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Original research article

Pleiotrophin: Analysis of the endothelialisation potential

Francesco Copes[a,b,1], Martina Ramella[a], Luca Fusaro[a,c], Diego Mantovani[b], Mario Cannas[a], Francesca Boccafoschi[a,*,1]

[a] Laboratory of Human Anatomy, Department of Health Sciences, University of Piemonte Orientale, Novara, Italy
[b] Laboratory for Biomaterials and Bioengineering, Canada Research Chair Tier I in Biomaterials and Bioengineering for the Innovation in Surgery, Department of Min-Met-Materials Engineering & CHU de Quebec Research Center, Laval University, Quebec City, Quebec, Canada
[c] Tissuegraft srl, Novara, Italy

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ABSTRACT

Purpose: Endothelialisation of vascular substitutes, in fact, remains one of the most unsolved problems in cardiovascular diseases treatment. Stromal Derived Factor 1 (SDF-1) has been largely investigated as an endothelialisation promoter and Pleiotrophin is a promising alternative. Although it has been known to exert beneficial effects on different cell types, its potential as an inducer of proliferation and migration of endothelial cells was not investigated. Therefore, this work is aimed to compare the effects of Pleiotrophin on proliferation and migration of endothelial cells with respect to SDF-1.

Materials/methods: Endothelial cell line EA.hy926 was treated with Pleiotrophin (50 ng/ml) or SDF-1 (50 ng/ml). Cell viability was evaluated by MTT assay and migration assays were performed in Transwell chambers. Wound healing potential was evaluated by scratch wound assay. CXCR4, RPTP β/ζ, PCNA and Rac1 expression was detected by Western Blot.

Results: Interestingly, Pleiotrophin significantly increased the viability of the treated endothelial cells with respects to SDF-1. The migratory ability of the endothelial cells was also improved in the presence of Pleiotrophin with reference to the SDF-1 treatment. Moreover, Western Blot analysis showed how the treatment with Pleiotrophin can induce an increase in the expression of RPTP β/ζ, PCNA and Rac1 compared to SDF-1.

Conclusion: Due to the significant effects exerted on viability, migration and repair ability of endothelial cells compared to SDF-1, Pleiotrophin can be considered as an interesting molecule to promote re-endothelialisation.

1. Introduction

Cardiovascular diseases (CVDs) are one of the leading causes of death worldwide. According to the World Health Organization (WHO), CVDs were responsible for 15 million deaths in 2015, almost 30% of the total global mortality [1]. Among the different diseases, vascular occlusion remains the leading cause of death in Western countries. Arterial bypass graft remains the primary effective surgical therapy for patients with advanced vascular occlusion. Autologous grafts are the ideal substitutes with a success rate of 90% at one year. However, often patients needing bypass surgery may not possess healthy available arteries useful as autograft [2]. In the absence of available natural substitutes, the simplest solution is the use of synthetic substitutes. Although many improvements have been made over the years, the clinical performance of these prostheses for small arteries bypass remains quite disappointing. In fact, lumen occlusion due to blood coagulation and platelet deposition under the relatively low flow conditions along with restenosis, that is commonly observed in the months or years following surgery [3,4], hamper their use in the clinic. The rapid formation of a functional endothelial layer on the luminal surface of vascular substitutes would significantly improve small-diameter graft patency by: preventing thrombus formation on the graft surface, enhancing internal healing and limiting intimal hyperplasia [5]. In recent years, the enrichment with bioactive molecules such as growth factors and cytokines able to efficiently recruit resident endothelial cells (ECs), promote their adhesion and growth has been proposed as an approach to overcome this problem [6,7]. Several molecules have been shown to be able to promote angiogenesis or ECs recruitment. Among them, stromal derived factor 1 (SDF-1) has been studied. It is known as C-X-C motif chemokine 12 (CXCL12), a chemokine protein that in humans is encoded by the *CXCL12* gene. SDF-1 is ubiquitously expressed in many tissues and cell types and its receptor, C-X-C chemokine receptor type 4

* Corresponding author at: Department of Health Sciences, University of Piemonte Orientale, Via Solaroli, 17, 28100, Novara, Italy.
E-mail address: francesca.boccafoschi@med.uniupo.it (F. Boccafoschi).
[1] These authors contributed equally to this work.

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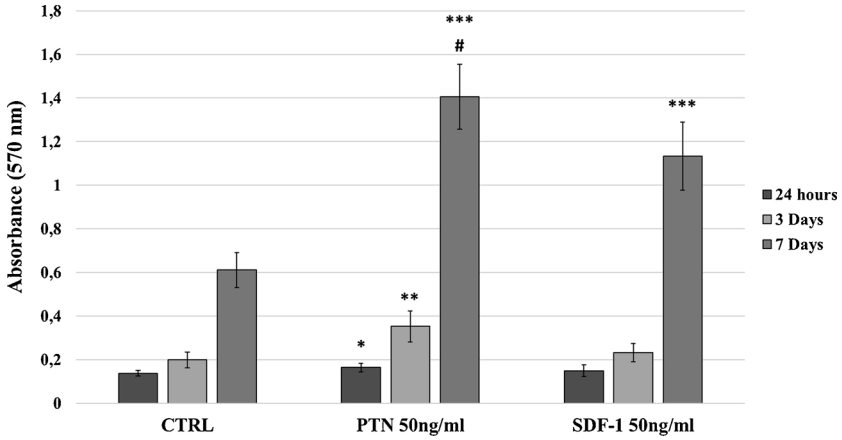

Fig. 1. Viability Assay. Ea.hy926 cells were treated up to 7 days with: basal growth medium (CTRL); growth medium enriched with 50 ng/ml PTN (PTN 50 ng/ml); growth medium enriched with 50 ng/ml SDF-1 (SDF-1 50 ng/ml). Cell viability was measured after 24 h, 3 and 7 days with MTT Assay. The graphic shows the mean absorbance recorded for each condition. * $p < 0.01$ vs. 24 h CTRL; ** $p < 0.001$ vs. 3 days CTRL and $p < 0.01$ vs. 3 days SDF-1 50 ng/ml; ***$p < 0.001$ vs. 7 days CTRL; # $p < 0.01$ vs. SDF-1 50 ng/ml.

(CXCR4), is widely expressed in different tissues like blood vessels, particularly by ECs [8]. SDF-1 is released into the circulation in response to ischemia [9] and is an initiating signal in the angiogenesis process, promoting ECs recruitment. For these reasons, SDF-1 has been widely used for the biological functionalization of vascular substitutes [10–15]. However, even if initially the application of SDF-1 on vascular prosthesis was regarded as very promising, clinical trials of angiogenic factor delivery have been mostly disappointing, underscoring the need to investigate a wider array of angiogenic factors [16].

Pleiotrophin (PTN) is an 18-kDa growth/differentiation cytokine with high affinity for heparin and it is structurally related to Midkine, the only other member of its protein family [17]. It has mitogenic, differentiating and angiogenic properties for various cell types and is expressed mainly, but not exclusively, during embryogenesis. Through the activation of its receptor, protein tyrosine phosphatase beta/zeta (RPTP β/ζ), PTN is able to regulate multiple functions including cell adhesion, cell migration, cell proliferation and cytoskeletal stability [18]. PTN was described as a potent pro-angiogenic factor acting on ECs during healing from ischemic brain injury, and was found to stabilize the formation of tube structures by cultured capillary ECs [19]. Interestingly, recent studies have also pointed out a PTN-induced transdifferentiation of monocytes into functional ECs suggesting a role for PTN in inflammation-mediated neovascularization [20] and a role in the recruitment of endothelial progenitor cells (EPC) during angiogenesis [21].

Considering these evidences, herein the *in vitro* effects of PTN on proliferation, migration and repair ability of the ECs were investigated and compared with those exerted by SDF-1, finally aiming to unravel the PTN potential as an endothelialisation enhancer.

2. Materials and methods

2.1. Cell culture

Human umbilical vein cell line EA.hy926 [22] was used in this study. Cells were supplied by the Vascular and Endovascular Surgery Unit, Research Laboratory of Experimental and Clinical Vascular Biology, DISC, University of Genoa, Italy. No Ethical Approval was needed for the use of this cell line. Briefly, EA.hy926 cells were cultured in a cell culture medium: Dulbecco's modified Eagle's medium (D-MEM) with 10% fetal bovine serum (FBS), penicillin (100U/ml), streptomycin (100U/ml) and L-glutamine (2 mM) (all products from Euroclone, Milan, Italy). This medium, that will be referred to as complete D-MEM (C-D-MEM), has been used in our experiments along with a serum free version of it (SF-D-MEM). The cells were maintained at 37 °C in saturated atmosphere at 5% CO_2. Cell culture media were renewed every two days. When 85%–90% of confluence was reached, cells were then enzymatically detached from the plate (0.05% trypsin) and then reseeded at a ratio of 1:10.

2.2. Cell viability assay

Cell viability was evaluated by MTT Assay. EA.hy926 cells were seeded at a density of 2500 cell/cm^2 in 96 well culture plates (5 wells for each condition). After an overnight incubation at 37 °C in saturated atmosphere at 5% CO_2, cells were treated respectively with: 1) C-D-MEM (CTRL); 2) C-D-MEM enriched with 50 ng/ml SDF-1 (Sigma-Aldrich, Milan, Italy); 3) C-D-MEM enriched with 50 ng/ml PTN (Sigma-Aldrich, Milan, Italy). The concentration used for the PTN has been chosen following a dose-response curve obtained in the preliminary steps of the study (data not shown). SDF-1 concentration was chosen accordingly to data present in the literature [23,24]. After 24 h, 3 and 7 days, cells were incubated with the MTT reagent (Sigma-Aldrich, Milan, Italy) for 3 h at 37 °C. After the incubation, the formazan product obtained by the reduction of MTT reagent by the way of the mitochondrial activity was solubilized using dimethyl sulfoxide (Carlo Erba Reagents, Milan, Italy). Then, the absorbance at 570 nm was measured with a SpectraCount Absorbance microplate reader (Packard, Connecticut, USA). Absorbance is proportional to cell viability.

2.3. Wound healing assay

Wound Healing Assay was performed to assess cell migration and reparatory ability *in vitro* in the presence of PTN and SDF-1. Briefly, cells were seeded in 6-well multi-plate to obtain a confluent monolayer. Then, cell monolayer was scraped in a straight line to create a "scratch" with a 200 µl pipet tip. Medium containing cellular debris was removed, and cells were washed 2 times with sterile PBS 1X and then treated as follows: 1) SF-D-MEM (CTRL), 2) SF-D-MEM enriched with SDF-1 (50 ng/ml) or 3) SF-D-MEM enriched with PTN (50 ng/ml). Markings were created to be used as reference points close to the scratch. Images at a magnification of 20X were collected at 0, 6, 12 and 24 h of incubation (n = 5). To quantify the scratch-area reduction over time, images acquired from each sample were further analysed by using ImageJ software (ImageJ 1.49v; Wayne Rasband; National Institute of Health, USA). Briefly, for each time point, the outline of the cell-free surface of the scratch was drawn and the inner area was calculated using the software. The percentage of wound closure was calculated by comparing the areas measured at the different time points with the area at T_0.

2.4. Transwell migration assay

To verify the migratory response of EA.hy926 cells to SDF-1 and PTN treatments, the transwell migration assay was used. 9×10^4 cells were seeded in the upper compartment of 24 well-format transwell with

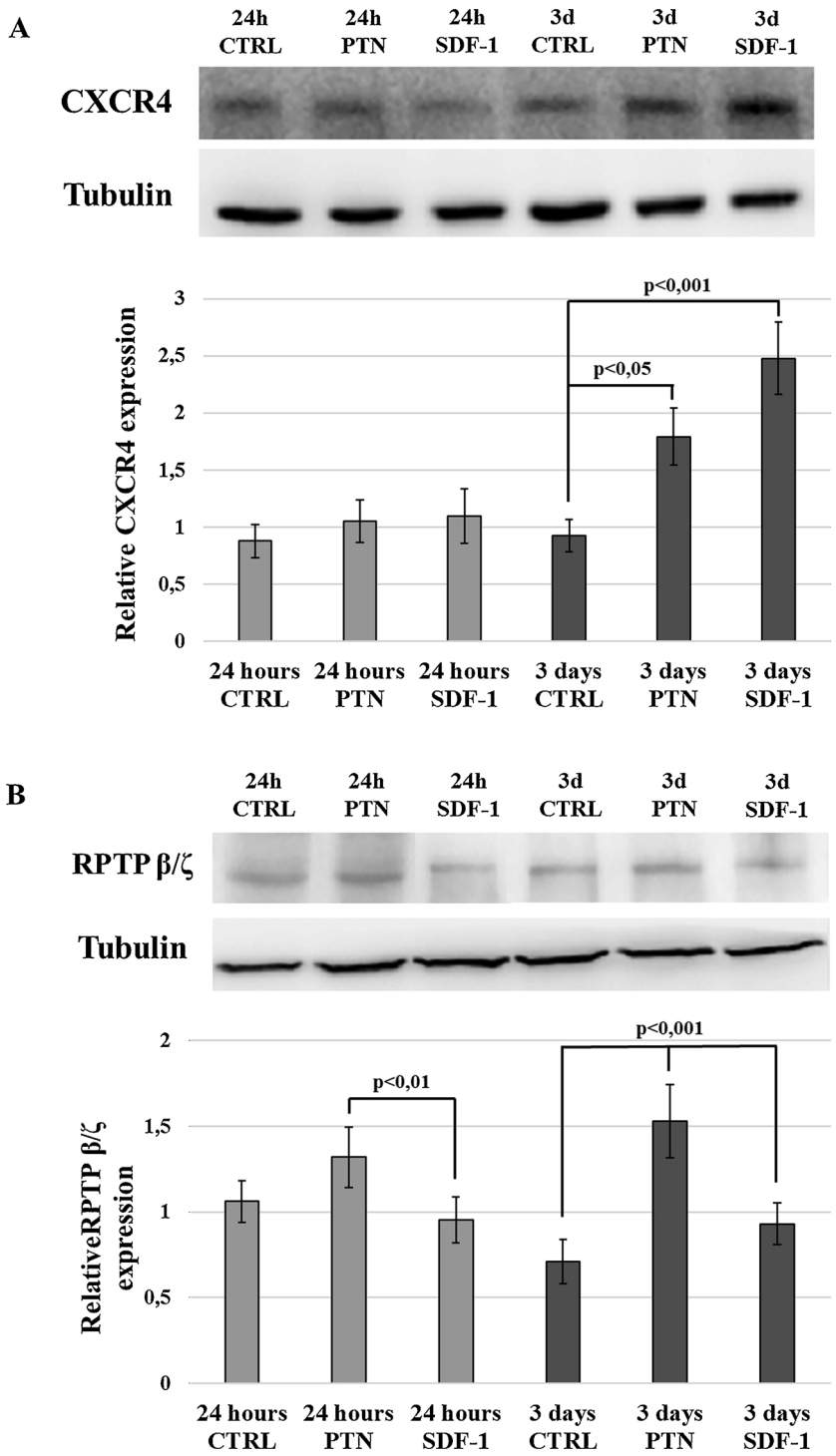

Fig. 2. Western Blot Analysis for RPTP β/ζ and CXCR4. The images show the results of the Western Blot analysis for the expression of the two receptors in samples obtained by EA.hy926 cells treated with: C-D-MEM (CTRL), C-D-MEM containing 50 ng/ml PTN (PTN 50 ng/ml) and C-D-MEM containing 50 ng/ml SDF-1 (SDF-1 50 ng/ml). Lysates were collected after 24 h (24 h) and 3 days (3d). Data were normalized over tubulin, used as loading control. **A**) Expression of SDF-1 receptor CXCR4. **B**) Expression of PTN receptor RPTP β/ζ.

8 μm pores (Corning, Amsterdam, the Netherlands) in 250 μl of D-MEM without FBS. In the lower compartment, the different treatment compounds were added: 1) SF-D-MEM (CTRL); 2) SF-D-MEM enriched with 50 ng/ml SDF-1; 3) SF-D-MEM enriched with 50 ng/ml PTN. Cells were incubated at 37 °C in saturated atmosphere at 5% CO_2 for 6 h. After the incubation, cells on either faces of the porous membrane were fixed by incubation with formalin 4% for 20 min at room temperature. Then, cells were stained with 1% Crystal Violet for 20 min at room temperature. Once stained, cells on the upper side of the porous membranes were gently removed using a cotton swab. The transwell inserts were then placed under a phase-contrast microscope and images of different fields (n = 5) were collected at 20X magnification. To assess the migration rate, for each condition stained cells were quantified.

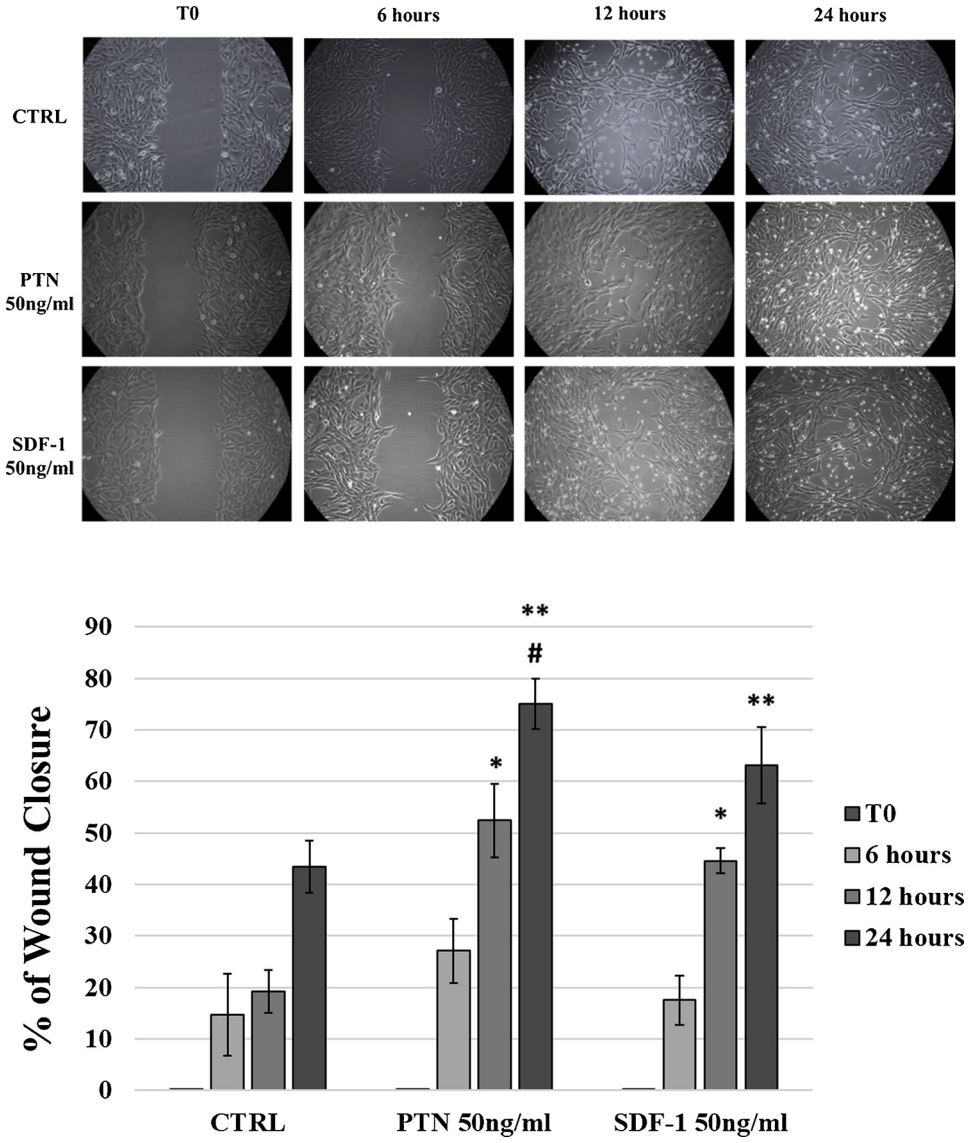

Fig. 3. Wound Healing Assay. **A)** Migration of EA.hy926 induced by treatment with: SF-D-MEM (CTRL), SF-D-MEM enriched with SDF-1 (SDF-1 50 ng/ml) or SF-D-MEM enriched with PTN (PTN 50 ng/ml). The pictures were acquired right after the scratch (T0) and 6, 12 and 24 h after the scratch (magnification: 20X). **B)** Percentage of wound closure. Graphic represents the area as mean ± SD of the percentage reduction of original wound at T0, 6, 12 and 24 h with the different treatments. * $p < 0.001$ vs 12 h CTRL; **$p < 0.001$ vs. 24 h CTRL; # $p < 0.01$ vs. 24 h SDF-1 50 ng/ml.

2.5. Western blot

Cells treated with C-D-MEM (CTRL), C-D-MEM containing 50 ng/ml PTN and C-D-MEM containing 50 ng/ml SDF-1 were lysed in adequate RIPA lysis buffer (Hepes 50 mM pH 7.4; NaCl 150 mM; SDS 0.1%; Triton X-100 1%; Na Deoxycolate 1%; Glicerol 10%, $MgCl_2$ 1.5 mM; EGTA 1 mM; NaF 1 mM) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, Milan, Italy). Quantification of the protein extract was carried out using the BCA Protein Assay Kit (Thermo-Scientific, Rockford IL, USA) according to the manufacturer's instructions. Electrophoretic analysis of 30 µg of total proteins was performed using 10% SDS–polyacrylamide gel. Gels were blotted onto nitrocellulose blotting membrane (GE Healthcare Life Sciences, Milan, Italy). Then, membranes were probed with the following primary antibody: mouse monoclonal anti-PCNA (1:1000; Millipore, Darmastadt, Germany), mouse monoclonal anti-Rac1 (1:1000), mouse monoclonal anti-PTPz (1:1000), rabbit monoclonal anti-CXCR4 (1:100) (all from Abcam, Cambridge, UK). For the analysis of PCNA and Rac-1, lysates were obtained by cells with both free receptors for PTN and SDF-1 and with blocked receptors. In the un-blocked receptor group, cells were grown as previously described for the viability and migration tests. In the blocked receptor group, the receptors for PTN and SDF-1 were blocked to impeach the interaction between the molecules and their receptors. To block the PTN receptor, RPTP β/ζ, cells were incubated with its primary antibody (anti-PTPz, 1:100, Abcam, Cambridge, United Kingdom). CXCR4, SDF-1 receptor, was blocked with 25 µg/ml AMD3100 octahydrochloride (Abcam, Cambridge, United Kingdom), a selective inhibitor of CXCR4. Primary antibodies were detected with species-specific horseradish peroxidase-conjugated secondary antibodies (Perkin Elmer, Milan, Italy). The bands were visualized using Western Lightning® Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, Milan, Italy). Results were revealed on membranes and acquired using the VersaDoc MP5000 System (Bio-Rad, Milan, Italy). The intensity of protein bands was measured with ImageJ software. Results were normalized to tubulin.

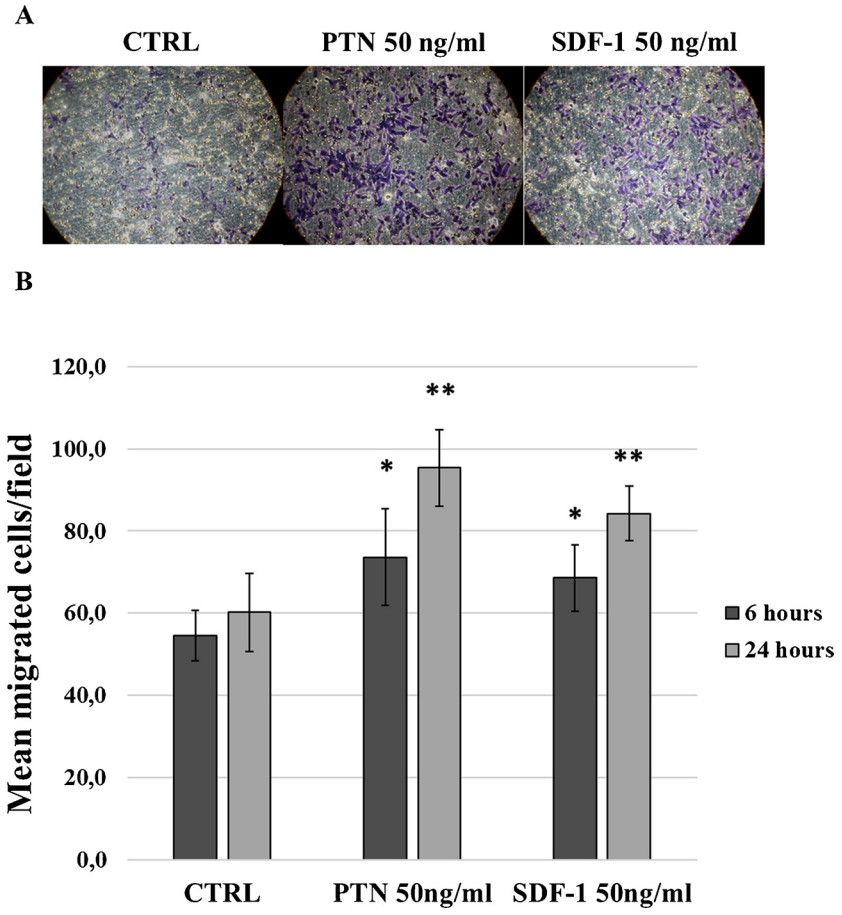

Fig. 4. Transwell Migration Assay. **A)** Brightfield images showing the migrated cells, stained with Crystal Violet, after 24 h of incubation with different treatments: SF-D-MEM (CTRL); SF-D-MEM enriched with 50 ng/ml SDF-1; SF-D-MEM enriched with 50 ng/ml PTN. **B)** Quantitative analysis of migrated cells expressed as mean ± SD of number of migrated cells per field. *p < 0.05 vs. 6 h CTRL; **p < 0.001 vs. 24 h CTRL.

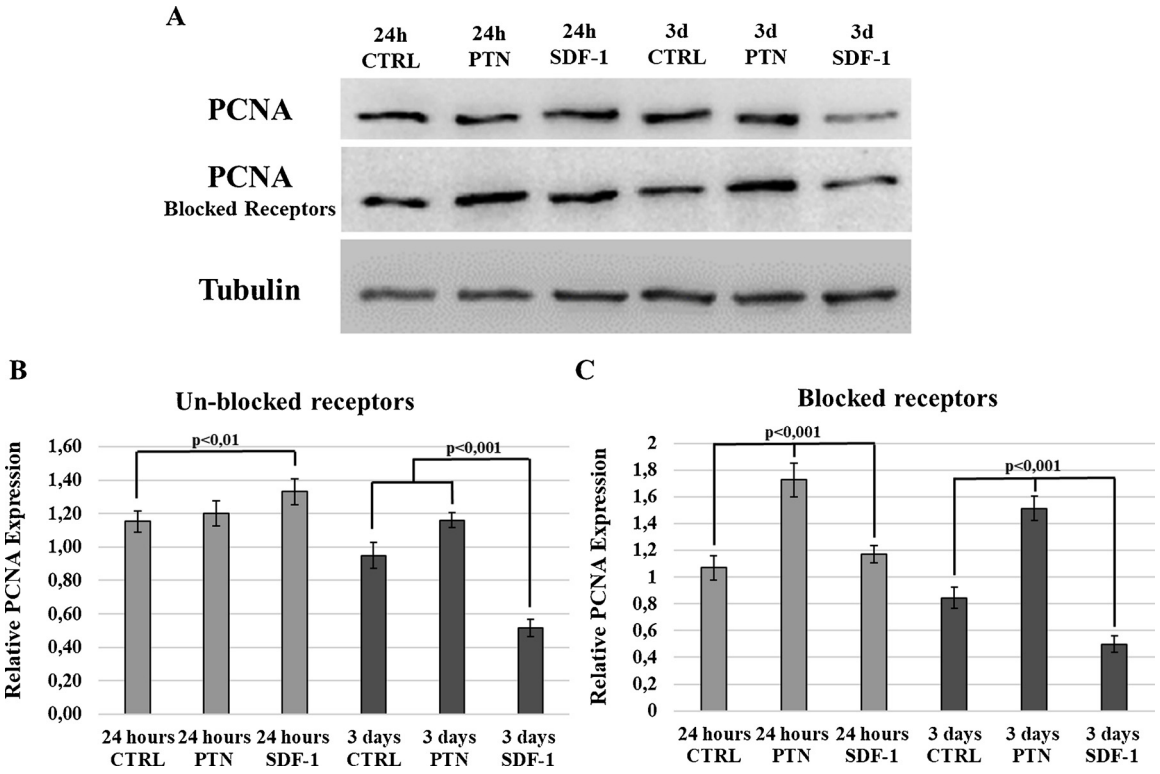

Fig. 5. Western Blot Analysis for PCNA on EA.hy926. **A)** Cells were treated with: C-D-MEM (CTRL), C-D-MEM containing 50 ng/ml PTN (PTN) and C-D-MEM containing 50 ng/ml SDF-1 (SDF-1). Lysates were collected after 24 h (24 h) and 3 days (3d). Data were normalized over tubulin, used as loading control. **B**) Densitometric analysis showing the absolute quantification for PCNA expression with un-blocked receptors for PTN and SDF-1. **C**) Densitometric analysis showing the absolute quantification for PCNA expression with the blocked receptors for PTN and SDF-1.

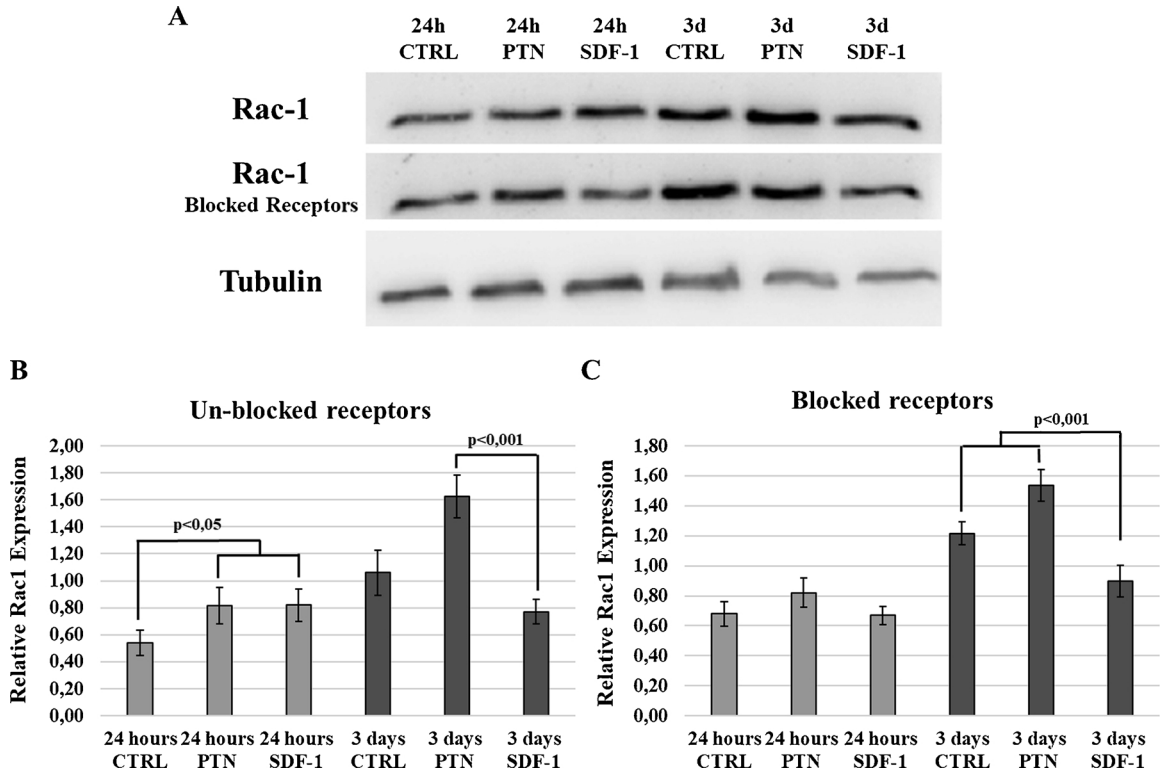

Fig. 6. Western Blot Analysis for Rac-1 on EA.hy926. **A)** Cell were treated with: C-D-MEM (CTRL), C-D-MEM containing 50 ng/ml PTN (PTN) and C-D-MEM containing 50 ng/ml SDF-1 (SDF-1). Lysates were collected after 24 h (24 h) and 3 days (3d). Data were normalized over tubulin, used as loading control. **B**) Densitometric analysis showing the absolute quantification for Rac-1 expression with un-blocked receptors for PTN and SDF-1. **C**) Densitometric analysis showing the absolute quantification for Rac-1 expression with the blocked receptors for PTN and SDF-1.

2.6. Statistical analysis

In each experiment, 5 replicates have been used for each condition. Each of the experiments performed was repeated three times. The data shown are means ± standard deviation (SD). Data have been analysed by two researchers through established protocol used in the laboratory. Statistical significance of the presented results was calculated using ANOVA non-parametric Kruskal-Wallis method through the software InStat ™ (GraphPad Software, La Jolla, CA, USA). Values of $p < 0.05$ or less were considered significant.

3. Results and discussion

The formation of a functional endothelial layer in newly implanted vascular grafts is of crucial importance for their functionality [25]. EC seeding on prosthetic materials was proposed as a solution, but several studies demonstrated a significant cell loss after implantation and exposure to blood flow [26]. The enrichment of vascular grafts with molecules acting as chemoattractant for ECs has been proposed as a method to speed-up the re-endothelialisation of the implants *in situ*. Herein we proposed PTN as a promising molecule capable of promoting and fastening the formation of a functional endothelial layer. PTN has been shown to be an effective angiogenic agent both *in vitro* and *in vivo* [19,21]. Therefore, we investigated whether PTN is able to exert beneficial effects on EC behaviour with respect to SDF-1, a chemokine known to play a key role in angiogenesis and EC chemo-attraction [27–29]. In our experiments, the human umbilical vein cell line EA.hy926 was used. This cell line demonstrates highly differentiated functions characteristic of human vascular endothelium, while offering the advantages of immortality and stability through passage number thus allowing a more consistent responses to specific variables and greater reproducibility of data [30–33].

Our results show that the treatment with PTN can significantly improve EC viability *in vitro* with respect to SDF-1 and to CTRL cells. The MTT assay showed that after 24 h, the treatment with both PTN (absorbance at 570 nm: 0.176 ± 0.009) and SDF-1 (0.174 ± 0.015) was able to significantly increase cell viability compared to CTRL (0.145 ± 0.012; $p < 0.01$ vs. CTRL). This effect was enhanced with time, especially for the treatment with PTN that after 3 days (0.404 ± 0.072) significantly increased cell viability with respect to both SDF-1 (0.243 ± 0.036; $p < 0.001$) and CTRL (0.223 ± 0.033; $p < 0.001$). After 7 days, again PTN (1.429 ± 0.151) significantly increased cells' viability with respect to CTRL (0.664 ± 0.061; $p < 0.001$) and SDF-1 (1.197 ± 0.203; $p < 0.05$) (Fig. 1). Contrary to the results presented by Palmieri et al. [34], where the treatments with PTN were not able to induce a better viability of the treated ECs, in our experiments PTN was able to efficiently increase the viability of the treated ECs, confirming the effects observed by Brewster et al. [35,36] with the use of a chimeric PTN fusion protein. Moreover, the results described in the present study show how the treatment with PTN offer better results compared to the treatment with SDF-1.

The expression of CXCR4, SDF-1 receptor, and RPTP β/ζ, PTN receptor, was evaluated by Western Blot analysis (Fig. 2). Concerning the expression of CXCR4, after 24 h of incubation no significant differences were shown. However, after 3 days, the expression of CXCR4 in the presence of SDF-1 (2.48 ± 0.31) significantly increased with respect to CTRL (0.93 ± 0.14; $p < 0.001$). Interestingly, the treatment with PTN (1.79 ± 0.25; $p < 0.05$) was able to significantly increase the expression of CXCR4 compared to CTRL ($p < 0.05$) (Fig. 2A). Concerning RPTP β/ζ, after 24 h PTN showed an increased expression (1.32 ± 0.17) of the receptor with respect to SDF-1 (0.95 ± 0.13; $p < 0.01$). After 3 days, the expression of RPTP β/ζ in the presence of PTN (1.53 ± 0.21) was significantly increased compared to CTRL (0.71 ± 0.13; $p < 0.001$) and SDF-1 (0.93 ± 0.12; $p < 0.001$). (Fig. 2B). The results obtained here show that the treatment of EC with PTN not only induces an increase in the expression of PTN own receptor

RPTP β/ζ, but also an increase in the expression of CXCR4, suggesting a possible role of PTN in potentiating the response of EC to SDF-1 by means of increasing the expression of its receptor. Similar effects of PTN induction of the overexpression of other growth factor /chemokine receptor has already been reported [34], suggesting a role for PTN in potentiating the pro-endothelialisation effects of other ECs enhancer molecules.

The migration ability of EA.hy926 cells following the treatments with PTN and SDF-1 have been tested by the Wound Healing assay. As shown in Fig. 3A, CTRL showed a 14.7 ± 7.9% scratch reduction after 6 h, 19.2 ± 0.4 after 12 h and a 43.3 ± 5% scratch reduction after 24 h. The enrichment with PTN and SDF-1 significantly increased the rate of wound closure compared to CTRL: with PTN, 27.1 ± 6.3% scratch reduction after 6 h; 52.4 ± 7.1% after 12 h ($p < 0.001$ vs CTRL) and 75 ± 5% after 24 h ($p < 0.001$ vs. CTRL and $p < 0.01$ vs. SDF-1) was achieved; SDF-1 induced a scratch reduction of 17.5 ± 4.8% after 6 h, a 44.5 ± 2.4% after 12 h ($p < 0.001$ vs. CTRL) and 63.1 ± 7.4% after 24 h ($p < 0.001$ vs. CTRL) (Fig. 3B). EC migration was further analysed by Transwell migration assay. After 6 h, the average number of migrated cells per field counted for CTRL was 54.6 ± 6.2. In response to the treatment with PTN or SDF-1, the average number of migrated cells was significantly higher with respect to CTRL: in the presence of PTN, the average number of migrated cells per field was 73.7 ± 11.8 ($p < 0.01$ vs. CTRL) while in the presence of SDF-1 the average number of cells was 68.6 ± 8 ($p < 0.05$ vs. CTRL). After 24 h, the average number of migrated cells was not significantly modified for CTRL, with an average of 60.3 ± 9.5 cells per field. Instead, both PTN (95.3 ± 9.2 cells/field; $p < 0.001$ vs. CTRL) and SDF-1 (84.2 ± 6.7; $p < 0.001$ vs. CTRL) significantly increased the number of migrated cells (Fig. 4). These results show how the treatment with PTN is able to improve the migration rate and repair ability of treated ECs compared to CTRL, as shown by other groups [34,37,38]. Of interest, the effects on migration exerted by PTN resulted being higher than the one obtained with the treatment with SDF-1, once again suggesting that the use of PTN for the enrichment of vascular substitute may play an important role in the re-endothelialisation process.

The expression of Proliferating Cell Nuclear Antigen (PCNA) - a marker of cell proliferation, and Ras-related C3 botulinum toxin substrate 1 (Rac1) - a known marker for cell migration, was analysed with both the free and blocked receptor for PTN and SDF-1. The analysis on the blocked receptor group, in which cells were treated with blockers specific for PTN receptor, RPTP β/ζ, and SDF-1 receptor, CXCR4, has been performed to evaluate if the effects of the two proteins were limited to the interaction with their own specific receptor. As shown in Fig. 5A, after 24 h, SDF-1 (1.33 ± 0.08) significantly increased the expression of PCNA compared to CTRL (1.15 ± 0.06; $p < 0.01$); however, after 3 days, SDF-1 treatment significantly inhibited PCNA expression (0.51 ± 0.05) with respect to CTRL (0.95 ± 0.08; $p < 0.001$) and PTN (1.16 ± 0.04; $p < 0.001$) (Fig. 5B). With the blocked receptors, after 24 h of incubation, the treatment with PTN can significantly increase the expression of PCNA (1.72 ± 0.13) compared to CTRL (1.07 ± 0.09; $p < 0.001$) and SDF-1 (1.17 ± 0.07; $p < 0.001$). After 3 days, PTN treatment again improved the expression of PCNA (1.51 ± 0.09) compared to CTRL (0.84 ± 0.08; $p < 0.001$) and SDF-1 (0.50 ± 0.06; $p < 0.001$). Still, the treatment with SDF-1 significantly inhibited the expression of PCNA with respect to CTRL ($p < 0.01$) (Fig. 5C). The Western Blot analysis for the expression of Rac-1 (Fig. 6A) showed that, after 24 h of incubation with the free receptors, PTN (0.81 ± 0.13) and SDF-1 (0.82 ± 0.12) were able to significantly increase the expression of Rac1 compared to the CTRL (0.54 ± 9; $p < 0.05$). Moreover, after 3 days of incubation with free receptors, Rac-1 was significantly higher in the PTN group (1.63 ± 0.16) compared to SDF-1 (0.77 ± 0.09; $p < 0.05$) (Fig. 6B). With the blocked receptors, after 24 h of incubation no significant difference between the treatments was observed. However, after 3 days of incubation, the treatment with PTN (1.54 ± 0.10), along with CTRL (1.22 ± 0.08), were able to significantly increase the expression of Rac-1 compared to the SDF-1 group (0.90 ± 0.11; $p < 0.001$) (Fig. 6C). As the data show, with the free receptors, the treatment with PTN was able to significantly increase the expression levels of both PCNA and Rac1 compared to SDF-1 and the CTRL conditions. The PCNA expression analysis with the unblocked receptor, as shown, does not show statistically significant difference in between the CTRL and PTN condition, suggesting that the statistically significant increase observed with the MTT assay is due to an amelioration of cells viability rather than an increase in cells proliferation. However, the Western Blot analysis was conducted over a 3-days period, while the MTT analysis was conducted over a longer period of 7 days. Thus, we cannot conclude if PTN, over longer periods, is able to induce a proliferation of the treated ECs. However, it is interesting to note how SDF-1, compared to PTN, downregulates PCNA expression, suggesting that the use of PTN could be preferred over SDF-1 for vascular grafts enrichment. Concerning Rac1 expression with the un-blocked receptor, the results obtained confirm the one shown by the Wound Healing and Transwell migration assays. Interestingly, the blockage of the receptors has no influence on the effects of PTN on the expression of PCNA and Rac-1, resulting in a statistically significant increase of their expression compared to the effects exerted by SDF-1 with its receptor blocked. In the present study, we examined the interaction of PTN with RPTP β/ζ - despite the fact that it is not the only receptor that is able to bind to PTN [39,40], RPTP β/ζ is known to be the main receptor for PTN [36,41,42]. Moreover, it has been suggested that for PTN to exert his effects through certain receptors, such as Anaplastic Lymphoma Kinase (ALK), a previous interaction between PTN and RPTP β/ζ is necessary [43]. However, as shown by our present results, even when RPTP β/ζ is blocked, PTN is still able to affect the expression of the two marker proteins (PCNA and Rac-1). Thus, the effect obtained with the blocked RPTP β/ζ could be explained by the interaction of PTN with other receptors, resulting in an increased expression of PCNA and Rac-1 markers and conferring an added value to the use of PTN for vascular grafts enrichment.

4. Conclusions

In conclusion, due to the effects exerted on viability, migration and repair ability of ECs, PTN could be an interesting molecule for vascular grafts enrichment. The comparison of the effects of PTN with the effects exerted by SDF-1, along with the effects of PTN on the expression of PCNA and Rac-1 suggesting that PTN can exert his beneficial effects on ECs even when its primary receptor RPTP β/ζ is blocked, can open the path to the use of PTN for vascular grafts enrichments applications.

Conflict of interest

The authors declare no conflict of interests.

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The author contributions

Study Design: Francesco Copes, Francesca Boccafoschi, Diego Mantovani.

Data Collection: Francesco Copes, Martina Ramella, Luca Fusaro.

Statistical Analysis: Francesco Copes, Luca Fusaro.

Data Interpretation: Francesco Copes, Francesca Boccafoschi.

Manuscript Preparation: Francesco Copes, Francesca Boccafoschi, Diego Mantovani.

Literature Research: Francesco Copes, Francesca Boccafoschi.

Funds Collection: Diego Mantovani, Francesca Boccafoschi.

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