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## Bioactive hydrogel scaffolds for controllable vascular differentiation of human embryonic stem cells

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### Abstract

We propose a new methodology to enhance the vascular differentiation of human embryonic stem cells (hESCs) by encapsulation in a bioactive hydrogel. hESCs were encapsulated in a dextran-based hydrogel with or without immobilized regulatory factors: a tethered RGD peptide and microencapsulated VEGF<sub>165</sub>. The fraction of cells expressing vascular endothelial growth factor (VEGF) receptor KDR/Flk-1, a vascular marker, increased up to 20-fold, as compared to spontaneously differentiated embryoid bodies (EBs). The percentage of encapsulated cells in hydrogels with regulatory factors expressing ectodermal markers including nestin or endodermal markers including  $\alpha$ -fetoprotein decreased 2 or 3 fold, respectively, as compared to EBs. When the cells were removed from these networks and cultured in media conditions conducive for further vascular differentiation, the number of vascular cells was higher than the number obtained through EBs, using the same media conditions. Functionalized dextran-based hydrogels could thus enable derivation of vascular cells in large quantities, particularly endothelial cells, for potential application in tissue engineering and regenerative medicine.

### 1- INTRODUCTION

During normal embryogenesis, human embryonic stem cells (hESCs) differentiate along different lineages in the context of complex three-dimensional tissue structures, where the extracellular matrix (ECM) and different growth factors play an important role in this process. The three-dimensional ECM provides structural support for higher level of tissue organization and remodelling [1]. Significant differences were found in the differentiation profile of ESCs when cultured in a three-dimensional (3D) versus two-dimensional (2D) system [2,3] or 3D scaffold system versus embryoid body (EB) system [4,5]. In this last case, mouse ESCs cultured within tantalum scaffolds differentiate at higher extent into hematopoietic cells than EBs [4], while hESCs cultured in alginate scaffolds express significantly more vascular markers than EBs [5]. Moreover, the culture of hESCs within 3D poly( $\alpha$ -hydroxy esters) scaffolds with media containing different growth factors induced their differentiation into 3D structures with

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characteristics of developing neural, cartilage or liver tissues [1,6]. However, these growth factors were supplied from outside the scaffold, and thus their activity may be affected by diffusion limitations within the scaffold. Furthermore this differentiation system does not allow the direct use of these cell constructs for *in vivo* applications since an *in vitro* differentiation step is required.

3D hydrogel scaffolds incorporating a compendium of bioactive molecules within the matrix may allow a better spatial control of stem cell differentiation and ultimately the direct use of these cells for *in vivo* conditions. For that purpose we developed a photopolymerizable dextran-based hydrogels comprising either insoluble (RGD sequences) or soluble [vascular endothelial growth factor (VEGF<sub>165</sub>)] factors for the preferential differentiation of hESCs into the vascular lineage. Dextran-based hydrogels are biocompatible [7,8], biodegradable [9] and cell-nonadhesive [7] which allows one to tailor its cell adhesiveness. The RGD peptide is the adhesive motif found in fibronectin, the earliest and most abundantly expressed extracellular matrix molecule during embryonic vascular development [10]. VEGF<sub>165</sub> has been reported to induce the differentiation of ESCs into endothelial and hematopoietic cells [11–14], and involved as a survival factor of ESCs during hypoxia [15].

Herein we report that dextran-based hydrogels with different compositions can support the differentiation of hESCs and lead to over a 22 fold increase in the percentage of cells expressing the VEGF receptor KDR/Flk-1, a vascular marker, as compared to spontaneously differentiated EBs. When the cells were removed from these networks and cultured in media promoting further vascular differentiation, they contained higher fraction of vascular cells than the spontaneously differentiating EBs.

## 2- MATERIALS and METHODS

### 2.1- Synthesis of dextran-acrylate macromonomer and acryloyl-poly(ethylene glycol)-Arg-Gly-Asp (Acr-PEG-RGD)

Dextran-acrylate with a degree of substitution of 13% was synthesized as described previously [16]. Acr-PEG-RGD was prepared by reacting GRGDS (2mg/mL, Bachem) with an equimolar amount of acryloyl-PEG-*N*-hydroxysuccinimide (3400 Da, Nektar) in 50 mM sodium bicarbonate solution, pH 8.4, overnight at room temperature [17]. After reaction, the reaction vials were centrifuged at 4000 rpm (5 min) and the supernatant dialysed (MWCUTOF 500 Da) against water. After two days, the dialyzed product was freeze-dried for 48 h.

### 2.2- Preparation and release from VEGF-loaded poly(lactic acid-co-glycolic acid) (PLGA) microparticles

**2.2.1- Preparation**—Microparticles were prepared using a double emulsion solvent evaporation procedure. PLGA (100 mg, inherent viscosities of 0.16–0.24 or 0.32–0.44 dl/g, Boheringer Mannheim) polymer was dissolved in methylene chloride (2 ml, Aldrich) with magnesium hydroxide (4 mg, Aldrich). Next, 50 µl of VEGF<sub>165</sub> (R&D Systems) (5µg; in 50 mg/mL BSA in 10 mM phosphate buffer, pH 7.4, with or without 4 mg of sucrose) was added to the organic polymer solution, and the aqueous and organic phases were emulsified by sonication (Vibra Cell, Sonics & Materials, Inc., Danbury, CT). Microparticles were prepared and characterized as previously described [18] and stored at –20 °C. The morphology of PLGA microparticles during VEGF<sub>165</sub> release was evaluated by scanning electron microscopy (SEM). For that purpose, the microparticles were mounted onto an aluminum stud, and gold coated by plasma vapor deposition.

**2.2.2- VEGF<sub>165</sub> release studies**—Microparticles (10 mg) were placed in PBS (0.5 mL) and incubated under mild agitation, at 37 °C. At specific intervals of time, the microparticle

suspension was centrifuged (4000 rpm for 2 min) and 0.4 mL of the release medium removed and replaced by a new one. The reserved supernatant was stored at  $-20^{\circ}\text{C}$  until the VEGF content in release samples was assessed using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems). The analyses were conducted in duplicate. Concentrations of VEGF were determined by comparison to a standard curve.

**2.2.3- Loading efficiency of VEGF<sub>165</sub>-containing microparticles**—To determine the loading efficiency of PLGA microparticles, 5 mg of microparticles were dissolved in 1 mL of 1 N NaOH overnight and the absorbance measured at 284 nm. The results are expressed as a percentage of the ratio of protein encapsulated to total protein used.

**2.2.4- Bioactivity of released VEGF<sub>165</sub>**—The bioactivity of released VEGF<sub>165</sub> was assessed over a 3-day period on the EOMA cell line (ATCC: CRL-2586). EOMA cells were plated at 10,000 cells per well in gelatin coated (1%, w/v) 24-well plates and maintained with Dulbecco's Modified Eagle Medium (DMEM) containing 20% PBS with 34 ngmL<sup>-1</sup> VEGF (obtained from the release experiment). DMEM containing 20% PBS with or without 34 ngmL<sup>-1</sup> extrinsic VEGF were used as controls. After that time, a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, Sigma) assay was performed to quantify cell viability.

### 2.3- Preparation and characterization of dextran-based hydrogels

Dextran-based hydrogels (10 mm diameter and 1 mm thickness, before swelling) were obtained by photopolymerization reaction of aqueous solutions of 10% (w/v) dextran-acrylate (100 mg in 0.9 mL of PBS) alone or either containing 0.5 mM Acr-PEG-RGD or 5 mM Acr-PEG-RGD or 0.5 mM Acr-PEG-RGD with 5 mg/mL of microparticles. In all cases, the ultraviolet photoinitiator Irgacure 2959 (100  $\mu\text{L}$ , 0.5% w/v in PBS, Cyba Chemicals) was added to the solution. The polymerization reaction was initiated by ultraviolet light (ca. 4 mW/cm<sup>2</sup>), between two glasses with a 1 mm spacer, for 10 min. The gel was subsequently removed from the glasses, punched using stainless steel bars to yield cylinders with 10 mm diameter, and the cylinders immersed in ca. 5 mL of PBS for 6 days, changing the buffer daily. After this period of time the hydrogels were weighed (Ws), lyophilised for 48 h, and again weighed to determine the dried weight (Wd). The swelling ratio at equilibrium (SRE) was calculated according to the equation:  $\text{SRE} = (\text{Ws} - \text{Wd}) / \text{Wd}$ .

Rheological experiments have been carried out using the parallel plate geometry (8 mm diameter, steel) of an AR1000-N rheometer (TA Instruments). Hydrogels were subjected to stress sweep experiments (frequency of 1 Hz) to optimize the applied stress used in the frequency-oscillation experiments. These last experiments were recorded over a frequency range from 0.1 to 10 Hz.

### 2.4- hESC culture and EB formation

**2.4.1- hESC culture**—Undifferentiated hESCs (H9, WiCell, Wisconsin) were grown on an inactivated mouse embryonic fibroblast (MEF) feeder layer, as previously described [19]. To assess the effect of VEGF<sub>165</sub> on the differentiation of hESCs, cell aggregates were cultured in tissue flasks with EGM-2 medium (Cambrex) supplemented with 50 ng/mL VEGF<sub>165</sub> for 24 h, and then trypsinized to obtain single cells. Then 50,000 cells were plated per well on a 24-well plate coated with gelatin and cultured in the same media for 15 additional days.

**2.4.2- Formation of EBs**—To induce the formation of EBs, undifferentiated hESCs were treated with 2 mg/mL type IV collagenase for 2 h, and then transferred (2:1) to low attachment plates ( $\varnothing = 10$  cm, Ref:3262, Corning) containing 10 mL of differentiation medium [80% knockout- Dulbecco's Modified Eagle Medium, supplemented with 20% fetal bovine serum

(FBS, Hyclone), 0.5% L-glutamine, 0.2%  $\beta$ -mercaptoethanol and 1% nonessential amino acids (all from Invitrogen)]. EBs were cultured for 10 days at 37°C and 5% CO<sub>2</sub>, in a humidified atmosphere, with changes of media every 3–4 days. To assess the effect of VEGF<sub>165</sub> on the differentiation profile of EBs, the differentiation medium was supplemented with 50 ngmL<sup>-1</sup> VEGF<sub>165</sub>.

### 2.5- Cell-material interactions studies in dextran-based hydrogels

Undifferentiated hESC aggregates (0.3 × 10<sup>6</sup> cells in 0.5 mL of differentiation medium) isolated after collagenase treatment (see above) were plated on the surface of autoclaved dextran-based hydrogels with different composition (Ø=1.5 cm). After 24 h, cell adhesion was evaluated by light microscopy.

### 2.6- Cell encapsulation studies in dextran-based hydrogels

Undifferentiating hESC aggregates were resuspended in PBS (30 × 10<sup>6</sup> cells per ml). The cell suspension (400 µL) was mixed with a dextran-acrylate solution (500 µL) containing either Acr-PEG-RGD or microparticles, and 100 µL of photoinitiator. The suspension (50 µL) was photopolymerized in a 5 mm syringe by using an ultraviolet light, for 10 min. The cell constructs were subsequently removed from the syringe and immersed in differentiation medium (VEGF<sub>165</sub> concentration below 20 pg/mL, as determined by ELISA). The differentiation process of cell aggregates proceeded for 10 days at 37 °C, and 5% CO<sub>2</sub> in a humidified atmosphere, with changes of media every 3–4 days. In some cases, after 10 days, the cell constructs were treated with 0.4 U/mL collagenase B for 2 h in a 37 °C incubator, followed by treatment with cell dissociation buffer for 10 minutes and dissociated by gentle pipetting. Using these conditions, ca. 2 × 10<sup>5</sup> cells (20–30% of all the cells) per construct were removed. Single cells were then grown on 24 well-plates (50,000 cells/well) coated with 1% gelatin in EGM-2 medium supplemented with VEGF<sub>165</sub> (50 ngmL<sup>-1</sup>).

### 2.7- MTT assay for hESCs encapsulated into dextran-based hydrogels

The metabolic activity of encapsulated hESCs was measured through a MTT assay after 1 and 10 days of culture. The MTT solution (0.5 mL, 0.45 mg/mL in differentiation medium) was added to each well for 4 h, at 37 °C. After that time, the cell-hydrogel constructs were placed in 1.5 mL polypropylene tubes, and 0.2 mL of DMSO was added. The constructs were broken apart using a tissue homogenizer to dissolve the formazan crystals formed by cells presenting mitochondrial metabolic activity. The absorbance was measured spectrophotometrically at 540 nm.

### 2.8- FACS analysis

Cell constructs or EBs were treated with 0.4 U/mL collagenase B for 2 h in a 37 °C incubator, followed by treatment with cell dissociation buffer for 10 minutes and dissociated by gentle pipetting. Single cell suspensions were washed with PBS supplemented with 5% FBS and filtered through a 85 µm mesh strainer to remove remaining clumps. The single cell suspensions were aliquoted (1.25–2.5 × 10<sup>5</sup> cells per condition) and stained with either isotype controls or antigen-specific antibodies: anti-human/mouse SSEA4-PE (R&D Systems), PECAM1-FITC (BD Pharmingen), CD34-PE/CD34-FITC (Miltenyi Biotec) and KDR/Flk1-PE (R&D Systems). Cells were analyzed without fixation on a FACScan (Becton Dickinson), using propidium iodide to exclude dead cells. Data analysis was carried out using CellQuest software.

### 2.9-Immunostaining

hESC aggregates encapsulated in dextran-based hydrogels were fixed with 4% (w/v) paraformaldehyde for 30 minutes at room temperature. After blocking with 3% BSA solution, the cells were stained for 1 h with the following primary anti-human antibodies: PECAM1

(Dako), CD34 (Dako), VE-cadherin and KDR/Flk-1 (all from Santa Cruz Biochemicals). In each immunofluorescence experiment, an isotype-matched IgG control was used. Binding of primary antibodies to specific cells was detected with anti-mouse IgG Cy3 conjugate (Sigma, Saint Louis, Missouri). The nucleus of cells was stained with Topro-3 (Sigma). After the indirect labelling the cells were examined with a Zeiss LSM 510 confocal microscope.

Cell constructs were also characterized by immunocytochemistry involving a peroxidase staining. For that purpose, the cell constructs after fixation were embedded in paraffin, and sectioned for examination. The cells were stained with anti-human nestin (1:20, R&D systems) and anti-human  $\alpha$ -fetoprotein (1:500, Sigma) primary antibodies or the corresponding isotype controls. Immunostaining was carried out using the Dako EnVision<sup>TM</sup>+/HRP kit (Dako) according to the manufacturer's instructions.

## 2.10- Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Cell constructs were smashed in an eppendorf tube and the total RNA was extracted using trizol (Invitrogen) according to manufacturer's instructions. Total RNA was quantified by a UV spectrophotometer, and 1  $\mu$ g was used for each RT sample. RNA was reversed transcribed with M-MLV and oligo (dT) primers (Promega) according to manufacturer's instructions. PCRs were done with BIOTAQ DNA Polymerase (Bioline, Randolph, MA) using 1  $\mu$ L of RT product per reaction. To ensure semi-quantitative results of the RT-PCR assays, the number of PCR cycles for each set of primers was verified to be in the linear range of the amplification. In addition, all RNA samples were adjusted to yield equal amplification of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an internal standard. Primer sequences, reaction conditions, and optimal cycle numbers are published as supporting information (Supplemental Table 1). The amplified products were separated on 2% agarose gels with ethidium bromide. Quantitative PCR (qPCR) was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and the detection using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster). Quantification of target genes was performed relatively to the reference GAPDH gene: relative expression =  $2^{[-(C_{t\text{sample}} - C_{t\text{GAPDH}})]}$ . The mean minimal cycle threshold values (Ct) were calculated from quadruplicate reactions.

## 2.11- Statistical analysis

An unpaired t test or one-way analysis of variance with Bonferroni post test was performed for statistical tests by using GraphPad Prism 4.0 (San Diego, CA). Results were considered significant when  $P \leq 0.05$ .

# 3- RESULTS

## 3.1- Design and properties of dextran-based hydrogels

To make dextran-based hydrogels conducive for vascular differentiation, the hydrogel precursor macromonomer (dextran-acrylate) was copolymerized with Acr-PEG-RGD, a cell adhesive epitope. The hydrogels were formed via a photoinitiated polymerisation reaction and during this process microparticles loaded with VEGF<sub>165</sub> were physically immobilized in the network (Fig. 1A). These networks were then characterized regarding their swelling and elastic modulus (Fig. 1B). These parameters are of utmost importance for the ultimate application of hydrogels as hESC differentiating matrices since they affect solute diffusion and mechanical properties. The swelling ratio of dextran-based hydrogels containing Acr-PEG-RGD increases with the increase of incorporation of this cell adhesion epitope. This may be either due to the ionisation of some aminoacids (glycine and aspartate) of RGD peptide (the net charge at pH 7.4 is negative), which promotes water uptake in order to achieve an electrostatic equilibrium, or the formation of a loose network as a result of the incorporation of increasing concentrations of Acr-PEG-RGD. As a consequence of high swelling, dextran-based hydrogels containing

Acr-PEG-RGD show a lower elastic (Young's) modulus than networks without these sequences.

To evaluate the bioactivity of the cell-adhesive RGD peptide, hESC interaction studies with hydrogel surfaces were performed. Figure 1C shows light micrographs of hESC colonies attached on hydrogels containing 0.5 mM and 5.0 mM Acr-PEG-RGD but not on the unmodified hydrogels, after 24 h of being seeded. This indicates that the incorporation of RGD sequence into dextran hydrogels was necessary to promote hESC attachment and spreading on top of hydrogels.

### 3.2- Preparation and release kinetics for VEGF-containing microparticles

A VEGF release system was developed with the ultimate goal to be incorporated within the hydrogel network. Two protocols were used to make microparticles (7  $\mu\text{m}$  and 20  $\mu\text{m}$  in size) with highly varied release kinetics (Table 1). After 10 days of release in PBS buffer, small size microparticles kept their initial surface morphology while the surface of large microparticles underwent degradation (Fig. 2A). The release kinetics in both VEGF-loaded microparticles show an initial burst followed by a lower steady-state release (Fig. 2B). After 10 days,  $21 \pm 0.5\%$  and  $1 \pm 0.01\%$  of initially encapsulated VEGF has been released from microparticles 7 and 20  $\mu\text{m}$ , respectively (Table 1). The bioactivity of VEGF released microparticles 7  $\mu\text{m}$  was evaluated *in vitro* by assessing its survival effect on an endothelial cell line (EOMA cells, see Materials and Methods). Cells grown in wells containing VEGF showed a significant higher viability (as measured by the MTT viability assay) than cells grown in wells without this growth factor ( $100 \pm 7.7\%$  versus  $67.7 \pm 5.7\%$ , respectively,  $n=6$ ,  $P<0.0001$ ). VEGF released for short times (4h) was  $101.1 \pm 7.8\%$  ( $n=3$ ,  $P=0.8166$ ) active as compared to control VEGF.

### 3.3- Distribution and viability of hESC aggregates within bioactive three-dimensional hydrogel networks

Undifferentiated hESCs colonies were suspended in a dextran-acrylate solution along with a cytocompatible photoinitiator, in the presence or absence of adhesive peptides and microparticles. hESC colonies were used instead of single hESCs for this process since the viability of single cells encapsulated in dextran hydrogels (without cell adhesion peptides or microparticles) for 10 days is only 26% of that observed at day 1. Phase contrast images of these constructs show that hESC aggregates distributed across the network albeit preferentially located at one of the sides of the hydrogel, i.e. the farthest one from the UV light (Fig. 2C). This is due to the deposition of the hESC aggregates before the crosslinking reaction takes place. When these constructs are incubated in differentiation medium, cell aggregates at the surface proliferate and clumps of cells start to proliferate outside the network (Figs. 2C.3 and 2C.4). Regarding aggregate distribution and morphology there was not significant differences among the systems tested. When microparticles were encapsulated in the hydrogels, they were distributed across the network and in some cases incorporated in the hESC aggregates (Fig. 2C.3).

MTT assay was used to determine the metabolic activity of hESC aggregates encapsulated into dextran-based hydrogels. The incorporation of RGD or VEGF in the networks did not statistically increase cell viability at day 1 as compared to hydrogels without these bioactive molecules (data not shown). In addition, in all the conditions tested, no statistical increase ( $P > 0.05$ ) in cell proliferation was observed from day 1 to day 10 (Fig. 2D).

### 3.4- hESCs encapsulated in dextran-based hydrogels for 10 days express higher levels of KDR/Flk-1 than EBs

The expression of endothelial markers such as PECAM1, CD34, VE-cadherin and KDR/Flk-1 [20–22] as well as the expression of undifferentiating marker SSEA4 [23] was compared to

the expression observed in EBs and undifferentiating cells (Fig. 3A and Fig. 4). At day 10, the encapsulated aggregates express CD34 ( $P < 0.05$  or  $P < 0.001$ , Fig. 3A) at higher levels than in undifferentiated hESCs, while the expression of PECAM1 ( $P > 0.05$ ), KDR/Flk-1 ( $P > 0.05$ , Fig. 3A; see below) and SSEA4 was not statistically different (except for 0 mM RGD *versus* undifferentiated cells,  $P < 0.05$ ).

The expression of endothelial markers in encapsulated aggregates also differs from the pattern observed in EBs. In this regard, the expression of KDR/Flk-1 is statistically higher ( $P < 0.05$  or  $P < 0.001$ , Fig. 3A) on encapsulated cells than in EBs, while the other markers remain relatively constant.

Incorporation of RGD in dextran-based hydrogels either decreased (0.5 mM,  $P < 0.05$ ) or maintained (5 mM RGD) the expression of KDR/Flk-1 as compared to the cells encapsulated in the hydrogel without this epitope (Fig. 3A). Incorporation of VEGF-loaded microparticles within hydrogels increased the expression of KDR/Flk-1 in the hESC aggregates slightly but not statistically ( $P > 0.05$ ) compared to the cells encapsulated into hydrogels containing microparticles without VEGF. Furthermore, this expression is not dependent on the concentration of VEGF released.

Analysis by RT-PCR demonstrated that hESC aggregates within dextran-based hydrogels express other common markers of endothelial cells or endothelial progenitor cells including vWF [22], angiopoietin-2 (a soluble ligand expressed by endothelial cells [24]), Tie-2 (a receptor for angiopoietin-1 secreted by cells such as smooth muscle cells [25]) and AC133 [26] (Fig. 3B.1). Interestingly, the release of high levels of VEGF<sub>165</sub> within the hydrogel network seems to down-regulate the gene expression of vWF and Tie-2, respectively.

To demonstrate that VEGF contributes to the vascular differentiation of hESCs, we evaluated the effects of VEGF in separate experiments. To this end, EBs were grown in medium supplemented with 50 ng/mL of VEGF<sub>165</sub> for 10 days and then the expression of vascular markers assessed by FACS (Fig. 3C). The supplementation of differentiation medium with 50 ngmL<sup>-1</sup> of VEGF<sub>165</sub> increased the expression levels of PECAM1 ( $P = 0.0299$ ) and KDR/Flk-1 ( $P = 0.0098$ ) but not CD34 ( $P = 0.588$ ) markers as compared to the values found in EBs cultured in the absence of VEGF<sub>165</sub> (Fig. 3A).

**3.4.1- Expression and localization of vascular markers on encapsulated hESCs or EBs**—The expression and localization of endothelial markers on encapsulated hESCs or EBs was also determined at day 10 by immunofluorescence (Fig. 4). In both cases, CD34<sup>+</sup> and PECAM1<sup>+</sup> cells formed a primitive vascular network. Either in EBs or encapsulated hESC aggregates, VE-CAD<sup>+</sup> and KDR/Flk-1<sup>+</sup> cells did not form vascular networks showing that the expression of these markers did not co-localize entirely with the expression of CD34 or PECAM1. Encapsulated cells express higher levels of KDR/Flk-1 marker than EBs (Fig. 4) confirming the results obtained previously by FACS.

**3.4.2- The role of Hypoxia Inducible Factors (HIF) in the up-regulation of KDR/Flk-1**—Since the up-regulation of KDR/Flk-1 cellular marker may be related to hypoxia within the hydrogel networks [15,27], the expression of hypoxia inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and 2 $\alpha$  (HIF-2 $\alpha$ ) was assessed by RT-PCR and qPCR, respectively. According to Figure 3B.1, the expression of HIF-1 $\alpha$  was up regulated in encapsulated cells, but also on undifferentiated hESCs and EBs. On the other side, the expression of HIF-2 $\alpha$  was up-regulated ( $P < 0.001$ ) in cells encapsulated in hydrogels containing 0 mM RGD, 0.5 mM RGD or VEGF-containing 7  $\mu$ m microparticles, as compared to EBs (Fig. 3B.2).

### 3.5-The encapsulation of hESC aggregates in dextran-based hydrogels limited their differentiation into other germ layers

Next, we assess whether the encapsulation of hESCs in dextran-based hydrogels could limit their differentiation toward other germ layers (i.e., ectoderm and endoderm germ layers). Like EBs, encapsulated hESCs express at gene and protein levels nestin and neurofilament 68 Kd [28], markers related to neural (ectodermal differentiation) differentiation [29] (Figs. 5A–5C). However, according to immunohistochemistry analysis, cells encapsulated within hydrogel networks containing VEGF-loaded microparticles express lower levels of nestin than networks without microparticles or EBs, yet in both cases not statistically significant ( $P > 0.05$ ) (Fig. 5B). In addition, cells encapsulated in hydrogels express lower levels of hepatic markers including albumin and  $\alpha$ -fetoprotein (endodermal differentiation) [30] than EBs, as shown by immunohistochemistry and RT-PCR results (Figs. 5A–5C). According to immunohistochemistry results, the expression of  $\alpha$ -fetoprotein is significantly ( $P < 0.05$ ) lower on cells encapsulated within hydrogel networks containing VEGF-loaded microparticles but not on networks without microparticles (Fig. 5B).

### 3.6- hESCs removed from hydrogels and cultured in endothelial differentiation media express high levels of endothelial markers

Bioactive dextran-based hydrogels can be envisioned as a cell differentiation system to generate vascular cells. For that purpose, the cells were removed from the hydrogel network by mechanical dissociation at day 10 and cultured on gelatin-coated petri dishes containing EGM-2 medium supplemented with VEGF (50 ng/mL) for 6 more days (Fig. 6A). hESCs encapsulated in dextran-based hydrogels containing microparticles with or without VEGF<sub>165</sub> were selected for this experiment. The differentiation profile of these cells were compared to the one observed for undifferentiating hESCs cultured for 16 days in VEGF-enriched EGM-2 medium and EBs cultured in differentiation medium for 10 days and then cultured in VEGF-enriched EGM-2 medium for an additional 6 days as a cell monolayer (Fig. 6A). The expression of vascular and undifferentiating stem cell markers was assessed by FACS (Fig. 6B). When hESCs were removed from dextran hydrogels and cultured in EGM-2-supplemented with VEGF, they expressed higher levels of endothelial markers (PECAM1, CD34 and KDR/Flk-1) than in the other conditions tested. Our results also indicate that cells previously encapsulated in hydrogels containing VEGF-loaded microparticles (particularly for 7  $\mu$ m microparticles) express higher levels of KDR/Flk-1 marker as compared to cells that were encapsulated with blank microparticles, however in both conditions the expression of the other markers (CD34, PECAM1) was similar. Finally, no statistical difference was observed in the expression of the undifferentiating stem cell marker SSEA4 in all the conditions tested.

## 4- Discussion

hESC aggregates survived during the encapsulation process in dextran-based hydrogels and remained viable during at least 10 days. The incorporation of RGD epitopes in the networks did not increase cell viability. Nuttelman *et al* [31] have shown that the viability of human mesenchymal stem cells increased when RGD was incorporated in PEG hydrogels. However, in that study, single cells were used for the encapsulation studies while we used cell aggregates which enabled higher viability of hESCs as compared to single cells. During the encapsulation process, hESC aggregates distributed preferentially in one side of the networks. This is a consequence of the low photopolymerization rate, which favours the deposition of the aggregates over time. To obtain a homogeneous distribution of cell aggregates within the hydrogel, future work should explore either the use of a faster polymerisation reaction or the increase of hydrogel precursor viscosity that may retard the deposition process of the cells.



When these cell constructs were cultured in differentiation medium for 10 days they show a different differentiation profile than EBs regarding the expression of different germ layer markers. Particularly, the cells encapsulated into hydrogel networks containing VEGF-loaded microparticles (7  $\mu\text{m}$  condition) express relatively lower levels of neural (NF68Kd and nestin; ectodermal differentiation) and hepatic (albumin and  $\alpha$ -fetoprotein; endodermal differentiation) markers than EBs. Furthermore, hESC aggregates encapsulated into dextran-based hydrogels express higher levels of KDR/Flk-1 than EBs (Fig. 3A). The up-regulation of KDR/Flk-1 in encapsulated cells for 10 days may be regarded as indication of vascular differentiation. Although this marker is upregulated in the undifferentiated hESCs, a down-regulation is observed over time (data not shown) and only  $0.95 \pm 0.5\%$  cells expressed this marker in EBs at day 10 (Fig. 3A). Previously, this marker was used to identify vascular progenitor cells in mouse ESCs [14]. These progenitor cells gave rise to endothelial cells when exposed to medium containing VEGF [14]. Furthermore, this marker is expressed by endothelial cells [22]. The incorporation of 0.5 mM RGD but not 5 mM RGD in the hydrogel network reduced the expression of KDR/Flk-1 marker as compared to cells encapsulated in the hydrogel without this epitope. Incorporation of VEGF-loaded microparticles within hydrogels did not increase the expression of KDR/Flk-1 in the hESC aggregates in a statistically significant manner ( $P > 0.05$ ), for any concentration of VEGF released.

It is known that the expression of KDR/Flk-1 [15,27] as well as other VEGF receptors including VEGFR3 [32] increases in response to hypoxia. In addition, VEGF is significantly up regulated in response to hypoxia via activation of HIF factors, which bind to the hypoxia-response element in the VEGF promoter [15]. RT-PCR results show that HIF-1 $\alpha$  expression is up regulated in hESC aggregates encapsulated in dextran-based hydrogels; however, such high expression was extensive to undifferentiating hESCs and EBs. The expression of HIF-2 $\alpha$  was statistically higher in cells encapsulated in hydrogels containing 0 mM RGD, 0.5 mM RGD or VEGF-containing microparticles 7  $\mu\text{m}$  as compared to EBs (Fig. 3B.2), but not in the other conditions assessed. Therefore, our results suggest that hypoxia can not entirely explain the up-regulation of KDR/Flk-1 marker, and that other regulatory mechanisms may also be involved. It should be noted that the mechanisms of the up- and downregulation of KDR/Flk-1 expression in human embryonic stem cells and endothelial cells remains largely unknown [33]. Upregulation of KDR/Flk-1 was reported for endothelial cells cultured in collagen gels as compared to cells cultured in polystyrene dishes [33]; however the mechanism is still unknown. In addition long-term exposure to high glucose up regulates KDR/Flk-1 expression in endothelial cells [33]. Dextran is composed by glucose units that may activate this regulatory mechanism. These hypotheses should be explored in future work.

The removal of the encapsulated cells from hydrogels and culturing them in EGM-2 media supplemented with VEGF increased the expression of endothelial markers including CD34, PECAM1 while slightly decreasing the expression of KDR/Flk-1 marker (Fig. 6). The expression of all endothelial markers compares favourably to that observed in undifferentiating hESCs cultured for 16 days in VEGF-enriched EGM-2 medium and EBs cultured in differentiation medium for 10 days and then cultured in VEGF-enriched EGM-2 medium for additional 6 days as a cell monolayer. The differences found between the encapsulated cells and the other conditions tested may be ascribed to the high expression of KDR/Flk-1 in the encapsulated cells, which would make these cells respond to VEGF. This growth factor has been reported to be important in the development of primitive vasculature in embryonic stem cells [11]. Given that the expression of KDR/Flk-1 in undifferentiated stem cells is high (ca. 17%), it seems that the developmental differentiation stage of cells is important for VEGF to direct the differentiation of hESCs into the endothelial lineage.

## 5- Conclusions

We developed a new methodology for enhancing the vascular differentiation of human embryonic stem cells (hESCs) by encapsulation in a bioactive hydrogel. This system dramatically increased the fraction of cells expressing vascular endothelial growth factor (VEGF) receptor KDR/Flk-1, as compared to spontaneously differentiated embryoid bodies (EBs). Our results show that the incorporation of RGD or VEGF-containing microparticles in the hydrogel network did not contribute significantly for the up-regulation of this marker. When the cells were released from hydrogel network and cultured under conditions conducive for vascular differentiation, the number of vascular cells was higher than the number obtained from EBs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

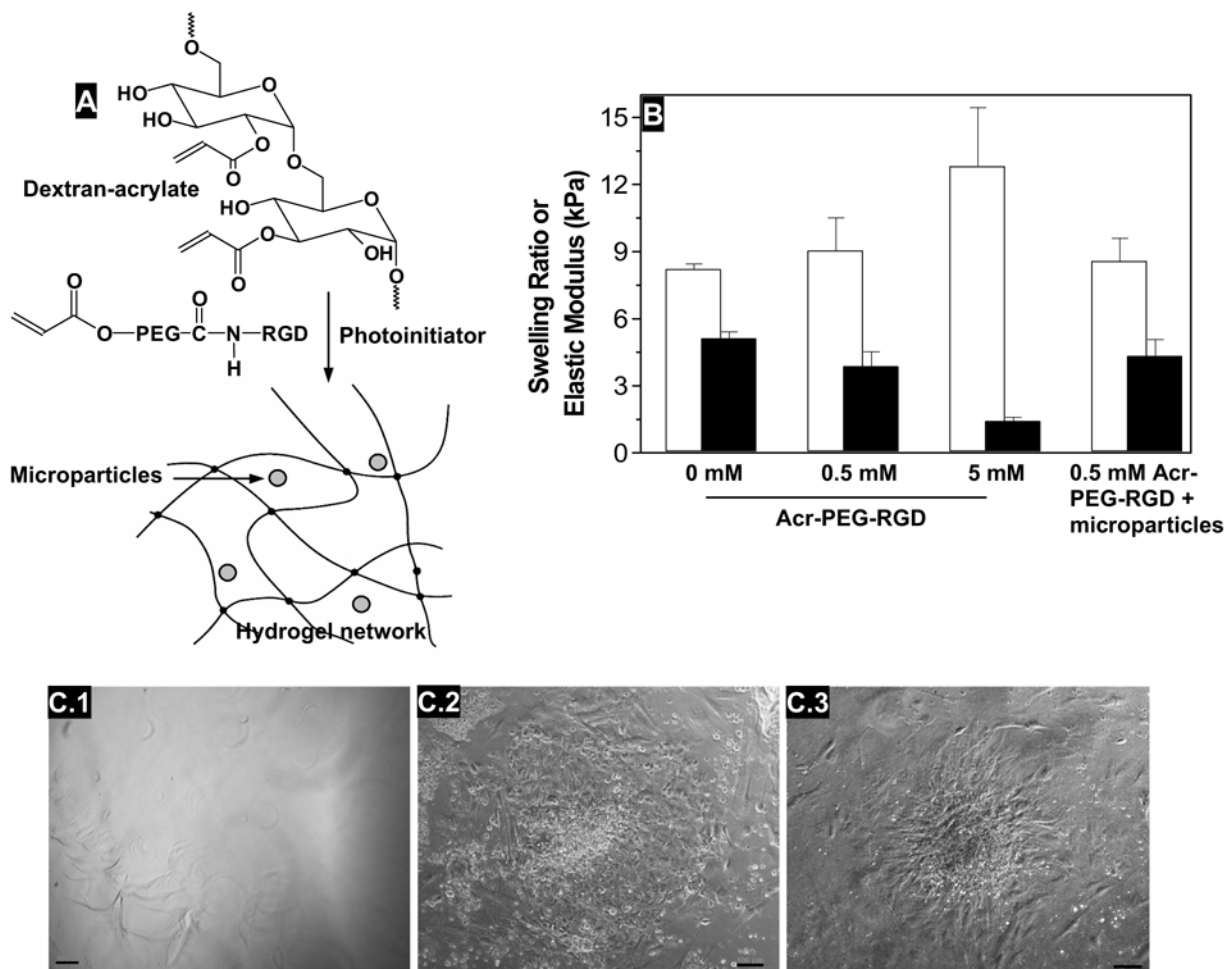
The authors would like to thank Dan Kohane for comments, Hyounghshin Park and Hannah Choe for help in qPCR analysis, and the financial support of Fundação para a Ciência e a Tecnologia (SFRH/BPD/14502/2003; fellowship to L.F), the Juvenile Diabetes Research Foundation (fellowship to S. G-N) and NIH (grants HL060435, DE13023 and HL076485).

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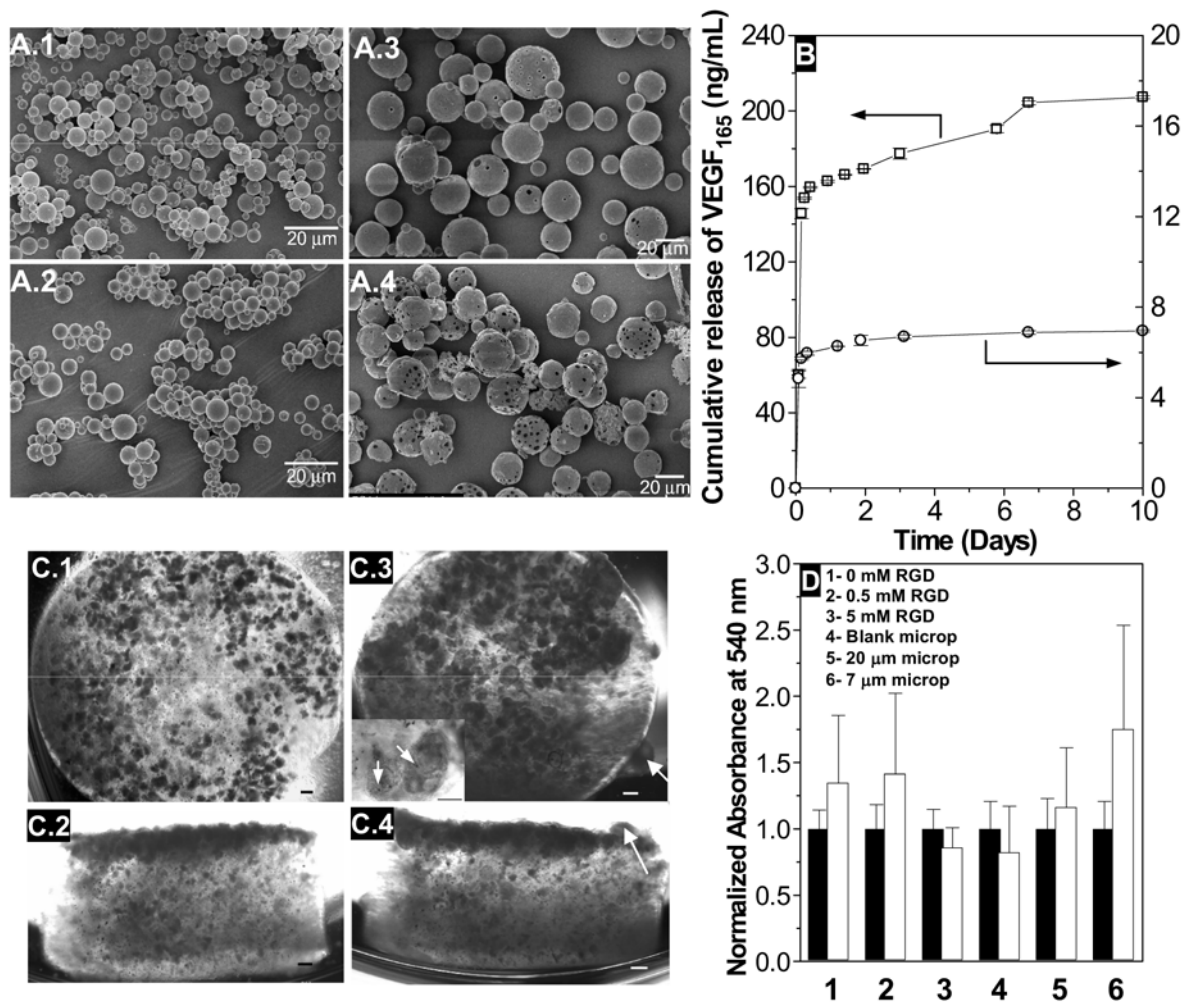
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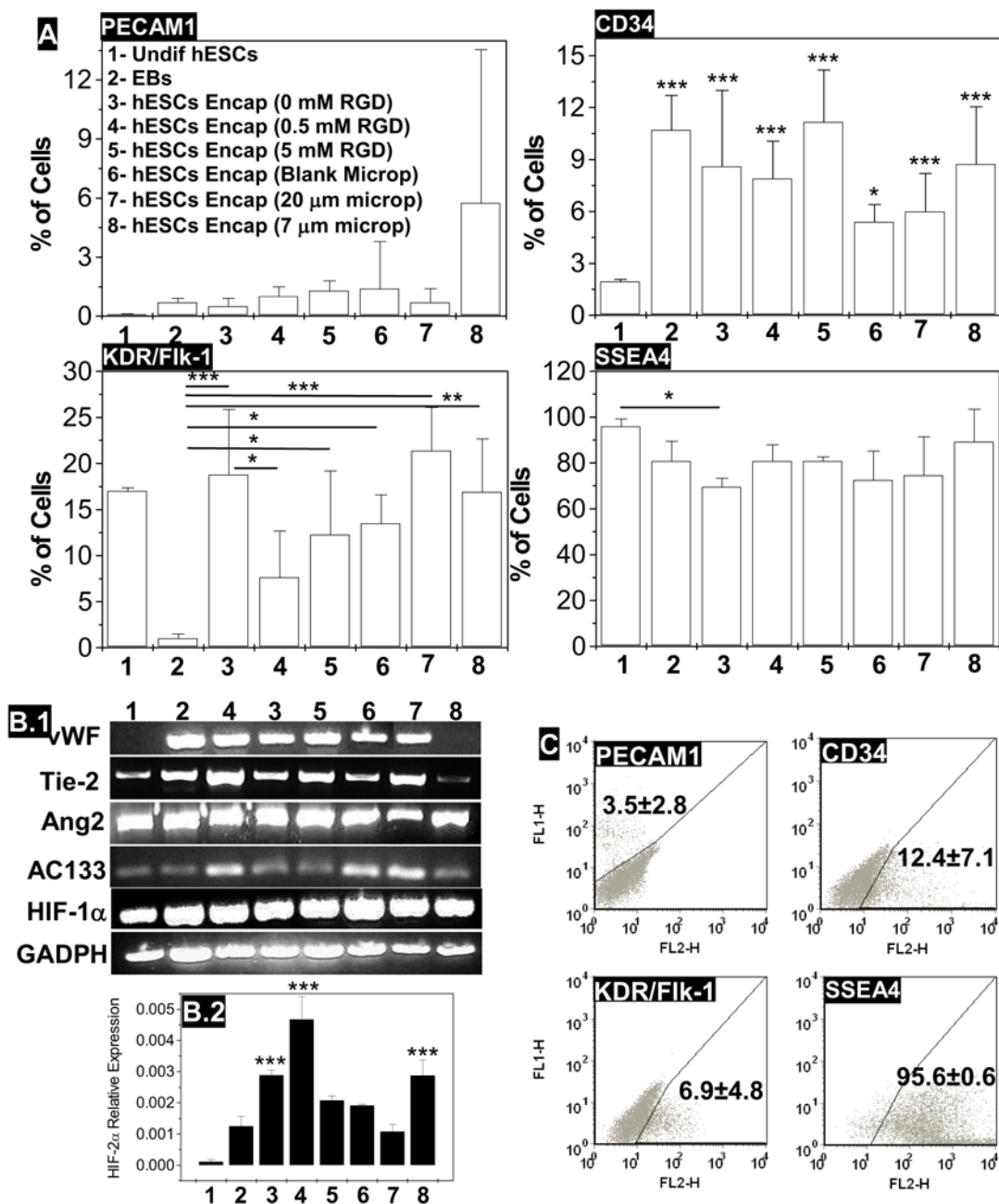
**Figure 1. Hydrogel properties and cell attachment in the surface of dextran-based hydrogels**

A) Schematic representation for the preparation of dextran-based hydrogels. B) Swelling (white columns) and elastic modulus (black columns) of hydrogels with different compositions (Average  $\pm$  S.D.,  $n=3$ ). 20  $\mu$ m microparticles were used in these assays. C) Representative light micrographs of attached hESCs after 24 h for dextran-based hydrogels with no Acr-PEG-RGD (C.1), 0.5 mM Acr-PEG-RGD (C.2), and 5 mM Acr-PEG-RGD (C.3). Bar corresponds to 100  $\mu$ m.



**Figure 2. Distribution and viability of hESC aggregates encapsulated within bioactive dextran-based hydrogels**

A) Scanning electron micrographs of 7  $\mu\text{m}$  (A.1, A.2) and 20  $\mu\text{m}$  (A.3, A.4) PLGA microparticles at day 0 (A.1, A.3) and 10 (A.2, A.4), during the release of VEGF<sub>165</sub>. B) Release profile of VEGF<sub>165</sub> from 7  $\mu\text{m}$  (□) and 20  $\mu\text{m}$  (○) microparticle formulations. C) Distribution of hESC aggregates on dextran-based hydrogels with 0.5 mM Acr-PEG-RGD and 5 mg/mL VEGF-loaded 20  $\mu\text{m}$  microparticles, at day 0 (C.1, C.2) and day 10 (C.3, C.4). Top (C.1, C.3) and side (C.2, C.4) views. Arrows in C.3 and C.4 indicate clump of cells proliferating outside the hydrogel. Inset in C.3 shows the incorporation of microparticles (arrow) within the hESC aggregates. Scale bar corresponds to 200  $\mu\text{m}$ . D) Mitochondrial metabolic activity (average  $\pm$  SD,  $n=6$ ) of hESC aggregates encapsulated in dextran-based hydrogels with no Acr-PEG-RGD (1), 0.5 mM Acr-PEG-RGD (2), 5 mM Acr-PEG-RGD (3), 0.5 mM Acr-PEG-RGD with 5 mg/mL of blank microparticles (4), and 0.5 mM Acr-PEG-RGD with 5 mg/mL of VEGF<sub>165</sub> loaded 20  $\mu\text{m}$  (5) or 7  $\mu\text{m}$  (6) microparticles. The absorbance at 540 nm was measured after 1 day (black bars) and 10 days (white bars). Both absorbances were normalized by day 1 absorbance.

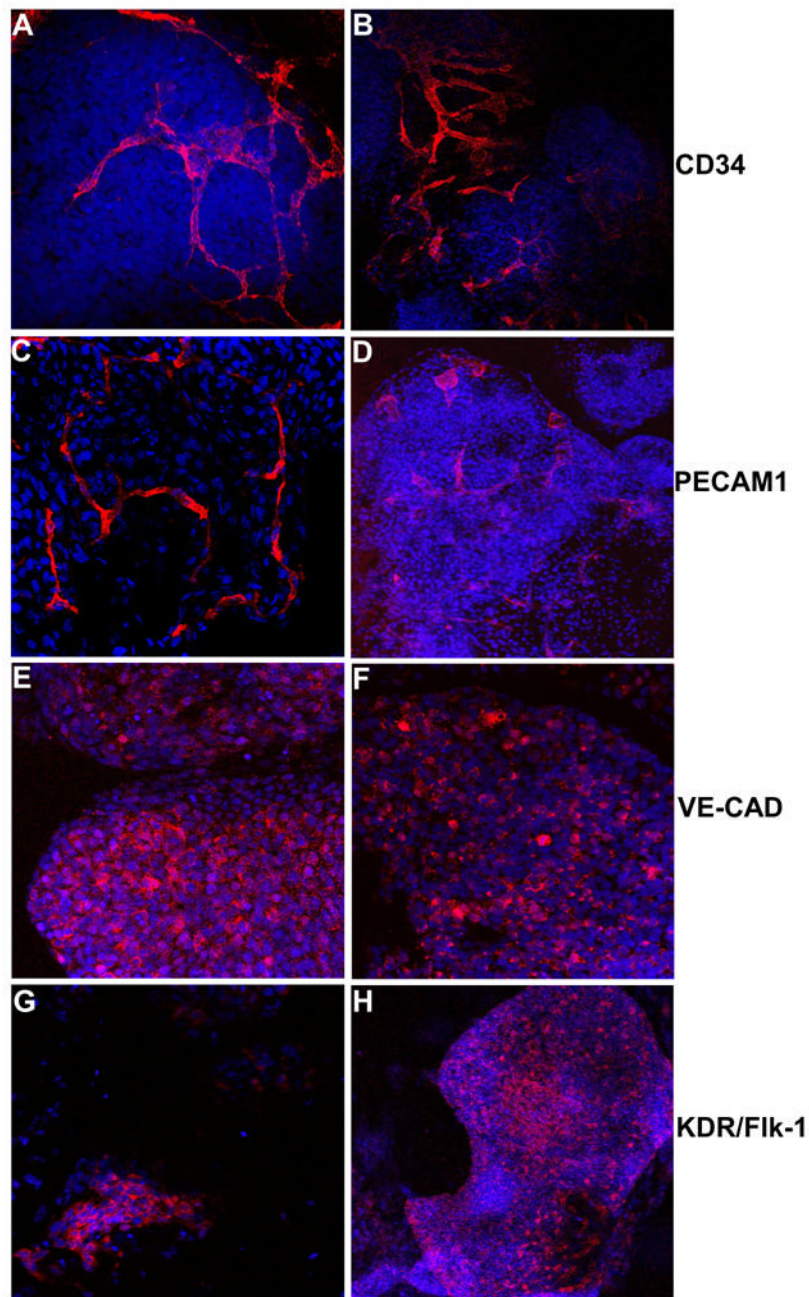


**Figure 3. Expression of endothelial and undifferentiating stem cell markers on hESC aggregates encapsulated in dextran-based hydrogels and on EBs**

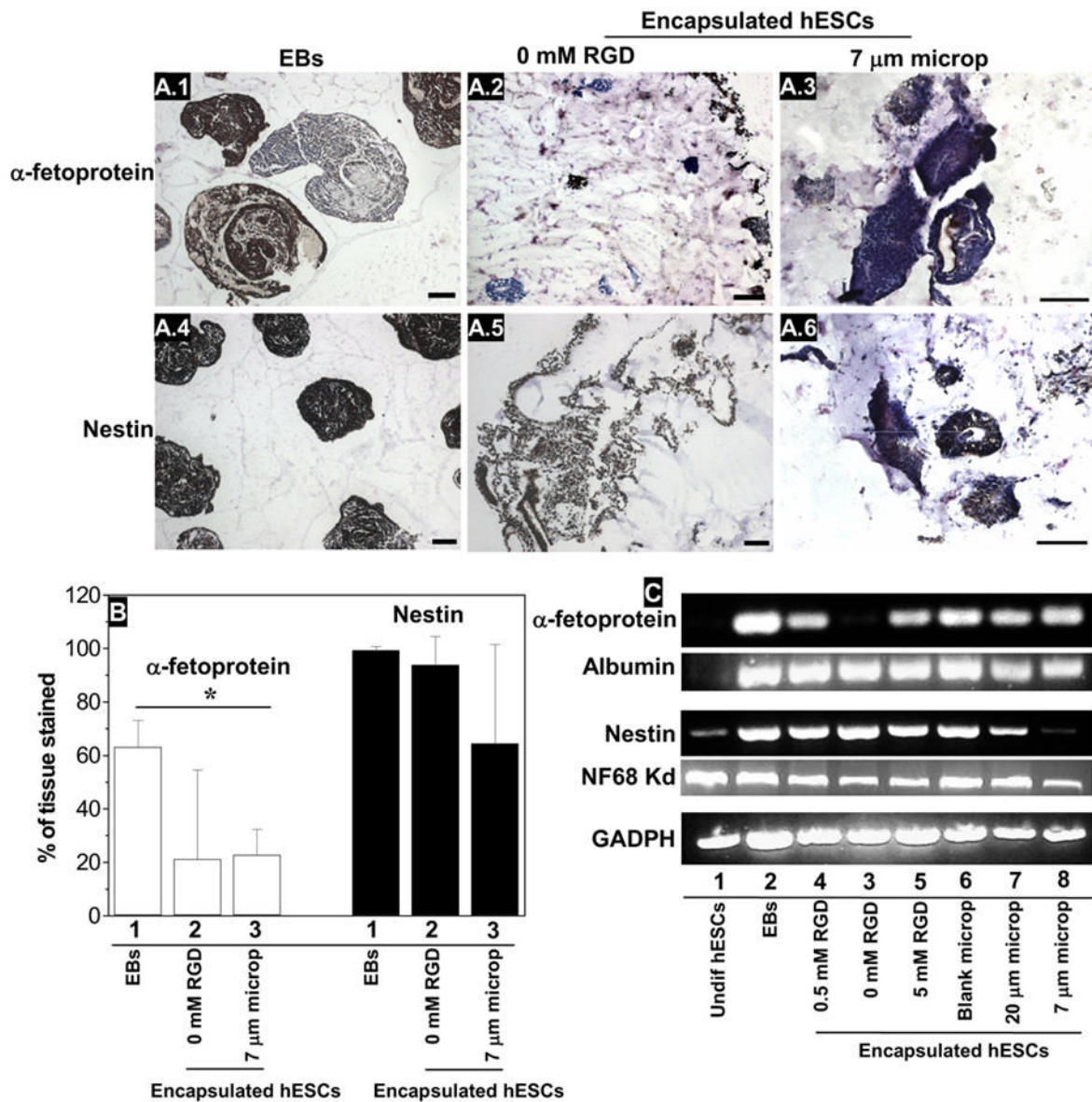
A,B) Summary of FACS (A), RT-PCR (B.1) and qPCR (B.2) analysis of undifferentiating cells (1), EBs at day 10 (2), hESC aggregates encapsulated into dextran-based hydrogels without (3) or containing 0.5 mM (4) or 5 mM Acr-PEG-RGD (5), hESC aggregates encapsulated into dextran-based hydrogels containing 0.5 mM Acr-PEG-RGD and 5 mg/mL of microparticles loaded with [20 μm (7) and 7 μm (8)] or without [20 μm (6)] VEGF<sub>165</sub>. Please note that samples 3 and 4 are reversed in all gels. C) FACS analysis of EBs grown in suspension for 10 days in differentiation medium containing 50 ngmL<sup>-1</sup> VEGF<sub>165</sub>. For all FACS and qPCR analysis, values indicate average ± S.D., from at least 3 independent experiments. \*, \*\* and \*\*\* denote statistical significance ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively). In the graph for the CD34

marker, statistical analyses were performed between all conditions and undifferentiating hESCs.



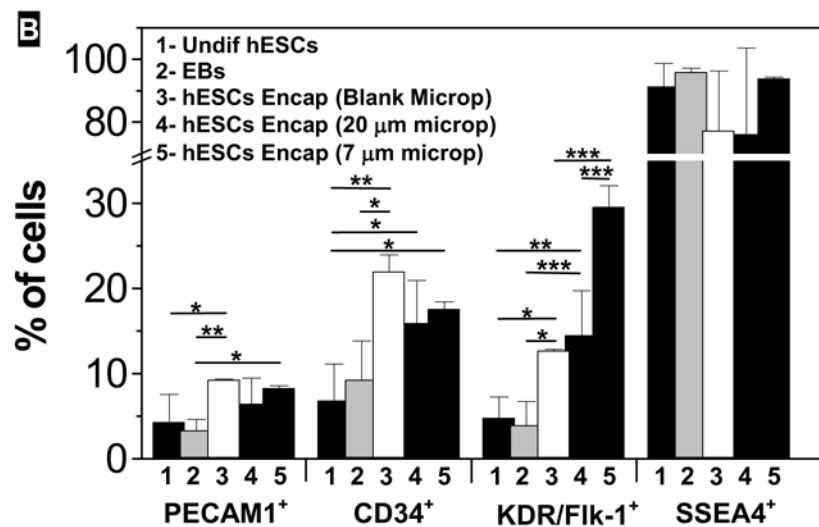
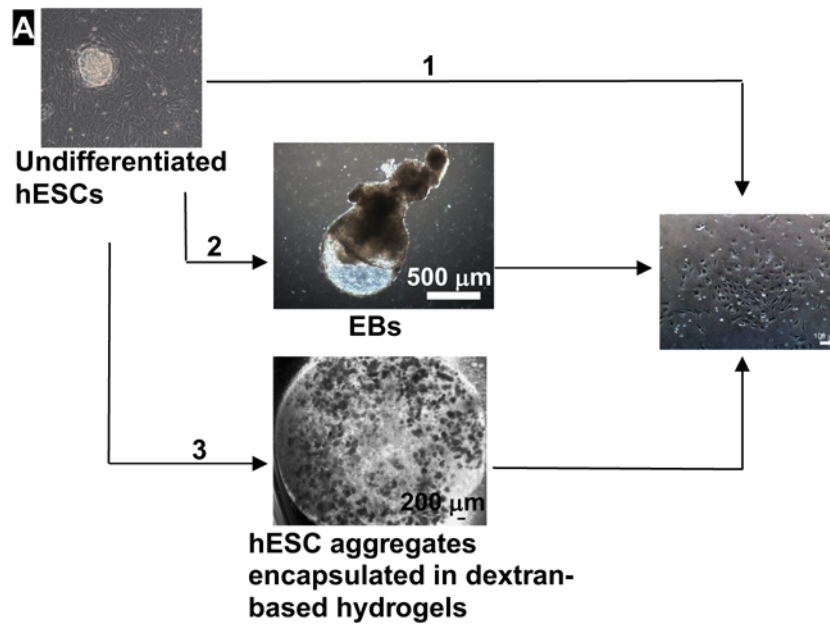


**Figure 4. Localization and organization of endothelial markers on EBs and on hESC aggregates encapsulated in dextran-based hydrogels**  
 Confocal images of CD34<sup>+</sup> (A,B), PECAM1<sup>+</sup> (C,D), VE-CAD<sup>+</sup> (E,F) and KDR/Flk-1<sup>+</sup> (G,H) cells from EBs (A,C,E,G) and from hESC aggregates encapsulated in dextran-based hydrogels with 0.5 mM Acr-PEG-RGD [B ( $\times 10$ ); D ( $\times 10$ )], with 5.0 mM Acr-PEG-RGD [F ( $\times 25$ )] and with 0.5 mM Acr-PEG-RGD containing 5 mgmL<sup>-1</sup> of 7  $\mu$ m microparticles loaded with VEGF<sub>165</sub> [H ( $\times 10$ )].



**Figure 5. Ectodermal and endodermal differentiation of hESC aggregates encapsulated in dextran-based hydrogels**

A, B) Expression of  $\alpha$ -fetoprotein (1,2,3) and nestin (4,5,6) as revealed by immunohistochemistry, in EBs (1,4) and hESC aggregates encapsulated in dextran-based hydrogels (2,5) or in networks containing 0.5 mM Acr-PEG-RGD and 5 mgmL<sup>-1</sup> 7  $\mu$ m microparticles (3,6). Bar corresponds to 100  $\mu$ m. For the quantitative analysis of antibody staining (B), the results are average  $\pm$  S.D. from two different samples and the counts were obtained in at least two different sections per sample. C) RT-PCR analysis of endoderm (albumin and  $\alpha$ -fetoprotein) and ectoderm markers (nestin, neurofilament 68 Kd) in undifferentiated cells (1), EBs at day 10 (2), hESC aggregates encapsulated in dextran-based hydrogels with no Acr-PEG-RGD (3), 0.5 mM Acr-PEG-RGD (4), 5 mM Acr-PEG-RGD (5), 0.5 mM Acr-PEG-RGD with 5 mg/mL of 20  $\mu$ m blank microparticles (6), and 0.5 mM Acr-PEG-RGD with 5 mg/mL of 20 (7) or 7  $\mu$ m (8) microparticles loaded with VEGF<sub>165</sub>.



**Figure 6. Expression of endothelial and undifferentiating stem cell markers in cells removed from hydrogel networks and further cultured in EGM-2 medium supplemented with VEGF<sub>165</sub>**

A) Scheme showing the protocols adopted to differentiate hESCs into the vascular lineage. (1) Undifferentiated hESCs cultured in EGM-2 medium supplemented with 50 ngmL<sup>-1</sup> VEGF<sub>165</sub> for 16 days. (2) Undifferentiated hESCs were induced to form EBs (see Materials and Methods), and the EBs grown in differentiation medium for 10 days. Then, single cells isolated from EBs were cultured in EGM-2 medium supplemented with 50 ngmL<sup>-1</sup> VEGF<sub>165</sub> for 6 additional days. (3). Undifferentiated hESCs were encapsulated in dextran-based hydrogels containing 0.5 mM Acr-PEG-RGD and 5 mgmL<sup>-1</sup> of microparticles with [20 μm (4) and 7 μm (5)] or without VEGF<sub>165</sub> [20 μm (3)], and cultured in differentiation medium for 10 days. Subsequently, single cells isolated from the encapsulated aggregates were cultured in EGM-2 medium supplemented with 50 ngmL<sup>-1</sup> VEGF<sub>165</sub> for 6 additional days. B) Summary of FACS analysis for endothelial and undifferentiating stem cells markers. Cells were differentiated according to protocol 1 (dashed columns), protocol 2 (grey columns) and

protocol 3 (white (3), black (4) and dashed (5) columns). Values indicate average  $\pm$  S.D. from two or three independent experiments (3 replicates per run). \*, \*\* and \*\*\* denote statistical significance ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively).

**Table 1**  
Characteristics of VEGF-loaded PLGA microparticles.

Microparticle characteristics	VEGF –loaded PLGA microparticles	
	7 $\mu$ m	20 $\mu$ m
Polymer inherent viscosity (dl/g) <sup>a</sup>	0.16–0.24	0.32–0.44
Encapsulated molecules <sup>b</sup>	VEGF-BSA-MgOH <sub>2</sub>	VEGF-BSA-MgOH <sub>2</sub> -sucrose
VEGF loading (ng) per mg of microparticles <sup>c</sup>	50	50
Experimental VEGF loading (ng) per mg of microparticles <sup>d</sup>	50	39.5
VEGF released after 10 days (ng) <sup>e</sup>	10.5	0.37

<sup>a</sup>Information obtained by the manufacturer.

<sup>b</sup>2.5 mg of BSA, 4 mg of MgOH<sub>2</sub> and 4 mg of sucrose were used per 100 mg of polymer.

<sup>c</sup>Theoretical mass of VEGF dispersed through 1 mg of microparticles.

<sup>d</sup>Mass of VEGF dispersed through 1 mg of microparticles assessed experimentally.

<sup>e</sup>Mass of VEGF released from 1 mg of microparticles placed in buffered saline for 10 days.