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Glycosaminoglycan mimetic improves enrichment and cell functions of human endothelial progenitor cell colonies



Fabien Chevalier^{a,b}, Mélanie Lavergne^{c,1}, Elisa Negroni^{b,1},
Ségolène Ferratge^b, Gilles Carpentier^a, Marie Gilbert-Sirieix^d,
Fernando Siñeriz^d, Georges Uzan^b, Patricia Albanese^{a,*}

^a EAC CNRS 7149, Laboratoire CRRET, Université Paris Est Créteil, Faculté des Sciences et Technologies, 61 Avenue du Général de Gaulle, 94010 Créteil Cedex, France

^b INSERM U972, Hôpital Paul Brousse, Villejuif, France

^c ABCell-Bio, 6 rue Pierre Haret, 75009 Paris, France

^d OTR3 company, 4 rue Française, 75001 Paris, France

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Abstract Human circulating endothelial progenitor cells isolated from peripheral blood generate in culture cells with features of endothelial cells named late-outgrowth endothelial colony-forming cells (ECFC). In adult blood, ECFC display a constant quantitative and qualitative decline during life span. Even after expansion, it is difficult to reach the cell dose required for cell therapy of vascular diseases, thus limiting the clinical use of these cells. Glycosaminoglycans (GAG) are components from the extracellular matrix (ECM) that are able to interact and potentiate heparin binding growth factor (HBGF) activities. According to these relevant biological properties of GAG, we designed a GAG mimetic having the capacity to increase the yield of ECFC production from blood and to improve functionality of their endothelial outgrowth. We demonstrate that the addition of [OTR₄₁₃₁] mimetic during the isolation process of ECFC from Cord Blood induces a 3 fold increase in the number of colonies. Moreover, addition of [OTR₄₁₃₁] to cell culture media improves adhesion, proliferation, migration and self-renewal of ECFC. We provide evidence showing that GAG mimetics may have great interest for cell therapy applied to vascular regeneration therapy and represent an alternative to exogenous growth factor treatments to optimize potential therapeutic properties of ECFC.

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Abbreviations: GAG, glycosaminoglycan; HBGF, heparin binding growth factor; HS, heparan sulfate; CS, chondroitin sulfate.

* Corresponding author at: Université Paris Est Créteil, Faculté des Sciences et Technologie, CRRET Laboratory, 61 avenue du Général de Gaulle, 94010 Créteil Cedex, France.

E-mail addresses: fabien.chevalier@gmail.com (F. Chevalier), melanie.lavergne@abcell-bio.com (M. Lavergne), elisa.negroni@upmc.fr (E. Negroni), segolene.ferratge@inserm.fr (S. Ferratge), carpentier@u-pec.fr (G. Carpentier), marie.gilbert@otr3.com (M. Gilbert-Sirieix), fernando.sineriz@otr3.com (F. Siñeriz), georges.uzan@inserm.fr (G. Uzan), albanese@u-pec.fr (P. Albanese).

¹ Equal contribution.

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Introduction

Vascular diseases are the leading causes of death in the world (Libby et al., 2011; Murray and Lopez, 1997). Current therapies including life style management, pharmacological control of risk factors and surgical revascularization do not completely reverse the pathology. Since the initial discovery in 1997 of endothelial progenitor cells (EPC) by Asahara et al. (1997), these cells are considered as a promising therapeutic tool for vascular repair. Culture of blood mononuclear cells reveals two distinct populations of EPC that have been classified according to the time at which they appear in culture: early endothelial cell colony-forming units (CFU-EC) and late-outgrowth endothelial colony-forming cells (ECFC), both expressing specific endothelial markers and with capabilities to stimulate angiogenesis (Yoder et al., 2007). On the one hand, CFU-EC differentiate into phagocytic macrophages and fail to form vessels *in vivo*. On the other hand, ECFC display high proliferative potential when compared to mature endothelial cells (Bompais et al., 2004), form vessels *in vivo* and are incorporated into pre-existing vascular networks (Yoder et al., 2007; Au et al., 2008; Sieveking et al., 2008). Moreover, ECFC are more sensitive to angiogenic factors, such as VEGF and FGF-2, which confer to these cells greater protection against cell death. Their functional features indicate that although these cells have undergone some differentiation steps, they still have the properties of immature cells, suggesting greater tissue repair capabilities (Bompais et al., 2004). However, the use of autologous ECFC in cell therapy is limited by their rarity in adult peripheral blood (PB) as they constitute less than 0.01% of circulating mononuclear cells (Khan et al., 2005; Ingram et al., 2004). Cord blood (CB) contains more ECFC than PB, and following a crucial step of adhesion *in vitro*, it forms primary colonies of well-circumscribed monolayers of cobblestone appearing cells. After expansion, ECFC can give rise to secondary colonies from a single cell (Yoder et al., 2007).

For clinical application, it is important to reach the therapeutic cell dose with a limited number of cell division and limited culture time. This will limit occurrence of genetic abnormalities that may occur during long term cultures as observed for the use of mesenchymal stem cells (MSC) (Tarte et al., 2010). To improve production of ECFC, two parameters are very important: 1) the initial number of colony obtained and 2) the proliferation rate of endothelial outgrowth obtained from these colonies. In this study we propose a new strategy for improving these two parameters, based on the use of glycosaminoglycan (GAG) type compounds in culture media to potentiate endothelial cell properties.

Sulfated GAG, such as heparan sulfate (HS) or chondroitin sulfate (CS), are polysaccharidic molecules associated to a core protein to form proteoglycans, which interact with proteins to strengthen the structure and stability of the extracellular matrix (ECM). GAG also have functional roles since they regulate numerous biological processes through their abilities to interact with heparin-binding growth factors (HBGF) (Gandhi and Mancera, 2008). The structure of GAG is highly complex due to heterogeneity in glycosidic linkage, sulfation pattern and hydrophobic regions regulating their interactions with surrounding molecules. It is now accepted that particular structural characteristics of GAG are involved in their ability to modulate the activities of HBGF which include growth factors,

chemokines, cytokines and enzymes (Gallagher, 2006). GAG thus provide matrix-bound or cell surface-bound reservoirs for HBGF, protecting them from proteolytic degradation and increasing their half-life, providing storage and protection sites (Handel et al., 2005; Friedl et al., 1997). Moreover these structural features evolve during physiological and pathological processes and are involved in tissue homeostasis (Huynh et al., 2012a; Huynh et al., 2012b). According to such interest, a family of structural and functional analogs of natural GAG has been developed allowing availability of well-characterized GAG mimetics (Papy-Garcia et al., 2005). These compounds, also named RGTA (for ReGeneraTing Agents), greatly increase the rate and quality of wound healing in various preclinical models (for review, Barritault and Caruelle, 2006). It was demonstrated that a RGTA improves *in vitro* endothelialization of vascular prosthesis (Desgranges et al., 2001) and partly prevents *in vivo* skeletal muscle damage caused by ischemia (Desgranges et al., 1999). This regenerative effect could be explained by RGTA resistance to glycanase degradation which improves HBGF protection and function (Meddahi et al., 2002). It was indeed demonstrated that RGTA binds VEGF, potentiates its binding to specific receptors, improves its biological activity on HUVEC and modulates *in vivo* angiogenesis (Rouet et al., 2005). Finally, recent data support the interest of these GAG mimetics according to their effects on the mobilization and the potentiation of progenitors and stem cells from hematopoietic and mesenchymal lineages (Albanese et al., 2009; Frescaline et al., 2011; Frescaline et al., 2013).

According to the relevance of GAG functions we investigate here whether a selected GAG mimetic [OTR₄₁₃₁] would be able to potentiate *in vitro* different properties of human ECFC from CB, such as adhesion, growth, migration, primary and secondary colony formation. This should enable optimization of the expansion capacity of these cells for further therapeutic applications.

Materials and methods

Materials

The GAG mimetic [OTR₄₁₃₁] was obtained from OTR3 Inc. (Paris, France). This sulfated dextran derivative, composed of about 250 glucosidic units, presents degrees of substitution of carboxylates, and sulfate residues close to heparin, but differing from this one by a lower acetyl group ratio with a homogeneous repartition (Papy-Garcia et al., 2005). Heparin used as natural GAG control was cell culture grade (Sigma-Aldrich, France, H9399 lot 125K1400). Recombinant human FGF-2 and human VEGF were purchased from Promokine (PromoCell, GmbH, Heidelberg, Germany).

Human endothelial progenitor cell isolation and culture

Human umbilical CB samples were collected in a citrate phosphate dextrose solution from healthy full-term newborns after maternal informed consent according to approved institutional guidelines (Assistance Publique Hôpitaux de Paris, Paris, France). ECFC were isolated from Cord Blood MonoNuclear Cells (CBMNC) as previously described (Vanneaux et al., 2010). To assess an effect of [OTR₄₁₃₁] on

appearance of ECFC colonies, CBMNC resuspended in EGM2-MV complete media (Lonza, Ermerainville, France) were divided into 2 equal volumes, in which either phosphate buffered saline (PBS) or 100 ng/mL of [OTR₄₁₃₁] (final concentration) was added. Cell suspensions were both gently mixed and left for 30 min at room temperature (RT) before seeding. First media change, following a washing with PBS, was performed after 24 h to eliminate nonadherent cells and daily for 7 days. Thereafter, media was then changed every 2 days. ECFC appeared between days 7 and 25 and were visually identified as well-circumscribed monolayers of cobblestone appearing cells.

Adhesion assay

ECFC at sub-confluence were trypsinized and incubated in EGM2-MV complete media supplemented with or without 100 ng/mL of heparin or [OTR₄₁₃₁] for 15 min at RT. Cells were seeded onto non-coated plates for 2 h at 37 °C and the amount of attached viable cells was evaluated by a MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt)-based colorimetric method (Cory et al., 1991). Briefly, after a washing step to remove unattached cells, adherent cells were incubated for 2 h and 30 min at 37 °C with 20% (v:v) MTS reagent (Promega, Charbonnières, France) in a fresh EBM2 basal medium (without FBS and growth factors). The amount of soluble formazan product converted from MTS by dehydrogenase enzymes is directly proportional to the number of metabolically active cells. The absorbance of the obtained formazan dye is measured at 492 nm using a spectrophotometric TECAN Infinite M1000 plate reader (Tecan, Durham, NC).

Growth factor dependent mitogenic activity assay

ECFC were seeded into 48 well plates at a density of 10,000 cells/cm² and left for 5 h in complete EGM2-MV medium to allow cell adhesion. To investigate the effect of GAG on growth factor-induced proliferation, medium was changed by an EBM2 basal medium supplemented with 0.5% FBS and SingleQuots of growth factors devoid of FGF-2 and VEGF (EGM2 modified medium–0.5% FBS). After overnight incubation, medium was replaced by the same medium supplemented with FGF-2 or VEGF. GAG, [OTR₄₁₃₁] or heparin, was added to wells from 0.1 to 100 µg/mL (final concentration). Cells were incubated for 5 days, with a renewal of medium, GAG and growth factors at day 3. The amount of viable cells was evaluated by a MTS-based colorimetric method as previously described.

Chemotactic migration assay

ECFC migration in response to VEGF gradient was assessed in a 24-well modified Boyden chamber, on polycarbonate membrane filter with 8 µm pore diameter (BD Biosciences) coated with 50 µg/mL rat tail collagen type I (BD Biosciences). After an overnight starvation in EBM2 basal medium supplemented with 0.2% FBS, ECFC were loaded in starving medium into the upper part of the microchamber at 200,000 cells/well, with the same medium containing or not 2 ng/mL

of VEGF with or without 1 µg/mL of [OTR₄₁₃₁] or heparin on the lower chambers. After 12 h of incubation cells on the upper surface of the membrane filter were removed by wiping with a cotton swab. Then, all membranes were MGG-stained, mounted and photographed through an Aristoplan Leitz microscope coupled to a CoolSnap color CCD camera (Photometrics). The number of cells per field was automatically counted using a previously described procedure (Destouches et al., 2012) programmed with the macro language of the ImageJ software (Rasband, 1997–2013). Briefly, the red component of RGB images was extracted and segmented using the so called "Otsu" method, and then subsegmented with an 8-bit watershed method. Object count was then automatically performed to get the cell number. Results are reported as the mean of 18 areas from three pictures by filter, in duplicate during 3 independent experiments.

Clonogenicity frequency

Serial dilutions of ECFC were performed to obtain 32, 16, 8, 4, 2, 1 and 0.5 cells per well – with 12 to 36 replicate wells per cell dilution. Cells were maintained in EGM2-MV complete medium supplemented or not with 1 µg/mL of [OTR₄₁₃₁] or heparin (final concentration). Each medium was renewed every 3 days. Individual wells were scored for the presence of colony after 15 days of culture. The percentage of negative wells as a function of the number of cells/well overlaid was used to estimate the frequency of the clonogenicity, as the 37% log intercept according to Poisson statistics.

Flow cytometry

Fluorescence activated flow cytometry was performed with BD Accuri C6 flow cytometer (BD) and a minimum of 10,000 events were analyzed for each sample. The cells were harvested, washed in BSA 0.5% in PBS and incubated for 45 min at 4 °C, with primary antibodies to analyze the expression of endothelial cell surface marker proteins: FITC-conjugated mouse anti-human CD31 (PECAM-1) IgG1 (BD), PE-conjugated mouse anti-human CD144 IgG1 (Beckman Coulter, France) and Alexa Fluor 647-conjugated mouse anti-human CD309 (VEGFR2) IgG1 (BD). All the antibodies were used in the concentrations suggested by the suppliers. Living cells were discriminated by adding the 7AAD dye (BD). Background fluorescence was obtained from the negative control cells stained with FITC-conjugated mouse IgG1, PE-conjugated mouse IgG1 and APC-conjugated mouse IgG1 antibodies (Beckman Coulter) and subtracted from the mean channel fluorescence of the specific staining patterns. The expression of each antigen was expressed as the mean channel fluorescence.

Endothelial function assays

The ability to express adhesion molecules ICAM-1 and VCAM-1 in inflammatory conditions and the ability to uptake acetylated LDL are two main endothelial functional assays. In order to explore these functional capacities, ECFC were cultured with either 10 ng/mL of TNF α (R&D Systems, France) for 18 h or with 15 µg/mL of Ac-LDL Alexa Fluor 488 (Life technologies, Cergy Pontoise, France) for 4 h at 37 °C. Expression of ICAM-1 and

VCAM-1 (Biolegend, France) was analyzed by flow cytometry and internalization of Ac-LDL was visualized using a fluorescent microscope, as previously described (Lavergne et al., 2012). The number of Ac-LDL positive cell was measured by manual counting under a fluorescent microscope.

Immunocytochemistry

ECFC were fixed in 4% paraformaldehyde for 10 min and washed in PBS–BSA 0.5%. Saturation and permeabilization were performed by incubating fixed cells in PBS–FCS 2%–Triton 0.1% solution for 1 h at RT. After washing steps, staining of specific endothelial cell markers was performed using mouse anti-human CD31 (BD) and rabbit anti-human von Willebrand Factor (vWF; Dako, Trappes, France) primary antibodies followed by Alexa Fluor 488-conjugated goat anti-mouse and Alexa Fluor 647-conjugated goat anti-rabbit secondary antibody (Life Technologies) staining. Negative control samples included cells treated with secondary antibodies only. All the antibodies were used in the concentrations suggested by the suppliers. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Images were visualized using an Olympus BX60 microscope (Olympus Optical, Hamburg, Germany), digitalized using a CCD camera (Photometrics CoolSNAP fx; Roper Scientific, Tucson, AZ) and analyzed using MetaView image analysis system (Universal Imaging, Downingtown, PA).

Endothelial tube formation in Matrigel™ *in vitro*

Ability of ECFC to organize into capillary-like structures in the presence of basement membrane matrix was analyzed using Matrigel™ (BD, France) as previously described (Lavergne et al., 2012). To assess the pro-angiogenic effect of GAG, ECFC were cultured on Matrigel™ with complete EGM2-MV medium supplemented or not with 1 µg/mL of [OTR₄₁₃₁] or heparin (final concentration). Four pictures per well were taken using a camera associated to an inverted microscope with a 4× objective in phase contrast mode. Images were analyzed using an original home-made plugin (Carpentier et al., 2012), developed for the ImageJ software (Carpentier, 2012). Briefly, phase contrast images were segmented and skeletonized. The trees were then analyzed by detection of junctions and extremities, defining the segments and branches of the cellular meshed network organization.

In vivo Matrigel™ plug assay

Eight week old female NOD/SCID mice (Janvier, CERJ, Laval, France) were injected subcutaneously into inner flank region with 0.5 mL of Matrigel™ containing both 750,000 ECFC and 250,000 human bone marrow mesenchymal stem cells (MSC, ABCell-Bio, Paris, France) with or without [OTR₄₁₃₁] (1 or 10 µg/mL final concentration). The injected Matrigel™ rapidly formed a single solid gel plug. After 7 days, the mouse skins were pulled back to expose the plugs which were then dissected. The intact plugs were frozen in isopentane precooled in liquid nitrogen. Sections of 8 µm thickness were cut using a cryostat, fixed with a 4% paraformaldehyde solution for 10 min at RT, and stained with specific antibodies to detect human ECFC into the plugs. Cell

permeabilization was performed by incubating fixed sections in PBS–Triton 0.1% solution for 10 min at RT, and saturation in PBS–goat serum 5% solution for 1 h at RT. After washing steps, staining of specific endothelial cell markers was performed using mouse anti-human CD31 (BD) and rabbit anti-human von Willebrand Factor (Dako) primary antibodies followed by Alexa Fluor 488-conjugated goat anti-mouse and Alexa Fluor 647-conjugated goat anti-rabbit secondary antibody (Life Technologies) staining. Nuclei were counterstained with DAPI. Images were visualized using an Olympus BX60 microscope (Olympus Optical, Hamburg, Germany), digitalized using a CCD camera (Photometrics CoolSNAP fx; Roper Scientific, Tucson, AZ) and analyzed using MetaView image analysis system (Universal Imaging, Downingtown, PA). This experiment was repeated twice, and included 3 groups of 4 mice: ECFC + MSC without GAG, ECFC + MSC + GAG at a concentration of 1 µg/mL and ECFC + MSC + GAG at a concentration of 10 µg/mL.

Statistical analysis

Each experiment was performed at least three times and each sample was tested in triplicate. Data are expressed as mean ± SEM. Statistically significant difference was determined with one way ANOVA analysis followed by the Dunnett post-test when there were more than two groups. For analysis between two groups Student's-t test was used.

Results

[OTR₄₁₃₁] improves colony forming efficiency of human ECFC

The colony forming efficiency (CFE) of ECFC is a very critical step in production of therapeutic endothelial cells, as it will determine the first pool of endothelial outgrowth from which further expansion steps will proceed. The capacity of the [OTR₄₁₃₁] mimetic to improve this step was evaluated on ECFC from human CBMNC (Fig. 1). A single 24 h exposure of cells to a dose of 100 ng/mL of [OTR₄₁₃₁] added in EGM2-MV medium at day 0 is associated to a significant 1.8-fold increase of the CFE as compared to PBS-treated cells (Fig. 1A). A continuous exposure to 100 ng/mL of [OTR₄₁₃₁] during the first 7 days of culture, corresponding to the daily renewing medium needed to obtain ECFC, significantly increases by 2.7-fold the CFE compared to the PBS-treated group (Fig. 1B), with larger colony size corresponding to an increased number of cells per colony (data not shown). Same results were obtained with 1 µg/mL dose of [OTR₄₁₃₁] (data not shown). These data clearly indicate that [OTR₄₁₃₁] is able to increase the number of ECFC obtained from a fixed cord blood (CB) volume. Moreover this could be due to [OTR₄₁₃₁] effects not only on early steps such as adhesion of ECFC but also on later steps such as self-renewing and/or proliferation of ECFC.

Phenotype and functionality of ECFC isolated with [OTR₄₁₃₁]

Characterization of ECFC populations obtained in [OTR₄₁₃₁]-supplemented culture conditions was performed in single shot or 7 day continuous additions by flow cytometry (Fig. 2A) and

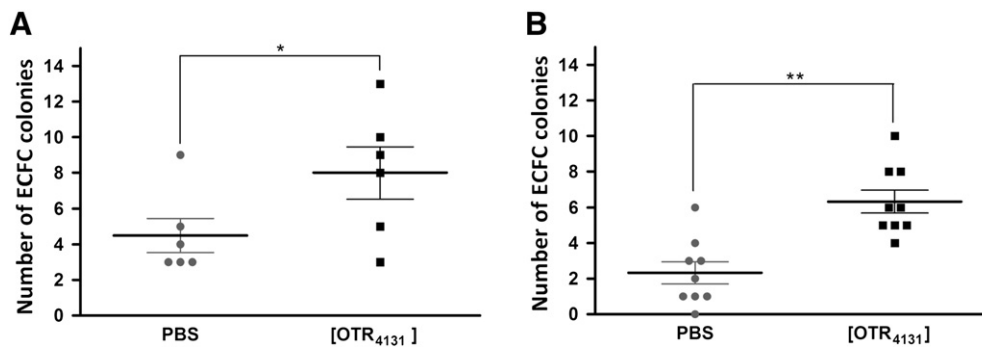


Figure 1 Effect of [OTR₄₁₃₁] on human ECFC colony formation efficiency. Number of ECFC colonies, identified as well-circumscribed monolayers of cobblestone appearing cells by visual inspection, were counted after 25 days of culture of purified CBMNC fraction, divided into 2 equal volumes and incubated either in PBS or [OTR₄₁₃₁] solutions, for 24 h (panel A) or 7 days (panel B). Values are the mean and SEM calculated from either 6 (24 h) or 9 (7 days) individual blood samples processed in independent experiments (n = 6 or n = 9, *p < 0.05; **p < 0.01).

immunofluorescence (Fig. 2B) analysis. ECFC were initially isolated with 7 day continuous additions of [OTR₄₁₃₁] and further amplified in medium without GAG express cell surface endothelial markers, including PECAM-1 (CD31), VE-Cadherin (CD144), VEGF-R2 (CD309) and vWF to the same extent than cells isolated and cultured without [OTR₄₁₃₁]. Moreover, Tumor Necrosis Factor alpha (TNF-alpha) activation (Fig. 2C) and acetylated low density lipoprotein (LDL-Ac) uptake (Fig. 2D) assays indicated that ECFC isolated in the presence of [OTR₄₁₃₁] are fully functional, since they are able to uptake in comparable extent LDL-Ac through specific receptor (PBS-treated positive cells: 94.9% ± 1.2, [OTR₄₁₃₁]-treated positive cells: 97.8% ± 1.2), and can be activated through TNF-alpha-pathways as efficiently as observed for classically isolated cells without [OTR₄₁₃₁]. Same results were obtained from ECFC treated by single shot of [OTR₄₁₃₁] (data not shown). Finally the two kinds of isolation procedures lead to the production of ECFC able to form a vascular-like network on Matrigel™ (Fig. 2E).

Effect of [OTR₄₁₃₁] on adhesion of ECFC

Because cell adhesion is a crucial property involved in CFE of ECFC, we explored the effect of [OTR₄₁₃₁] in this process. Since data collected from ECFC are limited by the small number of cells in colonies at passage 0, we analyzed the effect of pre-incubating [OTR₄₁₃₁] with ECFC on their capacities to attach to culture dish with cells at P3. In contrast to heparin, a natural GAG used as control, which inhibited adhesion by 15%, the [OTR₄₁₃₁] compound significantly improved ECFC adhesion by 25% (Fig. 3).

[OTR₄₁₃₁] potentiates heparin binding growth factor effects on ECFC proliferation

To further explore the effect of GAG mimetic and because data collected from ECFC are limited by the low number of cells at passage 0, we tested the effect of GAG on ECFC at P2 induced to proliferate for 5 days. First experiments were performed with addition of both GAG, natural or mimetic, in EGM2-MV complete medium and no effect was observed

(data not shown), probably due to a saturation of mitogenic signals in this complete medium. In order to measure a possible effect of GAG on ECFC growth, we used modified EGM2-MV medium with only 0.5% of FBS (0.5% EGM2-MV) in which we controlled the concentration of VEGF and FGF-2, the two most potent cytokines for ECFC proliferation. Figs. 4A and B show results of VEGF and FGF-2 dose-effect, respectively on proliferation rate of ECFC in 0.5% EGM2-MV for 5 days as compared to medium without neither FGF-2 nor VEGF. We confirm that an ECFC proliferation rate increases according to increasing dose of the growth factor added in the medium and reaches saturation levels of 2 ng/mL and 10 ng/mL for FGF-2 and VEGF respectively. We clearly observed that suboptimal concentration of both growth factors is around 1 ng/mL and decided to use this dose to test potential synergic effect of heparin and [OTR₄₁₃₁] (Figs. 4C, D).

ECFC proliferation rate in 0.5% EGM2-MV critically decreased when compared to EGM2-MV complete medium (7 fold). Addition of increasing doses of GAG in 0.5% EGM2-MV did not affect the ECFC growth (data not shown), suggesting that GAG do not promote proliferation properties of other growth factors present in the medium (neither IGF-1 nor EGF) or secreted by ECFC. Whereas addition of the suboptimal dose of 1 ng/mL VEGF alone didn't have significant effect, addition of 1 ng/mL FGF-2 alone increased 1.7 fold the proliferation rate of ECFC (Figs. 4C, D). In the case of proliferation associated to VEGF, whereas heparin significantly decreased the proliferation rate of ECFC at high doses, the mimetic [OTR₄₁₃₁] induced a significant 1.35 fold increase of ECFC proliferation, only at 0.1 µg/mL. In the case of ECFC proliferation induced by 1 ng/ml of FGF-2, a growth effect was observed with addition of 0.1 µg/mL of heparin (1.4-fold increase) but which decreased with higher doses. A lower increase of ECFC proliferation rate was obtained with 0.1 µg/mL of [OTR₄₁₃₁], but the FGF-2-induced proliferation was significantly potentiated in a dose-dependent manner since at higher doses of 1 µg/mL and 10 µg/mL, increases of 1.4-fold and 1.8-fold respectively were observed. These data clearly indicate that [OTR₄₁₃₁] is able to potentiate HBGF activity on ECFC proliferation in low serum condition.

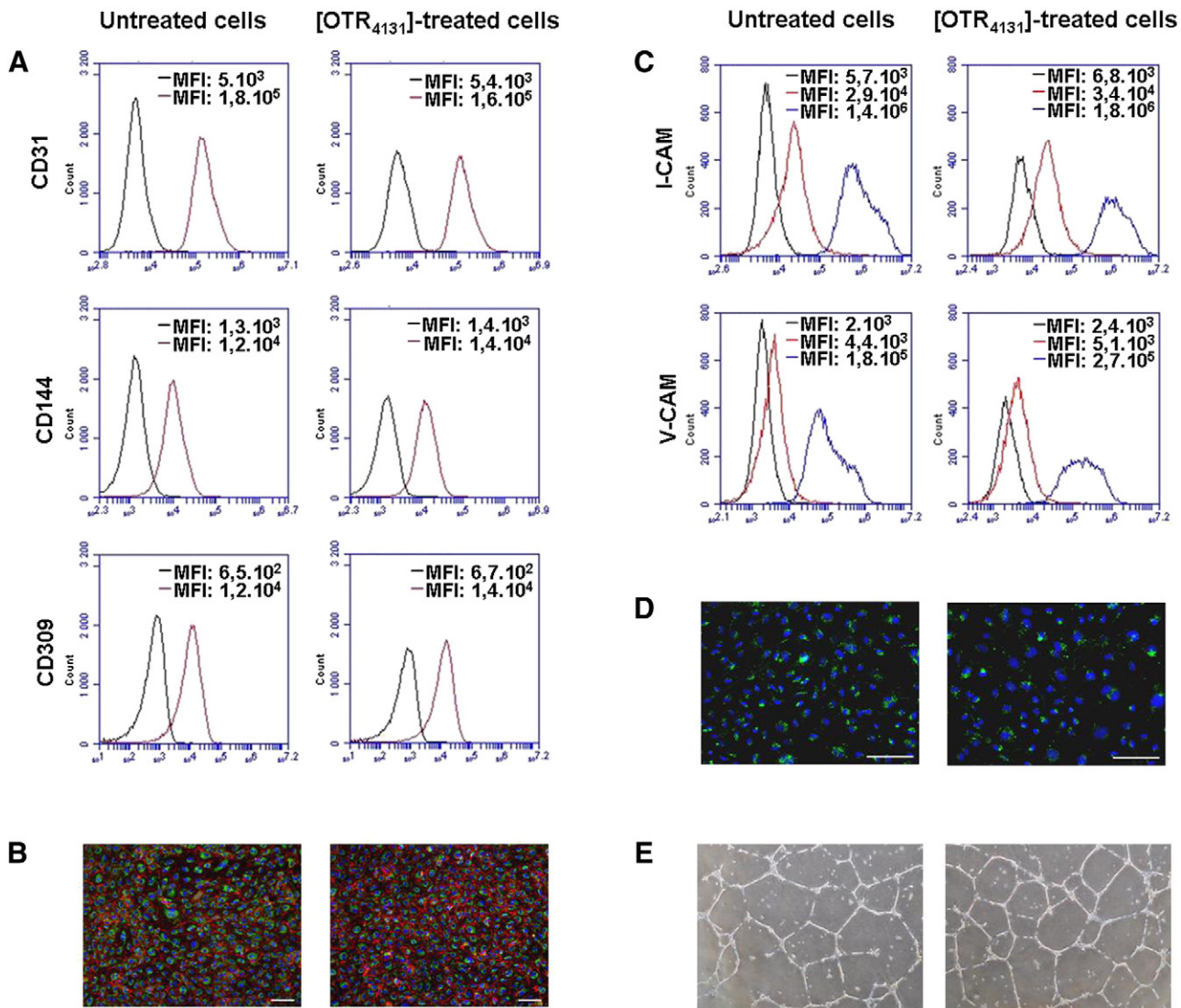


Figure 2 Phenotype and functionality of human ECFC isolated with [OTR₄₁₃₁]. Phenotypical considerations: (A) Flow cytometry analysis of the expression of specific endothelial markers CD31, CD144 and CD309 (IgG: black line; expression: red line; MFI: Mean Fluorescence Intensity in arbitrary unit). (B) ICC analysis of specific endothelial markers CD31 (red color) and vWF (green color) associated to nuclei staining (blue color); scale bar = 100 μ m. Functional considerations: (C) Flow cytometry analysis of ICAM-1 and VCAM-1 surexpression according to TNF alpha activation (IgG: black line; basal expression: red line; surexpression: blue line). (D) LDL-Ac (green color) uptake associated to nuclei staining (blue color); scale bar = 100 μ m. (E) Tubular network formation on MatrigelTM.

[OTR₄₁₃₁] doubles the capacity of a single ECFC to give rise to a clonal progeny

ECFC derived from EPC retain some immature properties after adhesion and proliferation *in vitro* such as the ability to form secondary colonies from a single cell. In order to evaluate this secondary colony forming capacity of ECFC, we adapted a serial dilution protocol, as described for hematopoietic stem cells for the quantification of cobblestone area-forming cells (Pettengell et al., 1994). This method permits evaluation of the influence of GAG on ECFC self-renewal capacity. In the absence of GAG, ECFC secondary colony frequency ranged between 1/2 and 1/40. According to this inter-individual variability, and to be able to compare

the frequency obtained in the presence of GAG, we defined a clonogenicity index which corresponds to the ratio of the frequency with/without GAG. Thereby, while heparin had no significant effect with a colony forming index at 1.3, [OTR₄₁₃₁] increased the colony forming index by 2-fold (Fig. 5).

[OTR₄₁₃₁] improves migration of ECFC

GAG mimetic effect was tested *in vitro* on ECFC migration in Boyden chambers using VEGF as chemoattractant and compared to heparin (Fig. 6). A basal migration level was defined in EBM2 basal medium with 0.2% serum in the absence of both VEGF and GAG (dotted line) and was enumerated around 100

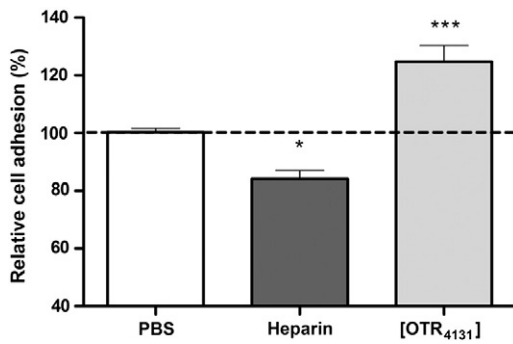


Figure 3 Effect of heparin and [OTR₄₁₃₁] on human ECFC adhesion. Relative adhesion capacity of ECFC pre-incubated with or without 100 ng/mL heparin or [OTR₄₁₃₁]. The relative adhesion performance of each condition was expressed as fold changes as compared to the control condition without GAG (PBS). Values are the mean and SEM calculated from triplicates in 4 independent experiments (n = 4, *p < 0.05; ***p < 0.001).

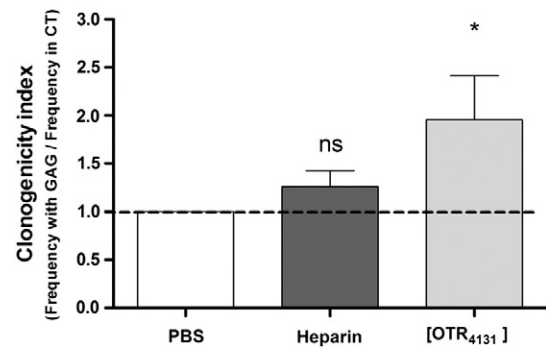


Figure 5 Effect of heparin and [OTR₄₁₃₁] on human ECFC secondary clonogenicity. Clonogenicity index (Frequency with GAG / Frequency CT) of ECFC after 15 days of culture with or without 1 μg/mL final concentration of heparin or [OTR₄₁₃₁]. Values are the mean and SEM calculated from 6 individual blood samples in 6 independent experiments (n = 6, *p < 0.05).

cells per field. Interestingly, addition of heparin or mimetic [OTR₄₁₃₁] alone to basal medium (0.2% serum) induced a 2-fold increase of ECFC migration. This increase was similar to that induced by addition of VEGF (2 ng/mL). This suggests that

heparin and [OTR₄₁₃₁] are able to potentiate chemoattraction of ECFC mediated by growth factors present in the 0.2% serum medium. Finally, in the presence of VEGF, addition of heparin or [OTR₄₁₃₁] statistically improves the migration rate by 1.5

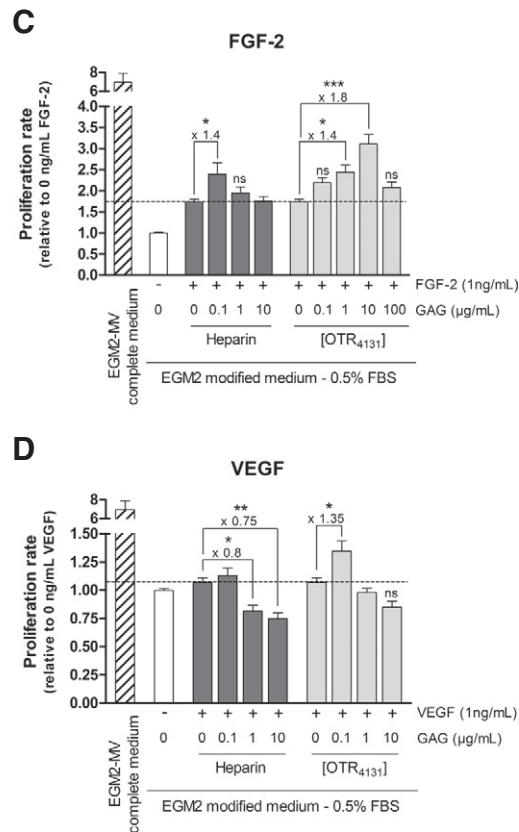
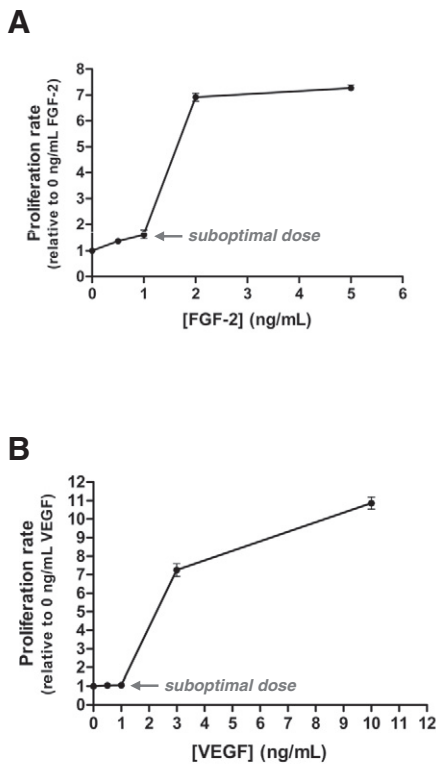


Figure 4 Effect of heparin and [OTR₄₁₃₁] on human ECFC proliferation. Proliferation rate of ECFC with increasing concentrations of either FGF-2 (panel A) or VEGF (panel B) and amplified with 1 ng/mL of FGF-2 (panel C) or VEGF (panel D) with the indicated concentration of [OTR₄₁₃₁] or heparin in EGM2 modified medium–0.5% FBS for 5 days. The proliferation rate of each condition was expressed as fold changes as compared to condition without cytokines and without GAG (=1). In order to see the effect of GAG alone, the dotted line represents the proliferation rate with cytokine alone. Values are the mean and SEM calculated from triplicates in 4 independent experiments (n = 4, *p < 0.05; **p < 0.01; ***p < 0.001).

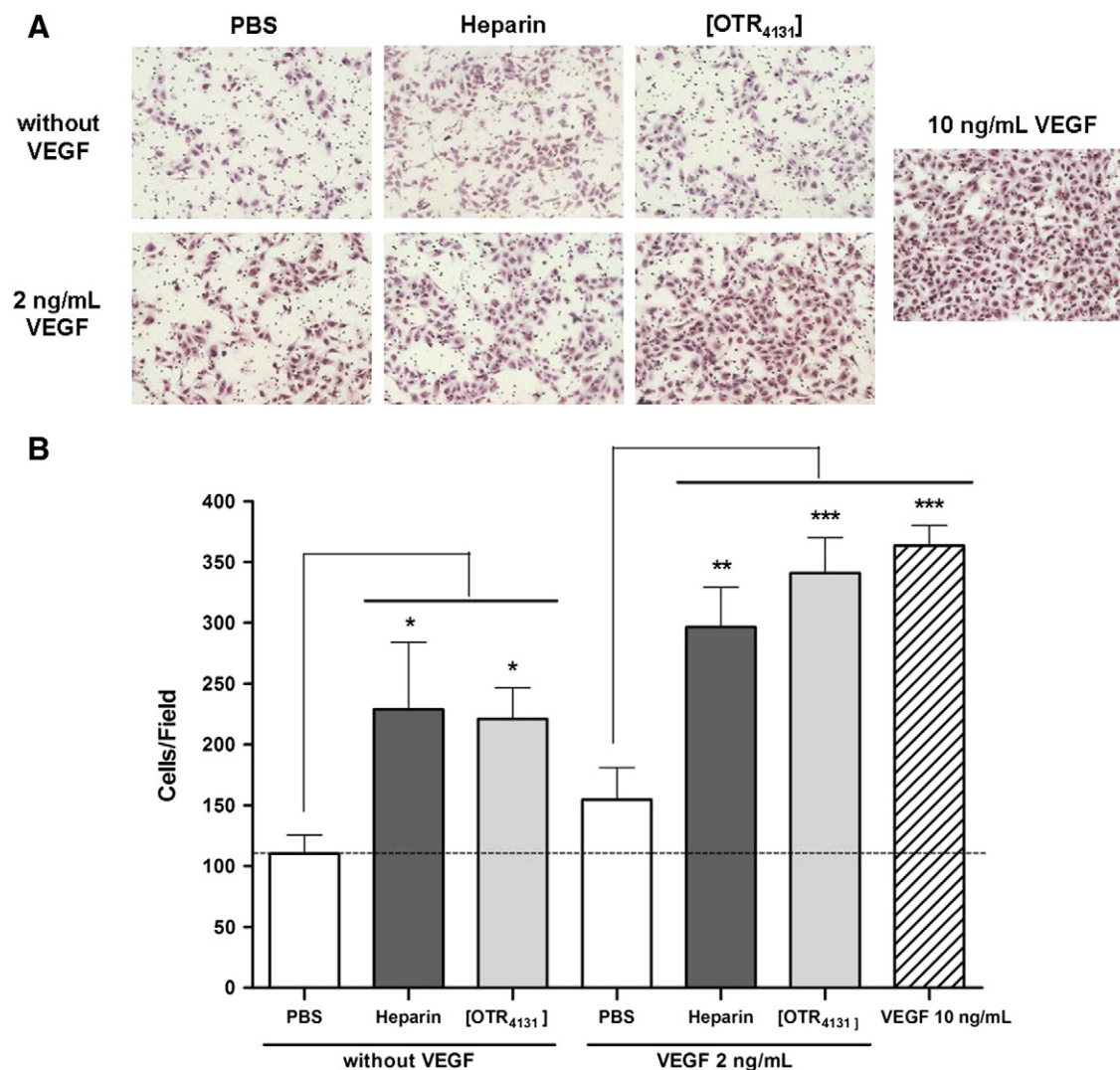


Figure 6 Effect of heparin and [OTR₄₁₃₁] on human ECFC migration capacity. (A) Representative pictures of MGG-stained transwell membranes. (B) Migration levels of ECFC exposed to 1 $\mu\text{g}/\text{mL}$ of heparin or [OTR₄₁₃₁] with or without VEGF. Data represent the mean and SEM from 3 independent experiments with each condition in duplicate (each value was determined by the mean of three different representative photographs) ($n = 3$, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

fold and 2 fold, respectively, reaching similar level obtained with 10 ng/mL of VEGF in the absence of GAG. This clearly indicates that [OTR₄₁₃₁] and heparin are able to potentiate chemoattractant effect of VEGF.

[OTR₄₁₃₁] does not modify vascular-like structure formation on MatrigelTM *in vitro* or *in vivo*

In vitro MatrigelTM assay allows investigating a functional ability of ECFC to form vascular-like tubes on a pro-angiogenic substrate. As shown in Figs. 7A and B, ECFC cultured in the presence of 1 $\mu\text{g}/\text{mL}$ of [OTR₄₁₃₁] displayed a similar capacity to form tubes on MatrigelTM as control ECFC. Interestingly, 1 $\mu\text{g}/\text{mL}$ of heparin induces a better network of vascular-like structures including 40% more segments, nodes and mesh with a total length of the network 20% higher. *In vivo* MatrigelTM implantation experiments permit to evaluate the capacity of [OTR₄₁₃₁] to improve ECFC-induced angiogenesis in the

presence of MSC used as supportive cells. As for the *in vitro* assay, the presence of 1 $\mu\text{g}/\text{mL}$ (Fig. 7C) or 10 $\mu\text{g}/\text{mL}$ (data not shown) of [OTR₄₁₃₁] GAG mimetic does not induce significant differences in the formation of vascular structures as compared to control plugs with same number of cells but without GAG.

Discussion

EPC are considered as a promising cell therapy tool for ischemic disease, but these cells represent a scarce population in blood and furthermore, many studies on circulating cell populations expressing combination of CD133, KDR and CD34 markers demonstrate that an inverse correlation exists between risk factors for cardiovascular diseases and potential EPC number and functionality (for review: Alaiti et al., 2010), suggesting that ECFC could be affected too. This is reinforced by recent published data of Shelley et al. (2012) demonstrating that the

number and functionality of ECFC are affected in aged individuals, which represent the population with highest cardiovascular risk. To further improve the efficacy of EPC transplantation, several approaches are currently being developed to enhance EPC expansion and bioactivities. These are mainly based on the combination of several growth factors such as SCF, FGF-2, VEGF, TPO, G-CSF, and IL-3 and 6 in culture media (Schlechta et al., 2010; Janic et al., 2010; Kalka et al., 2000; Lippross et al., 2011; O. E. et al., 2011; Senegaglia et al., 2010). Most of them are heparin-binding cytokines. Nevertheless, despite progress in growth factor combination needed for endothelial system, the optimal expansion conditions compatible with clinical use in serum-free medium still need to be defined. Since GAG are polysaccharide components of the ECM, known to potentiate the bioactivity of numerous HBGF on cells, we hypothesize that they will potentiate the activity of exogenous growth factors added to culture medium and/or secreted by the cells. However, GAG mixtures from natural origin are not structurally well characterized and carry high risk of contamination avoiding their clinical use for therapeutic application. Here, we studied the effect of a synthetic

GAG-like product, the [OTR₄₁₃₁] compound, synthesized in reproducible and standardized methods (Papy-Garcia et al., 2005) compatible with clinical use, on the properties of ECFC.

[OTR₄₁₃₁] optimizes the essential steps of *in vitro* ECFC colony formation frequency

Most patients with vascular diseases, eligible for autologous ECFC cell therapy are aged and display decreased frequency of these progenitor cells. Use of allogenic ECFC from CB may represent an efficient alternative strategy. To access existing CB banks, because the freezing conditions are not adapted to EPC and yield lesser number of ECFC (Vanneaux et al., 2010), the ECFC production from frozen CB units must be optimized. Thus, whatever the source of blood selected for obtaining ECFC, improvement of the number of colonies is an important issue for further reaching the cellular therapeutic dose. We demonstrate here that addition of a GAG mimetic during the first 24 h of CBMNC plating results in a 2 fold increase of the number of ECFC colonies (Fig. 1A).

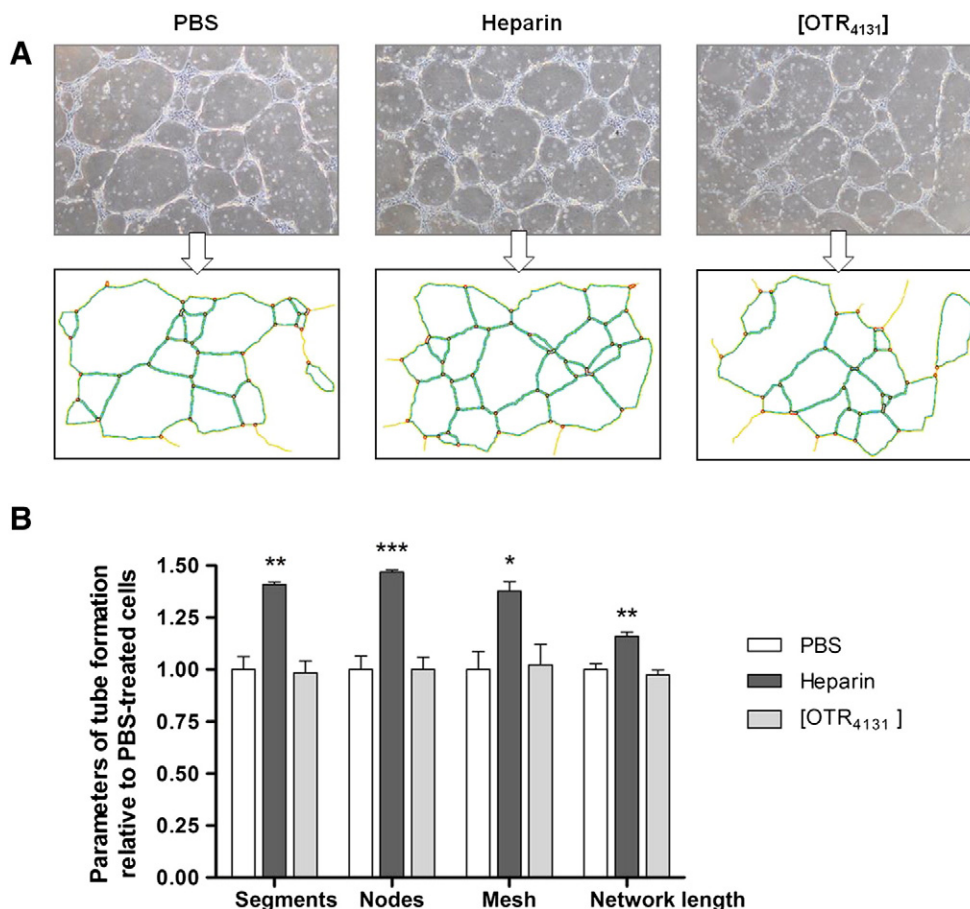


Figure 7 Effect of GAG on human ECFC tube formation. (A) Representative phase contrast pictures of tubular network on Matrigel™ formed by ECFC *in vitro* in the presence or not of 1 µg/mL of heparin or [OTR₄₁₃₁] and corresponding extracted skeletons of tubular networks identifying segments (yellow color), mesh (blue color) and nodes (red color). (B) ImageJ plugin analyzed parameters of tube formation. Values are the mean and SEM calculated from triplicates in 3 independent experiments (n = 3, *p < 0.05; **p < 0.01; ***p < 0.001). (C) Representative *in vivo* Matrigel™ plug sections labeled with an anti-von Willebrand Factor antibody (red) and an anti-human specific CD31 antibody (green) with DAPI staining (blue) to detect human ECFC, magnification ×25 (a and c) or ×63 (b and d). Cells were implanted alone (a and b) or with 1 µg/mL of [OTR₄₁₃₁] (c and d).

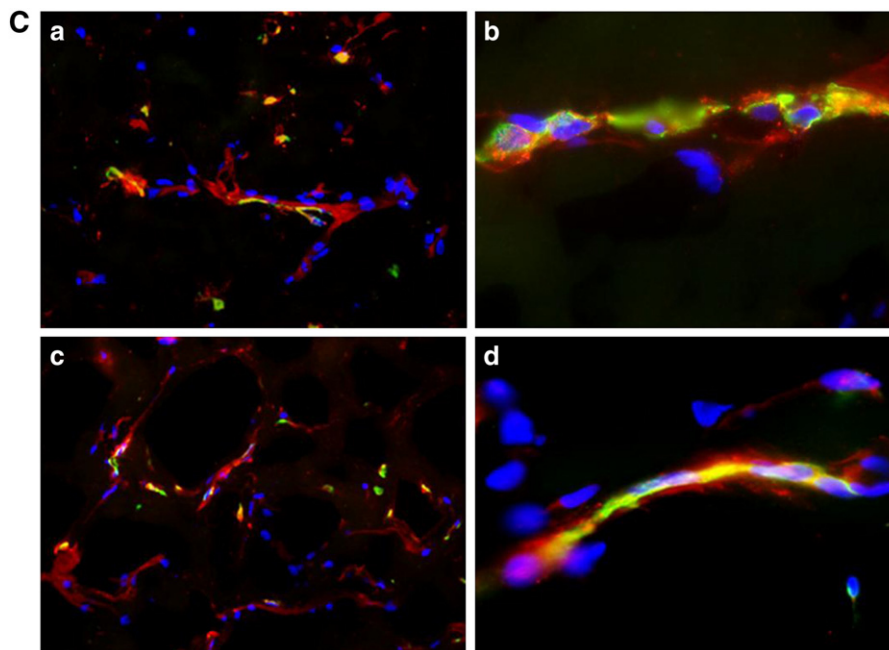


Figure 7 (continued).

One of the possible mechanisms of action of the GAG mimetic at this very early step of colony formation is the improvement of adhesion properties of EPC. This hypothesis is supported by the ability of the same GAG mimetic to improve cell adhesion of ECFC (Fig. 3). This result is very promising since it proves that we are able to optimize the essential step of *in vitro* ECFC colony formation. This is reinforced by previous similar data demonstrating that treatment of rat bone marrow mononuclear cells with GAG mimetics during the first step of culture increases the colony forming efficiency of MSC (Frescaline et al., 2011).

Furthermore, addition of the [OTR₄₁₃₁] mimetic not only for the first 24 h after plating but also all along the first 7 days of culture improves almost 3 fold the number of colonies (Fig. 1B). These data clearly suggest that the GAG mimetic acts not only on early steps of colony formation but also on later steps such as self-renewal and/or cell proliferation of ECFC. This hypothesis is supported by our results demonstrating an improving effect of this GAG mimetic on ECFC proliferation and secondary colony frequency (Figs. 4 and 5). In the past few years, evidences about the regulation of stem cell behavior by ubiquitous GAG such HS and CS have been accumulated. In mouse embryonic stem cell, siRNA knockdown of the EXT1 gene, encoding the enzyme involved in the elongation of the HS chains, leads to a decrease by half of the number of colonies remaining undifferentiated, even in the presence of LIF and serum, and to a critical decrease of the proliferation rate (Sasaki et al., 2008). CS is also involved in stem cell fate as demonstrated by its involvement in the regulation of signaling pathways essential for the proliferation, self-renewal, and cell lineage commitment of neural stem/progenitor cells (Purushothaman et al., 2012). All these studies clearly highlight the importance of HS and CS in the modulation of stem cell self-renewal

and lineage fate (Kraushaar et al., 2013; Smith et al., 2011). Our data clearly demonstrate for the first time that [OTR₄₁₃₁] GAG mimetic, by acting on ECFC clonogenicity, is able to impact stem cell properties as it was demonstrated for natural GAG.

[OTR₄₁₃₁] is able to potentiate the *in vitro* amplification of ECFC

GAG are essential for HBGF signaling implicated in endothelial cell proliferation since it has been shown that heparin potentiates mitogenic effect of both FGF-1 and VEGF₁₆₅ on endothelial cell (Thornton et al., 1983; Ono et al., 1999; Ashikari-Hada et al., 2005) and HS is required for binding FGF-2 and VEGF₁₆₅ to their high affinity receptors (Yayon et al., 1991; Gitay-Goren et al., 1992). We show here that [OTR₄₁₃₁] mimetic is able to potentiate ECFC proliferation induced by VEGF and FGF-2, in a cell culture medium with a low (0.5%) concentration of serum (Fig. 4). Interestingly, our data also indicate that the [OTR₄₁₃₁] GAG mimetic action is much higher on FGF-2 effect than on VEGF. This is probably due to different affinities of this GAG toward each growth factor. This hypothesis is supported by several studies analyzing GAG structure relationships with specific binding affinity and different biological effects of HBGF (Ashikari-Hada et al., 2005; Cole et al., 2010; Goger et al., 2002; Ouidja et al., 2007; Zhao et al., 2012). Moreover, our data are in accordance with previously published results demonstrating that another GAG mimetic [OTR₄₁₂₀] binds to VEGF₁₆₅ and potentiates the affinity for its receptors, leading to better *in vitro* VEGF-induced proliferation and migration of endothelial cells such as HUVEC (Rouet et al., 2005). Otherwise, we had already observed that only [OTR₄₁₃₁] significantly potentiates the growth of rat MSC

through a FGF-2/FGFR1 pathway, when compared to [OTR₄₁₂₀] or heparin (Frescaline et al., 2011).

[OTR₄₁₃₁] promotes the migration of ECFC

In this study, we show that [OTR₄₁₃₁] is able to promote the VEGF-dependent migration of ECFC (Fig. 6). Moreover, in the absence of VEGF it also potentiates migration, probably through interaction with different chemo-attractant molecules secreted by cells or present in the medium. Likewise, we previously demonstrated that [OTR₄₁₃₁] is able to potentiate the migration of rat MSC (Frescaline et al., 2011). In addition, [OTR₄₁₃₁] is able to mobilize immature population of hematopoietic stem cells according to a process that involves SDF-1 interactions (Albanese et al., 2009). These observations are supported by the demonstration that some HS carrying acetyl groups, like [OTR₄₁₃₁] GAG mimetic, display a better binding of chemokines such CCR2 (MCP-1) (Schenauer et al., 2007) which is involved in EPC homing to a neovascularization site (Hristov et al., 2007). Finally, such migratory effect of [OTR₄₁₃₁] suggests that this GAG mimetic could be of great interest if injected during a regenerative process, to attract therapeutic stem cells and mobilize endogenous circulating or resident ones into the injury site.

Role of [OTR₄₁₃₁] in angiogenesis

We demonstrate that the [OTR₄₁₃₁] mimetic doesn't improve significantly ECFC angiogenic capacity on Matrigel™ neither *in vitro* nor *in vivo* (Fig. 7). However, it has been documented that other GAG mimetics with structure close to that of [OTR₄₁₃₁], are able to potentiate angiogenesis *in vivo* in chicken embryo chorioallantoic membrane development (Rouet et al., 2005) or in pig model of myocardial ischemia (Yamauchi et al., 2000). Our hypothesis is that such GAG mimetics potentiate angiogenesis by reinforcing and stabilizing damaged extracellular matrix during tissue remodeling that occurs for example during embryonic development or in the regeneration process following injury. Moreover, GAG mimetics may also enforce the action of heparin binding growth factor, which may be present at limited concentration in the injured tissues. In Matrigel™ experiments, there is no tissue remodeling, and thus, the GAG mimetics' role in replacement of degraded endogenous GAG is not required. Moreover, growth factors (VEGF, FGF-2) are present at high concentrations, and potentialization of their action by GAG mimetics is not needed. Indeed, in our *in vitro* experiments, we had to adapt significantly culture conditions in order to make apparent GAG mimetic effects on ECFC (low serum concentration and growth factors at minimal concentrations), whereas in the *in vivo* Matrigel™ assay such flexibility in the experimental conditions is difficult to obtain. Moreover, we demonstrate here that [OTR₄₁₃₁] is able to reinforce cell adhesion *in vitro* whereas heparin inhibits it. However, it is well known that partial disruption of endothelial cell–matrix contact is a pre-requisite for triggering angiogenesis (Ingber, 2002), suggesting that such anti-adhesive effect of heparin could explain GAG-stimulated angiogenesis in Matrigel™ *in vitro*. Thus, the Matrigel™ model does not fully represent an *in vivo* situation in which GAG mimetic effects could be

evidenced. The putative synergy between ECFC and GAG mimetics, especially the [OTR₄₁₃₁] compound for inducing vascularization *in vivo* remains to be demonstrated in a more physiological model of angiogenesis.

Conclusion

Our results demonstrate that the GAG mimetic [OTR₄₁₃₁] is able to enhance the critical human ECFC colony formation frequency, required to improve the amplification of ECFC, and to promote cell properties such as cell adhesion, migration and self-renewal. Moreover, [OTR₄₁₃₁] is able to potentiate the mitogenic effect of angiogenic growth factors such as FGF-2 and VEGF in low serum condition. Considering cell therapy for ischemic diseases by ECFC isolated from CB or PB, [OTR₄₁₃₁] appears to be a promising tool to increase the cell recovery from these blood sources without modifying their properties and to optimize the development of clinical grade serum-free medium. Moreover, these data suggest that matrix therapeutic strategy based on GAG mimetics will be in accordance with innovative tissue engineering approaches to synergize exogenous growth factor treatments and/or to potentiate *in vivo* effects of cell-based therapeutic products. The addition of [OTR₄₁₃₁] to injured tissue should constitute scaffolds for a proper micro-environment before cell transplantation that will be able, according to its migratory effect, to induce the *in situ* recruitment of stem/progenitor cells to the defective sites and increase again tissue repair.

Acknowledgments

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