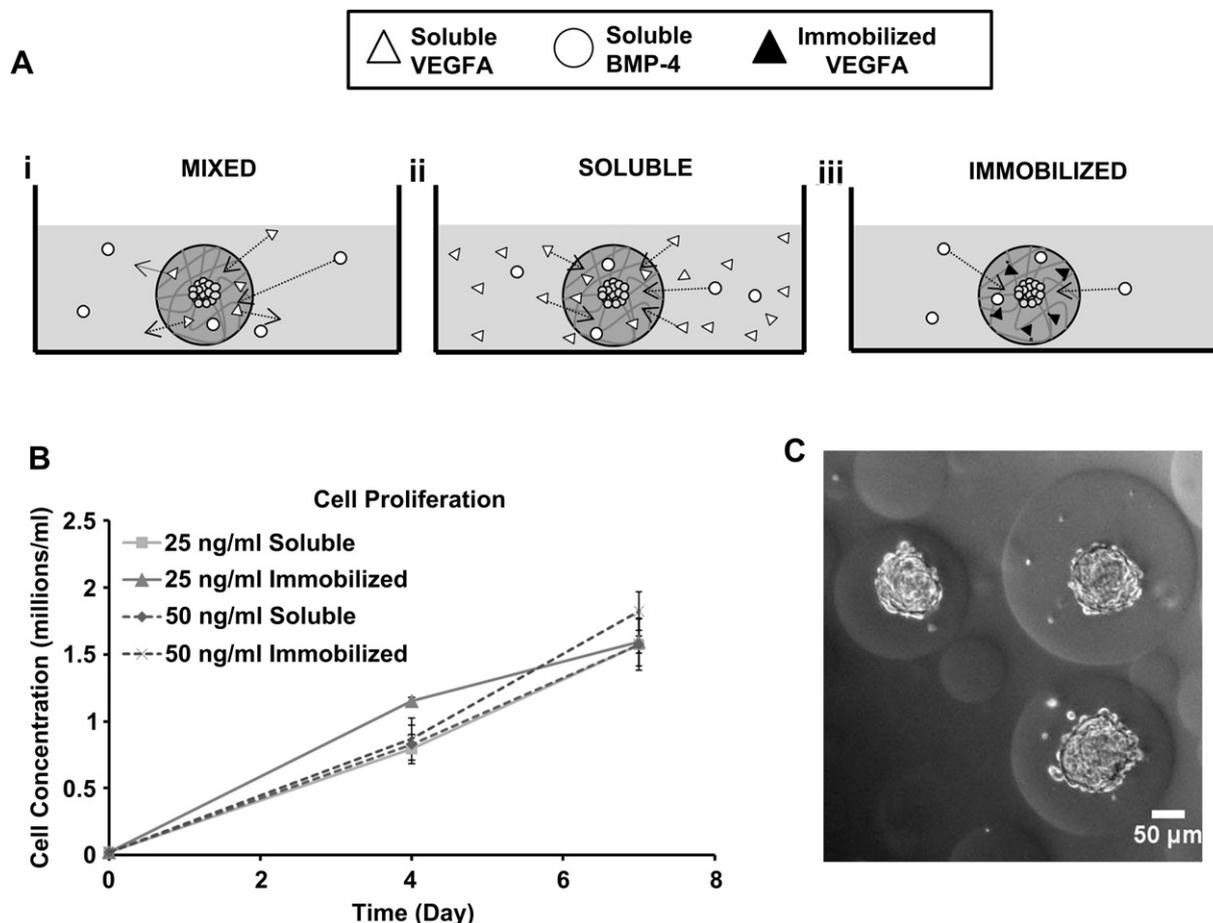


Fig. 2. Immobilized VEGFA exhibits similar cell proliferation compared to soluble VEGFA treatments. (A) Cartoon representations of EB encapsulations in (i) mixed, (ii) soluble and (iii) immobilized VEGFA treatments supplemented with soluble BMP-4. (B) Cell proliferation of encapsulated mouse EBs in soluble and immobilized VEGFA treatments at 25 ng/ml and 50 ng/ml. Cell counts were performed by mechanically dissociating encapsulated EBs into single cells using Trypsin-ethylenediaminetetraacetic acid (EDTA) followed by stop solution (10% fetal bovine serum in DMEM/F12). Cells were sampled prior to flow cytometry analysis on day 4 and day 7. (C) Representative light microscope picture of EBs encapsulated in VEGFA immobilized agarose. Scale bar = 50 μ m. Data are presented as mean \pm SD, $n = 3$.

centrifuged at 400 RCF for 5 min. The oil and HBSS layers were aspirated, leaving the agarose cell pellet behind. Encapsulated cell aggregates were re-suspended in 1 ml of HBSS and transferred to a fresh conical tube with 10 ml HBSS before centrifuging again at 400 RCF for 5 min. The agarose cell pellet was re-suspended in differentiation medium and encapsulated aggregates were filtered through a 350 μ m nylon mesh before seeding at a density of 20,000 cells/ml. Differentiation media was comprised of Iscove's Modified Dulbecco's Medium (IMDM, 71% v/v, Invitrogen), F12 (24% v/v, Invitrogen), N2-Supplement (0.5% v/v), B27 without retinoic acid (1% v/v, Invitrogen), BSA (0.5 mg/ml), glutamax (1.8% v/v), penicillin/streptomycin (0.8% v/v), ascorbic acid (50 μ g/ml) and MTG (4.5×10^{-4} M) supplemented with a combination of 5 ng/ml BMP-4 and 5 ng/ml, 25 ng/ml or 50 ng/ml VEGFA.

2.7. Flow cytometry

EBs were dissociated and test samples were re-suspended at a concentration of 2×10^5 cells/10 μ l in 2% v/v FBS in HBSS (HF). Samples were incubated for 35 min at 4 $^{\circ}$ C on day 4 or day 7 at a concentration of 1 μ l/100 μ l HF with the following antibodies as previously described [21]: allophycocyanin (APC) anti-mouse fetal liver kinase, phycoerythrin (PE) anti-mouse CD41 or allophycocyanin 647 (APC647) anti-mouse CD34. Negative control cells were incubated with fluorochrome-labelled isotype control antibodies: APC rat IgG_{2a,k}, PE rat IgG_{1,k} and APC647 Rat IgG_{2a,k}. All antibodies were obtained from BD Biosciences (Mississauga, ON, www.bdbiosciences.ca). Stained cells were washed twice in cold HF and placed in 1 μ l/ml 7-amino-actinomycin D (7AAD, Molecular Probes, Eugene, OR) as a viability indicator prior to analysis. Samples were analysed on a Becton Dickinson FACSCanto machine using BD FACS Diva Software version 5.0.1. Positive staining was gated as the fluorescence emission at >99.5% of the levels obtained by negative or isotype control cells from the same population.

2.8. Colony forming cell (CFC) assay

1.5×10^5 cells were seeded in duplicate on day 7 in 35 mm Greiner dishes in methylcellulose based M3434 media (Stem Cell Technologies, www.stemcell.com, Vancouver, BC). Samples were scored based on morphology 7 days after plating as previously described [22].

2.9. Statistical analysis

Statistics were performed using JMP IN 7 (SAS Institute, Cary, NC, USA). To identify significant trends between groups, comparisons were performed by one-way ANOVA with Tukey's post hoc analysis or student's *t*-test. An alpha level of 0.05 was used to determine significance between groups with three independent replicates. Data are reported as mean \pm standard deviation (SD).

3. Results

3.1. Functionalized agarose

We were interested in water-based chemistries to immobilize VEGFA on agarose as proteins are known to be most stable in physiological conditions [23]. The most reactive groups of the agarose backbone are the primary hydroxyls, which were modified using CDI chemistry to introduce amine, carboxylic acid or coumarin-protected thiol groups, thereby facilitating protein immobilization (Fig. 1Ai–iii). Agarose modification was confirmed for amines using the TNBSA assay, where we calculated 8% amine

substitution based on the number of moles of ethylenediamine per agarose repeat unit. The carboxylic acid substitution was difficult to quantify; however, the modification was confirmed by a characteristic carbonyl peak at 1730–1700 cm^{-1} by FTIR spectroscopy. This peak was absent in adsorbed or unmodified agarose samples. Coumarin substitution was calculated at 0.7% using UV absorbance ($\lambda_{\text{max}} = 373 \text{ nm}$, $\epsilon = 14\,500 \text{ M}^{-1} \text{ cm}^{-1}$ in 0.1 M HEPES, pH 7.4) [42].

3.2. Immobilization and quantification of α -chymotrypsinogen

α -chymotrypsinogen was used in initial experiments as a model protein for VEGFA to determine which functionalized agarose was most efficient for protein immobilization. α -chymotrypsinogen was activated using a heterobifunctional crosslinker and fluorescently tagged with FAMSE for quantification purposes. Protein was detected on all three functionalized agarose samples (Fig. 1B). By comparing the protein concentration of covalently-bound to adsorbed controls, it was clear that of the three functionalized agarose samples, covalent modification was most successful for both aminated agarose ($240 \pm 10 \text{ ng/ml}$ relative to adsorbed controls with $56 \pm 5 \text{ ng/ml}$) and coumarin-sulphide agarose ($204 \pm 11 \text{ ng/ml}$ relative to adsorbed controls of $36 \pm 2 \text{ ng/ml}$). However, the difference between covalently-bound ($480 \pm 70 \text{ ng/ml}$) and adsorbed controls ($410 \pm 88 \text{ ng/ml}$) for carboxylated agarose was not significantly different. While both aminated and coumarin-sulphide agarose were candidates for VEGFA conjugation, we were concerned that VEGFA carboxylic acids may crosslink with its own amine functional groups during the immobilization to aminated agarose. Given that VEGFA-thiols are unavailable for chemical reaction, coumarin-sulphide agarose was pursued for VEGFA immobilization.

3.3. Immobilization and quantification of VEGFA

VEGFA was modified with maleimide functional groups using sulpho-SMCC chemistry and reacted with thiol-modified agarose (Fig. 1C). Coumarin-sulphide agarose was exposed to UV light (275 nm–350 nm) thereby cleaving coumarin and exposing thiol groups that reacted with VEGFA-maleimide through Michael addition. Using Alexa-532 tagged VEGFA we determined that it was covalently immobilized to agarose-sulphide at a concentration of $254 + 44 \text{ ng/ml}$, which was significantly greater than adsorbed controls with a concentration of $81 + 54 \text{ ng/ml}$ (Fig. 1D, $p \leq 0.05$).

3.4. ESC differentiation in VEGFA functionalized agarose

Prior to testing bioactivity of the immobilized VEGFA, we compared ‘mixed’, ‘soluble’ and ‘immobilized’ agarose formulations where ‘mixed’ samples had VEGFA (5 ng/ml, 25 ng/ml, 50 ng/ml) homogeneously dispersed in C-SH agarose prior to cell encapsulation, and acted as an adsorbed control (Fig. 2). ‘Soluble’ samples had cells encapsulated in C-SH agarose immersed in media supplemented with VEGFA (5 ng/ml, 25 ng/ml or 50 ng/ml) whereas ‘immobilized’ samples had cells encapsulated in agarose with covalently-bound VEGFA (5 ng/ml, 25 ng/ml or 50 ng/ml). For all three conditions, diffusion of nutrients in agarose gels was likely similar [24,25]. Serum-free media was supplemented with BMP-4 (0 ng/ml or 5 ng/ml) for all conditions. After encapsulation, 95% of mouse EB aggregates were in agarose capsules (data not shown). By day 7, cell proliferation in soluble and immobilized VEGFA treatments for 25 ng/ml and 50 ng/ml were similar, suggesting that VEGFA functionalized agarose was not detrimental to EB proliferation compared to native agarose.

3.5. Day 4 differentiation with VEGFA and BMP-4

The bioactivity and efficacy of immobilized VEGFA in driving mouse EBs toward blood progenitor cells were compared to soluble VEGFA controls in the presence of soluble BMP-4. We used a Brachyury-GFP (T) cell line to assess early blood development and monitored the generation of T+VEGFR2+ cells in soluble, immobilized and mixed VEGFA treatments. This phenotype is associated with cells capable of both endothelial and hematopoietic development [20]. Serum-free treatments with 5 ng/ml soluble BMP-4 in the absence of VEGFA (soluble V0B5) resulted in an induction of $9.2 \pm 1.2\%$ T+VEGFR2+ and all other treatments were normalized to this baseline control (Fig. 3). EBs differentiated in the absence of both VEGFA and BMP-4 (V0B0) or BMP-4 (V25B0) did not induce T+VEGFR2+. This result confirmed that EBs required BMP-4 to express early mesodermal phenotypes [21,26]. For soluble VEGFA treatments, T+VEGFR2+ expression increased with VEGFA dose, peaking at 25 ng/ml (approximately 1.7 times the baseline control). However, the induction for immobilized VEGFA treatments was highest at 50 ng/ml (2.75 times the baseline control, Fig. 3, $p \leq 0.05$). Concentrations of immobilized VEGFA higher than 50 ng/ml resulted in lower T+VEGFR2+ induction (data not shown). Serum conditions induced T+VEGFR2+ approximately 2 times the baseline control (Fig. 3, $p \leq 0.05$). These results indicate that immobilized VEGFA was bioactive and could drive ESCs toward early blood development in conjunction with soluble BMP-4.

3.6. Day 7 differentiation with VEGFA and BMP-4

VEGFA immobilized treatments were characterized for their ability to give rise to blood progenitor cells on day 7 as detected by the CD34+CD41+ phenotype and functional CFC assays [21]. Serum-free conditions supplemented with 5 ng/ml BMP-4 in the

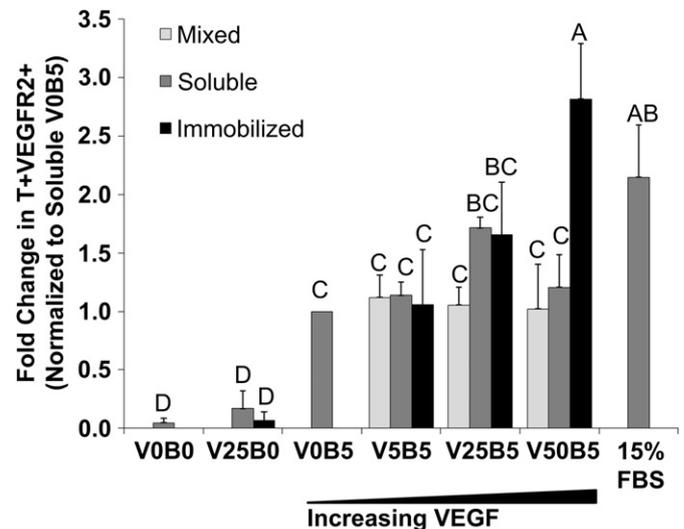


Fig. 3. Immobilized VEGFA enhances Brachyury-GFP/VEGFR2 expression on day 4. Brachyury-GFP and VEGFR2 expression were quantified on day 4 in mixed, soluble and immobilized VEGFA treatments at 5 ng/ml, 25 ng/ml and 50 ng/ml supplemented with soluble 5 ng/ml BMP-4 in serum-free media. 15% fetal bovine serum (FBS) was used as a positive control. Treatments were normalized to unsupplemented VEGFA treatments with 5 ng/ml BMP-4 (V0B5). EBs were encapsulated in their respective conditions and supplemented with factors on day 0. EB samples were dissociated to single cells, stained for VEGFR2 and analysed using flow cytometry on day 4. Data are presented as mean \pm SD, $n = 3$. Treatments that do not share the same letter are significantly different ($p \leq 0.05$). Notation used has V for VEGFA and B for BMP-4 and the number represents the concentration of each used. For example, V25B5 is VEGFA at 25 ng/ml and BMP-4 at 5 ng/ml.

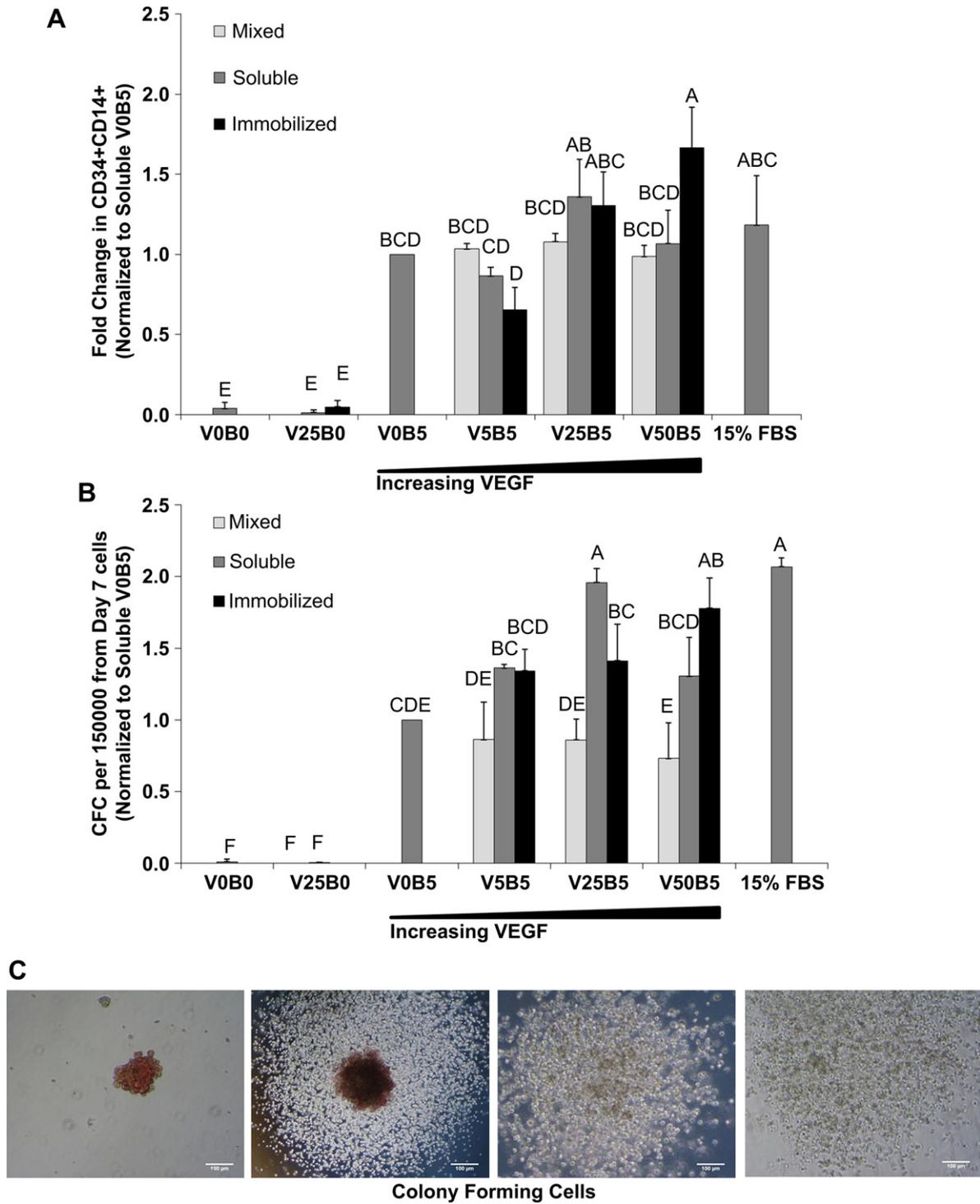


Fig. 4. Immobilized VEGFA enhances CD34 + CD41 + on day 7 and gives rise to colony forming cells. (A) CD34 + CD41 + expression on day 7 in mixed, soluble and immobilized VEGFA treatments. (B) CFCs counted on day 14 generated from day 7 EBs in mixed, soluble and immobilized VEGFA treatments. Treatments were normalized to unsupplemented VEGFA treatments with soluble 5 ng/ml BMP-4 (V0B5). EB samples were dissociated into single cells, stained for CD34/CD41 and analysed using flow cytometry on day 7. (C) Representative blood colony forming cells obtained from day 7 EBs in VEGFA immobilized treatments at 50 ng/ml. Scale bar = 100 μ m and data are presented as mean \pm SD, $n = 3$. Treatments that do not share the same letter are significantly different ($p \leq 0.05$). 15% FBS was used as a control. Notation used: V25B5 = VEGFA at 25 ng/ml and BMP-4 at 5 ng/ml.

absence of VEGFA generated $1.2 \pm 0.3\%$ CD34+CD41+ and 75 ± 10 CFCs per 1.5×10^5 cells and all samples were normalized to these values (Fig. 4A and B). The addition of increasing soluble VEGFA or serum containing media did not significantly increase CD34+CD41+ induction compared to the baseline control (Fig. 4A and B, $p \leq 0.05$). The highest CD34+CD41+ was observed with

immobilized VEGFA at 50 ng/ml with an induction of 1.5 times the control (Fig. 4A, $p \leq 0.05$). Interestingly, there were three treatments that resulted in higher CFCs compared to the control. These treatments were 25 ng/ml soluble VEGFA, 50 ng/ml immobilized VEGFA and serum containing media (Fig. 4B, $p \leq 0.05$). Mixed VEGFA treatments showed CD34+CD41+ induction and CFC

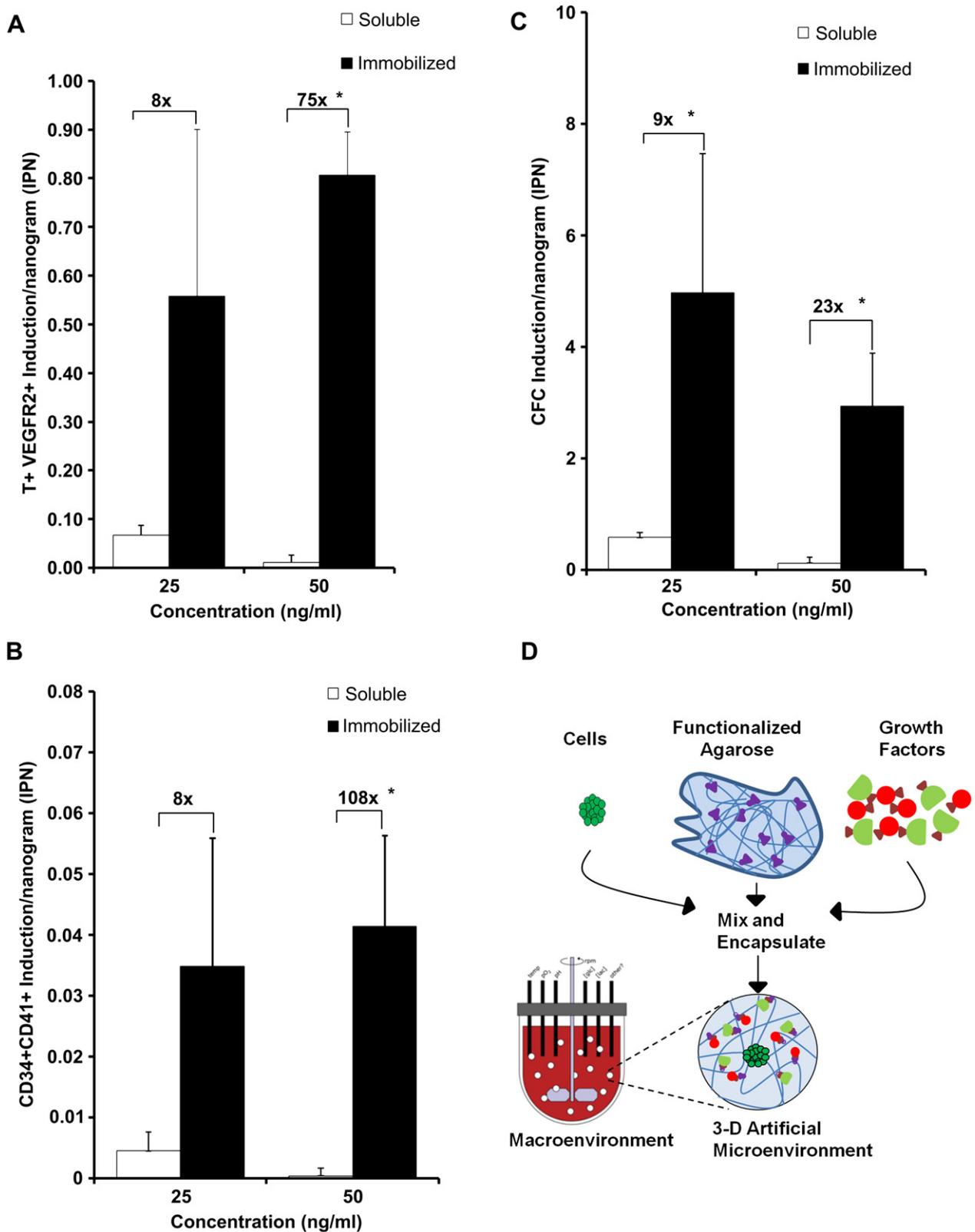


Fig. 5. Immobilized VEGFA treatments are more potent than equivalent soluble treatments for marker expression. Induction efficiency in VEGFA immobilized treatments are higher at 25 ng/ml and 50 ng/ml than soluble treatments for: (A) Day 4 brachyury+VEGFR2+ expression, (B) Day 7 CD34 + CD41 + expression and (C) CFC generation. Induction per nanogram for each condition was calculated by subtracting the induction of the baseline condition (V0B5) from the sample induction and dividing it by the total amount of VEGFA used. (D) Strategy of a cell-hydrogel system to incorporate micro- and macro-environmental control for directing cell differentiation. Bioreactor figure adapted from Kirouac D.C. and Zandstra P.W., Cell Stem Cell, 2008. Data are presented as mean + SD, $n = 3$. Groups with asterisks are significantly different ($p \leq 0.05$).

generation similar to the baseline control (Fig. 4B). These results suggest that immobilized VEGFA treatments were capable of generating blood progenitor cells by day 7 in serum-free media (Fig. 4B and C).

4. Discussion

Much work has been accomplished in recreating the adult stem cell microenvironment, but only recently has the niche been engineered to guide ESC differentiation [27,28]. Various studies have investigated different strategies to mimic the niche by controlling cell density [29], presenting motifs from the ECM [30], co-culturing different cell types [31] and altering mechanical stiffness [32,33]. In the current study, we investigated the effect of immobilized VEGFA on blood progenitor cell generation from encapsulated mouse EBs within agarose hydrogels cultured under serum-free conditions.

Using CDI chemistry, we synthesized three different functionalized forms of agarose – amines, carboxylic acids and coumarin-sulphides – and found that C-SH was the most suitable for VEGFA immobilization. Interestingly, while the degree of substitution of the aminated agarose (8%) was greater than that for coumarin-sulphide (0.7%), the concentration of immobilized α -chymotrypsinogen was similar, suggesting that the maleimide-thiol chemistry was efficient at protein conjugation. The coumarin-derivative, 6-bromo-7-hydroxycoumarin, has been previously used to synthesize thiol (C-SH) [42] and amine-protected [43] agarose for 3-D spatial patterning of molecules within agarose gels. For the first time, we demonstrate the use of C-SH chemistry to immobilize maleimide-modified proteins in aqueous solutions. While not pursued here, gradients of immobilized biomolecules can be synthesized using photo-patterning techniques [42], thereby providing a tool to replicate gradients of cytokines that are present in embryogenesis during early patterning of the germ layers [1].

To test the efficacy of immobilized VEGFA, aggregates were encapsulated within VEGFA immobilized agarose using a water in oil emulsification method. This process allows 3-D presentation of immobilized ligands similar to VEGFA presentation within the native ECM in vivo [34]. Cells grown in 3-D hydrogels better mimic physiological environments, providing more relevant cell–cell interactions than 2-D analogs [35].

Encapsulated EBs were characterized for early blood development by tracking T+VEGFR2+ expression [20]. Immobilized VEGFA treatments elicited similar differentiation responses compared to soluble and mixed conditions at 5 ng/ml and 25 ng/ml, but produced almost 3 times more T+VEGFR2+ cells at 50 ng/ml. It is known that immobilized growth factors increase local protein concentration by hindering receptor mediated endocytosis and preventing diffusion [28]. As a result of this phenomenon, immobilized VEGFA treatments are likely to have increased VEGFA/VEGFR2 complexes with encapsulated EBs thereby leading to greater mesoderm induction than soluble and adsorbed controls. Our results are consistent with other experiments that have shown inductive responses to immobilized VEGFA. For example, Anderson et al. reported that immobilized VEGFA on 2-D surfaces was biologically active and retained its ability to phosphorylate VEGFR2 in both an overexpressing KDR cell line and human umbilical vein endothelial cells (HUVECs) [36]. Alberti et al. have shown that immobilized leukemia inhibitory factor and stem cell factor on polymeric surfaces have the ability to phosphorylate their respective receptors and induce cell signalling [5]. Others have also shown enhanced induction resulting from immobilized growth factors upon comparison to similar levels of soluble supplementation [36–38,44]. To this end, we calculated an ‘induction per nanogram’ (IPN) factor for all VEGFA treatments. For soluble and immobilized

treatments, IPN was calculated by dividing the observed induction by the total amount of VEGFA (see Supplemental information, Sample Calculation of Induction per Nanogram). Treatments with 25 ng/ml and 50 ng/ml immobilized VEGFA were 8- and 75- times more efficient in T+VEGFR2+ induction than their soluble counterparts by day 4 (Fig. 5A).

Encapsulated cells were further characterized by CD34+CD41+ surface marker expression on day 7 and seeded in CFC assays for blood progenitor quantification. CD34 is a common blood marker [39] and CD41 cells are known to give rise to hematopoietic progenitor cells (HPCs) in functional assays [40]. Encapsulated EBs grew out of their hydrogel capsule by day 4–4.5, and BMP-4 was not supplemented to any treatments thereafter since it has been reported that BMP-4 alone does not enhance the generation of HPCs from mouse ESCs after this time [21,26]. It was also reported that VEGFA supplementation after day 5 preferentially guides mouse ESCs toward endothelial cells [10]. Thus, it is thought that early VEGFA treatment may have stimulated endogenous signals that support later blood development in this study. We found that 25 ng/ml and 50 ng/ml immobilized VEGFA treatments were 8- and 108- times more efficient at inducing CD34+CD41+ expression and 9- and 23-times more efficient in CFC generation compared to equivalent soluble concentrations (see Fig. 5B, C and Supplemental information for a sample calculation of Induction per Nanogram and Fig. 5B and C). These observations suggest that induction from immobilized VEGFA treatments is more efficient in guiding ESCs toward blood progenitor cells compared to soluble VEGFA.

This hydrogel system may be used as a framework to mimic the native stem cell niche and influence ESC differentiation. Through functionalized systems, as described herein, and culture conditions that allow control over macroscopic cues such as oxygen tension [41], pH and glucose; both micro- and macro-environments within which cells are cultured can be controlled. This extrinsic control will allow us to understand important mechanisms involved in stem cell regulation and provide a technique for the robust and effective differentiation of ESCs toward target cell types.

5. Conclusion

Agarose was successfully functionalized with VEGFA at a concentration of 254 + 44 ng/ml and was shown to elicit cellular activity. Under serum-free conditions, EBs were encapsulated within VEGFA immobilized agarose wherein they differentiated to express early and late blood phenotypes, giving rise to blood colonies in CFC assays. VEGFA immobilized treatments at 50 ng/ml resulted in cells that expressed 2.75 times more T+VEGFR2+ on day 4, had 1.5 times more CD34+CD41+ induction on day 7 and generated 1.7 times more blood progenitor colonies compared to unsupplemented VEGFA (VOBO). Consequently, immobilized treatments at 25 ng/ml and 50 ng/ml were highly efficient in their induction toward blood progenitor cells compared to soluble treatments. Ultimately, this hydrogel system can be tailored to functionalize biomolecules of interest and used as a platform to investigate ligand interactions in a combinatorial manner within a 3-D environment.

Acknowledgments

The authors thank Dr. Hannah Song for insightful discussions. The authors are grateful for funding support from Ontario Centre of Excellence (OCE), Canadian Institutes of Health Research (MOP-57885), the Stem Cell Network and Ontario Graduate Scholarship in Science and Technology (to NR).

Conflict of interests

The authors indicate no potential conflict of interests.

Appendix Supplementary information

Supplementary information associated with this article can be found in the on-line version, at [doi:10.1016/j.biomaterials.2010.07.040](https://doi.org/10.1016/j.biomaterials.2010.07.040).

Appendix

Figures with essential color discrimination. Figs. 4 and 5 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at [doi:10.1016/j.biomaterials.2010.07.040](https://doi.org/10.1016/j.biomaterials.2010.07.040).

References

- Gadue P, Huber TL, Nostro MC, Kattman S, Keller GM. Germ layer induction from embryonic stem cells. *Exp Hematol* 2005;33(9):955–64.
- Rider CC. Heparin/heparan sulphate binding in the TGF-beta cytokine superfamily. *Biochem Soc Trans* 2006;34(Pt 3):458–60.
- Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 2003;423(6938):448–52.
- Aizawa Y, Leipzig N, Zahir T, Shoichet M. The effect of immobilized platelet derived growth factor AA on neural stem/progenitor cell differentiation on cell-adhesive hydrogels. *Biomaterials* 2008;29(35):4676–83.
- Alberti K, Davey RE, Onishi K, George S, Salchert K, Seib FP, et al. Functional immobilization of signaling proteins enables control of stem cell fate. *Nat Methods* 2008;5(7):645–50.
- Ho JE, Chung EH, Wall S, Schaffer DV, Healy KE. Immobilized sonic hedgehog N-terminal signaling domain enhances differentiation of bone marrow-derived mesenchymal stem cells. *J Biomed Mater Res* 2007;83(4):1200–8.
- Kuhl PR, Griffith-Cima LG. Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase. *Nat Med* 1996;2(9):1022–7.
- Nagy JA, Dvorak AM, Dvorak HF. VEGF-A and the induction of pathological angiogenesis. *Annu Rev Pathol* 2007;2:251–75.
- Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling – in control of vascular function. *Nat Rev* 2006;7(5):359–71.
- Purpura KA, George SH, Dang SM, Choi K, Nagy A, Zandstra PW. Soluble Flt-1 regulates Flk-1 activation to control hematopoietic and endothelial development in an oxygen-responsive manner. *Stem Cells* 2008;26(11):2832–42.
- Ferreira LS, Gerecht S, Fuller J, Shieh HF, Vunjak-Novakovic G, Langer R. Bioactive hydrogel scaffolds for controllable vascular differentiation of human embryonic stem cells. *Biomaterials* 2007;28(17):2706–17.
- Hunt NC, Grover LM. Cell encapsulation using biopolymer gels for regenerative medicine. *Biotechnol Lett* 2010;32(6):733–42.
- Agudelo CA, Teramura Y, Iwata H. Cryopreserved agarose-encapsulated islets as bioartificial pancreas: a feasibility study. *Transplantation* 2009;87(1):29–34.
- Gazda LS, Vinerean HV, Laramore MA, Diehl CH, Hall RD, Rubin AL, et al. Encapsulation of porcine islets permits extended culture time and insulin independence in spontaneously diabetic BB rats. *Cell Transplant* 2007;16(6):609–20.
- Pelaez D, Huang CY, Cheung HS. Cyclic compression maintains viability and induces chondrogenesis of human mesenchymal stem cells in fibrin gel scaffolds. *Stem Cells Dev* 2009;18(1):93–102.
- Iwata H, Kobayashi K, Takagi T, Oka T, Yang H, Amemiya H, et al. Feasibility of agarose microbeads with xenogeneic islets as a bioartificial pancreas. *J Biomed Mater Res* 1994;28(9):1003–11.
- Lahooti S, Sefton MV. Agarose enhances the viability of intraperitoneally implanted microencapsulated L929 fibroblasts. *Cell Transplant* 2000;9(6):785–96.
- Yu X, Dillon GP, Bellamkonda RB. A laminin and nerve growth factor-laden three-dimensional scaffold for enhanced neurite extension. *Tissue Eng* 1999;5(4):291–304.
- Dang SM, Gerecht-Nir S, Chen J, Itskovitz-Eldor J, Zandstra PW. Controlled, scalable embryonic stem cell differentiation culture. *Stem Cells* 2004;22(3):275–82.
- Fehling HJ, Lacaud G, Kubo A, Kennedy M, Robertson S, Keller G, et al. Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development* 2003;130(17):4217–27.
- Purpura KA, Morin J, Zandstra PW. Analysis of the temporal and concentration-dependent effects of BMP-4, VEGF, and TPO on development of embryonic stem cell-derived mesoderm and blood progenitors in a defined, serum-free media. *Exp Hematol* 2008;36(9):1186–98.
- Eaves C, Fraser C, Udomsakdi C, Sutherland H, Barnett M, Szilvassy S, et al. Manipulation of the hematopoietic stem cell in vitro. *Leukemia* 1992;1(Suppl 6):27–30.
- Sletten EM, Bertozzi CR. Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. *Angew Chem Int Ed Engl* 2009;48(38):6974–98.
- Pluen A, Netti PA, Jain RK, Berk DA. Diffusion of macromolecules in agarose gels: comparison of linear and globular configurations. *Biophys J* 1999;77(1):542–52.
- Li YJ, Chung EH, Rodriguez RT, Firpo MT, Healy KE. Hydrogels as artificial matrices for human embryonic stem cell self-renewal. *J Biomed Mater Res* 2006;79(1):1–5.
- Nostro MC, Cheng X, Keller GM, Gadue P. Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood. *Cell Stem Cell* 2008;2(1):60–71.
- Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat Biotechnol* 2005;23(1):47–55.
- Peerani R, Zandstra PW. Enabling stem cell therapies through synthetic stem cell-niche engineering. *J Clin Invest* 2010;120(1):60–70.
- Peerani R, Rao BM, Bauwens C, Yin T, Wood GA, Nagy A, et al. Niche-mediated control of human embryonic stem cell self-renewal and differentiation. *EMBO J* 2007;26(22):4744–55.
- Flaim CJ, Chien S, Bhatia SN. An extracellular matrix microarray for probing cellular differentiation. *Nat Methods* 2005;2(2):119–25.
- Hui EE, Bhatia SN. Micromechanical control of cell-cell interactions. *Proc Natl Acad Sci U S A* 2007;104(14):5722–6.
- Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell* 2006;126(4):677–89.
- Leipzig ND, Shoichet MS. The effect of substrate stiffness on adult neural stem cell behavior. *Biomaterials* 2009;30(36):6867–78.
- Ferrara N. Binding to the extracellular matrix and proteolytic processing: two key mechanisms regulating vascular endothelial growth factor action. *Mol Biol Cell* 2010;21(5):687–90.
- Yamada KM, Cukierman E. Modeling tissue morphogenesis and cancer in 3D. *Cell* 2007;130(4):601–10.
- Anderson SM, Chen TT, Iruela-Arispe ML, Segura T. The phosphorylation of vascular endothelial growth factor receptor-2 (VEGFR-2) by engineered surfaces with electrostatically or covalently immobilized VEGF. *Biomaterials* 2009;30(27):4618–28.
- Shen YH, Shoichet MS, Radisic M. Vascular endothelial growth factor immobilized in collagen scaffold promotes penetration and proliferation of endothelial cells. *Acta Biomater* 2008;4(3):477–89.
- Suzuki T, Yokoyama Y, Kumano K, Takanashi M, Kozuma S, Takato T, et al. Highly efficient ex vivo expansion of human hematopoietic stem cells using Delta1-Fc chimeric protein. *Stem Cells* 2006;24(11):2456–65.
- Brown J, Greaves MF, Molgaard HV. The gene encoding the stem cell antigen, CD34, is conserved in mouse and expressed in haemopoietic progenitor cell lines, brain, and embryonic fibroblasts. *Int Immunol* 1991;3(2):175–84.
- Mitjavila-Garcia MT, Caillieret M, Godin I, Nogueira MM, Cohen-Solal K, Schiavon V, et al. Expression of CD41 on hematopoietic progenitors derived from embryonic hematopoietic cells. *Development* 2002;129(8):2003–13.
- Niebruegge S, Bauwens CL, Peerani R, Thavandiran N, Masse S, Sevaptisidis E, et al. Generation of human embryonic stem cell-derived mesoderm and cardiac cells using size-specified aggregates in an oxygen-controlled bioreactor. *Biotechnol Bioeng* 2009;102(2):493–507.
- Wosnick JH, Shoichet MS. Three dimensional chemical patterning of transparent hydrogels. *Chem Mater* 2008;20:55–60.
- Wylie RG, Shoichet MS. Two photon micropatterning of amines within an agarose hydrogel. *J Mater Chem* 2008;18:2716–21.
- Taguchi T, Kishida A, Akashi M, Maruyama I. Immobilization of human vascular endothelial growth factor (VEGF165) onto biomaterials: an evaluation of the biological activity of immobilized VEGF165. *J Bioact Compat Polym* 2000;15:309–20.
- Hayashi A, Kanzaki T. Swelling of agarose gel and its related changes. *Food Hydrocolloids* 1987;4:317–25.