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The atheroma plaque secretome stimulates the mobilization of endothelial progenitor cells *ex vivo*

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ABSTRACT

Endothelial progenitor cells (EPCs) constitute a promising alternative in cardiovascular regenerative medicine due to their assigned role in angiogenesis and vascular repair. In response to injury, EPCs promote vascular remodeling by replacement of damaged endothelial cells and/or by secreting angiogenic factors over the damaged tissue. Nevertheless, such mechanisms need to be further characterized. In the current approach we have evaluated the initial response of early EPCs (eEPCs) from healthy individuals after direct contact with the factors released by carotid arteries complicated with atherosclerotic plaques (AP), in order to understand the mechanisms underlying the neovascularization and remodeling properties assigned to these cells. Herein, we found that the AP secretome stimulated eEPCs proliferation and mobilization *ex vivo*, and such increase was accompanied by augmented permeability, cell contraction and also an increase of cell-cell adhesion in association with raised vinculin levels. Furthermore, a comparative mass spectrometry analysis of control *versus* stimulated eEPCs revealed a differential expression of proteins in the AP treated cells, mostly involved in cell migration, proliferation and vascular remodeling. Some of these protein changes were also detected in the eEPCs isolated from atherosclerotic patients compared to eEPCs from healthy donors.
We have shown, for the first time, that the AP released factors activate eEPCs *ex vivo* by inducing their mobilization together with the expression of vasculogenic related markers. The present approach could be taken as a *ex vivo* model to study the initial activation of vascular cells in atherosclerosis and also to evaluate strategies looking to potentiate the mobilization of EPCs prior to clinical applications.
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Abbreviations: AP, atherosclerotic plaque; eEPCs, early endothelial progenitor cells; KDR/VGFR2, vascular growth factor receptor 2; ox-LDL, oxidized low-density lipoprotein; GM-SCF, granulocyte macrophage colony-stimulating factor; SDF-1, stromal cell-derived factor 1; MA, mammary arteries; PBMCs, peripheral blood mononuclear cells; FBS, Fetal bovine serum; UEA-1, *Ulex europaeus* agglutinin-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HMOX1, heme oxygenase 1; VKORC1, vitamin K epoxide reductase complex subunit 1; KCD12, BTB/POZ domain-containing protein KCTD12; UBE2I, ubiquitin conjugating enzyme E2I; PARP1, poly(ADP-ribose) polymerase 1; MCTS1, malignant T-cell amplified sequence 1; AIMP1, aminoacyl TRNA synthetase complex-interacting multifunctional protein 1; SMVC, smooth muscle vascular cells; MMP2, matrix metalloproteinase-2; LPA, lipoprotein A; MIF, macrophage inhibitory factor; CORO1B, coronin 1B; ITGAX, integrin alpha X/CD11c.

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1. Introduction

Since the discovery of endothelial progenitor cells (EPCs) in 1997 [1], these cells have become potential candidates for therapeutic applications pursuing tissue revascularization due to their assigned vascular regenerative properties. They seem to play a pivotal role in the pathogenesis of atherosclerosis and arterial healing after injury [2], by actively participating in vascular repairing, promoting angiogenesis and maintaining a thrombo- and inflammatory resistant surface [3].

Several cardiovascular risk factors, including diabetes, hypertension and hypercholesterolemia, have devastating impacts on EPCs [4]. In atherosclerosis itself, a decrease in the number of circulating EPCs associates with the development of disease [5]. In addition, known protective factors against atherosclerosis have been correlated with increased numbers of EPCs, such as high-density lipoprotein, estrogen, statins and angiotensin II inhibitors [6]. Moreover, CD34, KDR and c-Kit positive cells (characteristics of EPCs) have been detected in human atherosclerotic lesions, suggesting their involvement in the remodeling process [7].

Recent research has highlighted the need to discriminate between several subsets of EPCs, depending on the differentiation status or the capability to form colonies [8–10]. Thus, circulating pro-angiogenic cells, also named “early” endothelial progenitor cells (eEPCs), have been identified in the adult human peripheral blood as cells expressing the CD34 progenitor cell marker [11]. These cells derive from hematopoietic progenitors and share phenotypic traits with monocytes such as CD14 expression. eEPCs are able to promote neovascularization into ischemic tissues [1–3], and participate in vascular homeostasis, contributing to cell turnover by homing in sites with damaged endothelium, where they replace damaged endothelial cells and protect vessels from injury. Moreover, these cells seem to exert their proangiogenic and vasoprotective function mainly in a paracrine fashion by secreting angiogenic hemocytokines [11].

Many studies have focused on the analysis of how eEPCs become activated and how they participate in the neovascularization process. The goal is to be able to manipulate them, increasing their mobilization and other related functions, and therefore to use them for cell therapy purposes. *In vitro* assays have shown that eEPCs are susceptible to inflammatory and pro-atherogenic factors such as ox-LDL, GM-CSF and SDF-1, which thereby augment the neovascularization of ischemic tissues [12,13]. All these assays have evaluated individual or combined inflammatory and atherogenic factors but, to our knowledge, the effect of directly released factors from atherosclerotic plaques (AP) onto eEPCs has not been evaluated. Accordingly, the development of an experimental model to study the molecular and cellular mechanisms by which the AP interacts directly with the eEPCs is of great interest, and it could provide new insights about their physiological or pathological relevance [14].

In the current work we describe a new approach to evaluate the initial response of eEPCs following atherosclerotic damage, by *ex vivo* incubation of eEPCs derived from healthy individuals with the AP secretome obtained from atherosclerotic patients undergoing carotid endarterectomy. Since we first demonstrated that the AP secrete proteins to the surrounding media [15], different approaches have been carried out in which several proteins have been identified in the AP secretome as potential markers of atherosclerosis [16,17] and the effect of different drugs on the secretome composition has been evaluated [18]. Ultimately, the AP secretome constitutes a rich source of information to understand de atherosclerotic phenomenon since it represents the first factors that plaques send to the circulating media as a sign of damage. Herein, we show how eEPCs are activated and mobilized in response to initial contact with the molecules secreted by the APs to the circulating media.

2. Materials and methods

A detailed description is available in the Supplementary material online.

2.1. Sample acquisition

eEPCs were isolated from the blood of healthy donors and atherosclerotic patients undergoing carotid endarterectomy. Carotid artery segments complicated with AP from the same patients were also collected. In addition, non-atherosclerotic mammary arteries (MA) were obtained from patients undergoing cardiac valve replacements. All volunteers provided informed consent prior sample collection. This study was approved by the local Ethic Committee, and it follows the principles outlined in the Declaration of Helsinki.

2.2. eEPCs isolation and culture

eEPCs were isolated from peripheral blood mononuclear cells (PBMCs) following standard procedures, as described [19]. Mononuclear cells were plated in fibronectin coated plates (10 μg/ml) and incubated in 2% FBS/EBM-2V media containing Single Quots growth factors (basal medium). After 4 days, non-adherent cells were discarded by removing cell media and attached cells were allowed to grow until day 7 in fresh media. Different sets of isolated eEPCs were used for the assays. By day 7, cells were confirmed as early EPCs by flow cytometric analysis, UEA-1 lectin labeling and DiI-acLDL up-take, as described [20]. Detailed description of cell isolation and culture are provided in the Supplementary material online.

2.3. Atherosclerotic plaque isolation and culture

Carotid endarterectomies complicated with AP and MA taken as non-atherosclerotic controls were cultured as described [15,21]. Briefly, samples were dissected with a scalpel, separating the non-complicated area, the area complicated with atheroma plaque but not thrombosed region and the ruptured and thrombosed region of the carotid material, as described [15]. For this study, we used the secretome of the complicated but not thrombosed segments, comparing it with the secreted factors of mammary arteries, taken as controls. The surgical pieces (about 0.3–0.4 cm length and 200 mg weight on average) were washed several times with RPMI-1640 medium containing Penicilin/Streptomycin and cultured for 72 h before collecting the supernatants, which were either used immediately or stored at −80 °C.

2.4. Viability and proliferation assays

At day 6, 5×10^4 cells were transferred to fibronectin pre-coated 96-well plates in 2%FBS medium. By day 7, cells were incubated with either fresh medium (eEPCs cont) or a mixture (1:1) of basal medium and AP (eEPCs + AP) or MA (eEPCs + MA) supernatants. Cell viability was measured 48 h and 72 h later by adding 20 μl MTT (5 mg/ml in PBS) and incubating for another 4 h at 37 °C, 5% CO_2. Cells were finally incubated with DMSO for 10 min before measuring optical density (OD).

eEPC proliferation in response to the AP secretome was measured by immunofluorescence staining of the proliferation marker KI67 [22]. Cells, 5×10^5 were plated by day 6 in fibronectin pre-treated coverslips into 24 well plates and incubated at day 7 with either 700 μl of basal media (eEPCs cont), or a mixture (1:1) of AP (eEPCs + AP) or MA secretome (eEPC + MA) with basal medium. After 48 h, supernatants were discarded and cells fixed with 4% PFA, and incubated with anti-human KI67 and the secondary Alexa 488- antibodies followed by nuclei staining with DAPI. Experiments were performed in duplicate for each condition and repeated 3 times with AP or MA secretomes and eEPCs control cells from different individuals (n:6). Quantification of KI67 positive staining was done manually (ten random fields per insert) under a fluorescence microscope.

2.5. Transwell migration assays

At day 7, 1×10^5 eEPCs from healthy individuals were resuspended in 0.2% FBS/EBM-2 V media and plated into the upper side of a fibronectin-pretreated Transwell chamber (8 μm pore size). In the outer chamber, 800 μl of either 5% FBS/EBM-2 V media (eEPCs cont) or a mixture (1:1) of AP (eEPCs + AP) or MA secretome (eEPC + MA) with 2%FBS basal medium, were added. After 6 h at 37 °C, 5% CO_2, cells on the bottom side of the filter were fixed with 4% PFA and cells at the top were removed. Experiments were performed in triplicate for each condition and repeated 3 times with AP secretomes and eEPCs control cells from different individuals (n:9). For quantification of cell migration, nuclei were stained with DAPI and manually counted (ten random fields per insert) under a fluorescence microscope.

2.6. Permeability assay

By day 6 cultured eEPCs (1×10^5) were plated onto fibronectin precoated transwell inserts, as described above, adding basal medium to the outer chamber. They were left overnight to create a stable monolayer. On day 7, the media in the outer chamber was replaced with fresh medium. In the inner chamber we added fresh medium (negative control), or 5 μg/μl of FITC-dextran 40 KDa in either basal medium (eEPCs cont), AP supernatant (eEPCs + AP) or MA supernatant (eEPCs + MA). Fluorescence levels in the outer chamber were measured after 30 min, 1 h and 2 h incubation.

2.7. Time lapse live microscopy and immunofluorescence

Isolated eEPCs (1×10^5) were seeded on fibronectin-coated plates. At day 7 phase contrast images were acquired at 37 °C, 5% CO_2 every 5 min on a fully monitorized, multifield Nikon Eclipse microscope. Cells were imaged for 1 h before stimulation, and then incubated with either 5%FBS basal medium (eEPCs cont), or a mixture (1:1) of basal media and the AP (eEPCs + AP) or MA secretome (eEPCs + MA). The same fields were further imaged for another 6 h.

For immunofluorescence microscopy, the following antibodies were used: anti-α-tubulin-FITC; anti-vinculin-FITC; Alexa-fluor phalloidin; and DAPI for nuclear staining. Fluorescent images were acquired on the same approximate fields. Cell morphology parameters and adhesions counting were obtained with the Cell Profiler analysis software [23].

2.8. Proteomic analysis of specifically released proteins by APs

The protein content from both the AP and MA secretome (100 μg) was precipitated with 100% acetone, overnight at −20 °C. Pellets were collected by centrifugation and resuspended in 6 M urea for tryptic in-solution digestion, prior mass spectrometry (MS) analysis. Samples from 7 different individuals were analyzed per condition (AP and MA secretome). Protein identification and further comparison was performed with the Protein Scape platform (Bruker). IPA software was applied to evaluate the functional roles of the identified proteins.

2.9. Evaluation of intracellular protein changes in eEPCs

At day 7, eEPCs from healthy individuals were incubated *ex vivo* with basal medium (eEPCs cont) or a mixture (1:1) of 5% FBS medium and AP secretome (eEPCs + AP). After 24 h at 37 °C, 5% CO_2, cells were collected, washed with PBS, centrifuged and the pellets were snap-frozen in liquid nitrogen. A third set of cells, isolated from the atherosclerotic donors (eEPCs pat), were also cultured and collected. eEPCs pellets were resuspended in lysis buffer and trypsin digested prior MS analysis. Quantitative analysis was performed by differential dimethyl labeling [24,25]. Samples from three different individuals were analyzed in triplicates and compared with the Proteome Discoverer software. Additional details are provided in the Supplementary materials online.

2.10. Validation of protein expression changes by Western blot

The levels of CD9 and HMOX1 were measured by Western blot (WB) following standard procedures. Proteins (20 μg) from cell lysates were separated on 12% polyacrylamide gels, transferred to a PVDF membrane and immunoblotted with the primary rabbit-anti-CD9 (1:200) and mouse-anti-HMOX1 (1:200) antibodies. The mouse-anti α-tubulin antibody (1:5000) was used for loading control. Blots were imaged employing a ChemiDoc Touch System (Biorad).

2.11. Statistical analysis

Graphs and data analysis of functional assays were obtained using the GraphPad Prism 6 software. For statistical significance analysis either an unpaired two-tailed *t*-test (for comparing two conditions) or a two-way ANOVA with Tukey's multiple comparison post-test (for comparing multiple conditions) was used. All significances indicated are compared with the eEPC control condition unless stated. Unless indicated, experiments were repeated three times at different dates with two replicates per condition each time, using AP and MA supernatants from two different individuals per experiment (n:6). Freshly isolated batches of healthy eEPCs were used each time. Significance levels: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Results are shown as mean ± SEM.

Protein related statistics were obtained with Proteome Discovery (Quantitative analysis) and IPA software. In addition, a hierarchical classification was done with the Gene-E software to represent the differential protein expression levels between eECP control, eEPC + AP and eEPC patient cells. For this purpose, fold change rate data obtained from the MS analysis (Proteome Discovery) were pre-processed with the Gene-E software and clustering itself was done with the following parameters: Euclidian distances for cluster conditions (upper tree) and Euclidean (normal) distance between proteins.

3. Results

3.1. Characterization of eEPCs ex vivo

eEPCs were isolated and cultured as described [19]. In addition of erythrocytes and other white cells, cultures initially (day 0–4) contained a population of small round cells that acquired a spindle-shape by day 7, with an endothelial cell-like morphology (Supplementary Fig. S1A). The majority of the *ex vivo* differentiated eEPCs were positive for the double labeling with ac-LDL up-take and UEA-1 binding (Fig. S1 B), as described [20]. In addition, at day 7 these cells were also positive for the markers CD34, CD45 and KDR (Fig. S1 C), in agreement with previous reports [20,26], confirming the identity of the eEPCs.

3.2. AP secretome affects the proliferation and activates migration of healthy eEPCs

The expansion of EPCs by proliferation and their mobilization to the injured tissues constitute two key processes in the promotion of vascular remodeling by these cells. We first evaluated the effect of the AP secretome on the viability (Supplementary Fig. S2) and proliferation of isolated eEPCs (Fig. 1A). Cell viability was higher after 48 h incubation of healthy eEPCs with the AP secretome (eEPC + AP) ($^{*}P < 0.001$) and less significantly with the MA secretome (eEPC + MA) ($^{*}P < 0.05$), compared to controls. Furthermore, the levels of KI67, a known marker of proliferation, increased significantly in both, eEPC + AP ($^{***}P < 0.001$) and eEPC + MA treated cells ($^{*}P < 0.5$) compared to eEPC controls. The percentage of eEPC proliferating cells was significantly higher in eEPC + AP and eEPC + MA treated cells ($^{**}P < 0.01$) after normalizing the number of KI67 positive cells *vs* the total number of cells. According

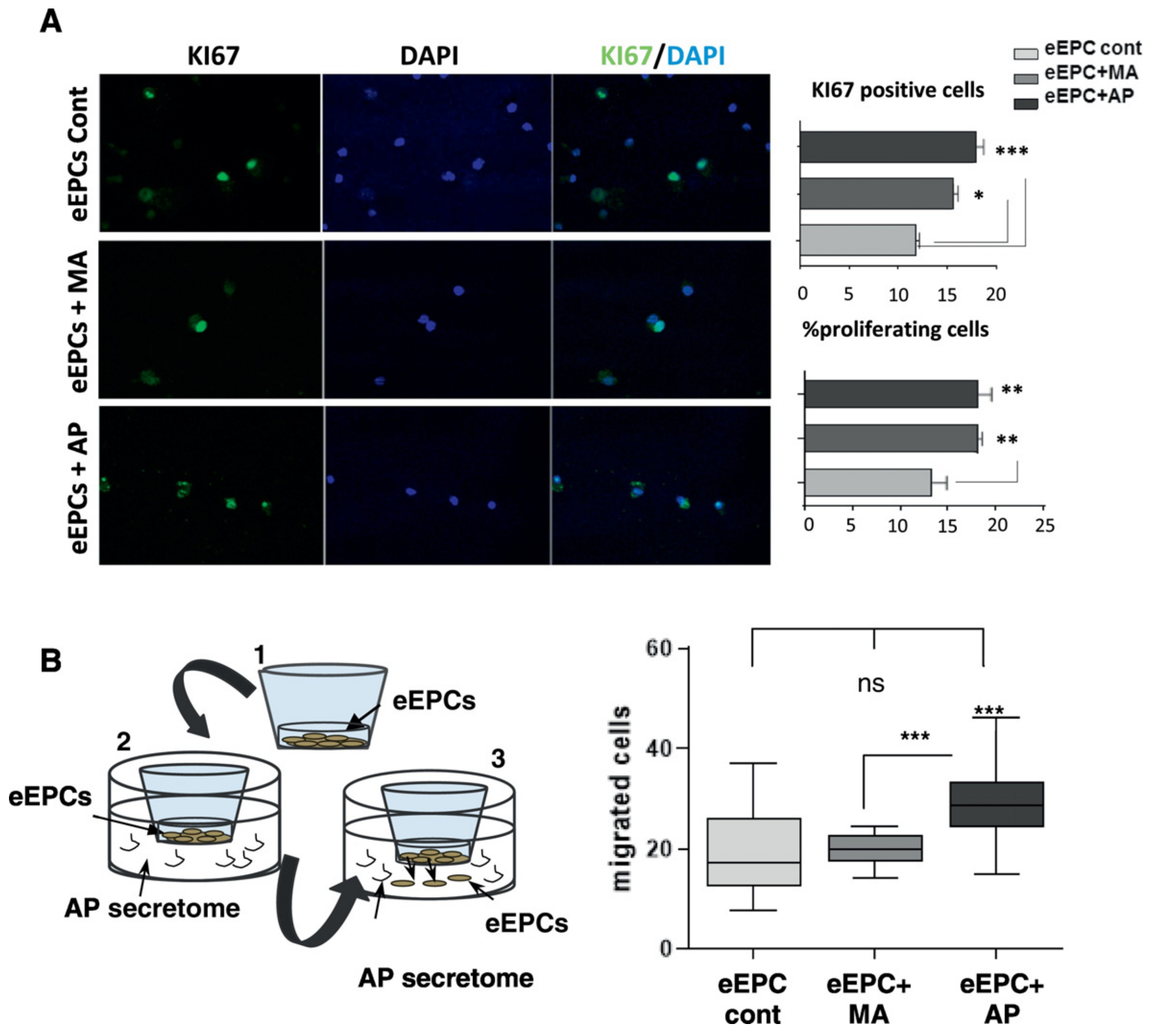

Fig. 1. AP secretome affects the proliferation and migration of healthy eEPCs. A) eEPCs proliferation significantly increased after 48 h incubation with the AP secretome (eEPC + AP) but also with the MA secretome (eEPC + MA) compared to the control group (eEPC cont) (n:6). B) eEPCs migration was evaluated with transwell chambers (left). After 6 h, cells that crossed the transwell membrane were counted. Cell migration was significantly induced in eEPC + AP treated cells compared to eEPC cont or to eEPCs incubated with the MA supernatants, which showed similar migration profile than control cells (n:9). Compared to eEPC cont: ns (not significant changes): *P < 0.05; **P < 0.01; ***P < 0.001.

to these results both, the AP and the MA secretome stimulated the proliferation of healthy eEPCs.

The effect of the AP secretome on the migratory properties of eEPCs was assessed by transwell assay (Fig. 1B). The AP supernatants significantly induced the migration of healthy eEPCs through the transwells compared with the non-treated cells (***P < 0.001) or with the same eEPCs incubated with the MA supernatant (***P < 0.001), which provoked a similar migratory response than the control situation. Thus, only the factors secreted by the atherosclerotic carotids, and not the mammary arteries, specifically stimulated the migration of healthy eEPCs, presumably by exerting a chemo-attractant effect over the cells.

3.3. AP secretome affects eEPCs permeability and induces contraction and morphological changes

The influence of the AP secretome over the permeability of eEPCs was evaluated by incubating them in transwell chambers in presence of FITC-dextran (Fig. 2A), as described [27]. Cultured eEPCs did not form mature, VE-cadherin expressing, cell-cell contacts (not shown), but a homogeneous monolayer could be observed on tested conditions. Our results indicated a significant increase in the permeability of eEPCs after 30 min (**P < 0.01) and also after 1 h (*P < 0.05) of incubation with the AP secretome, compared to eEPC controls. Further analysis of the time-lapse movies of eEPCs 1 h before and after 6 h of AP secretome incubation, showed that eEPCs rapidly contracted and changed morphology in response to the AP secretome (eEPC + AP) compared to non-treated (eEPC cont) or eEPC + MA treated cells (Fig. 2B and Electronic Supplementary Material, Video S1).

We then evaluated whether the increase seen in permeability could be related to changes in cell morphology, by quantifying different shape and morphological parameters, right after time-lapse. eEPCs exposed to the AP secretome showed a more compact and round shape regarding non-treated (eEPC cont) or eEPC + MA treated cells, indicative of cell contraction (Fig. 2C). The area occupied by the cells after incubation with the AP secretome was reduced (***P < 0.001), and as a consequence there was a reduction in cell-cell contacts (***P < 0.001), as confirmed by quantitative neighbor analysis [28]. These changes were not seen in eEPC cont or cells incubated with non-atherosclerotic secretomes (eEPC + MA).

3.4. AP secretome induces an increase in eEPCs migratory adhesions

Given the role of vinculin as an intracellular component of focal adhesions in cell migration [29,30], we next analyzed migration related adhesions by vinculin inmuno-staining in eEPCs cont or in the treated cells (Fig. 3A). We measured the number and area of vinculin rich adhesions in eEPCs, and found that cells exposed to the AP secretome presented a significantly higher number of adhesions per cell (**P < 0.01;

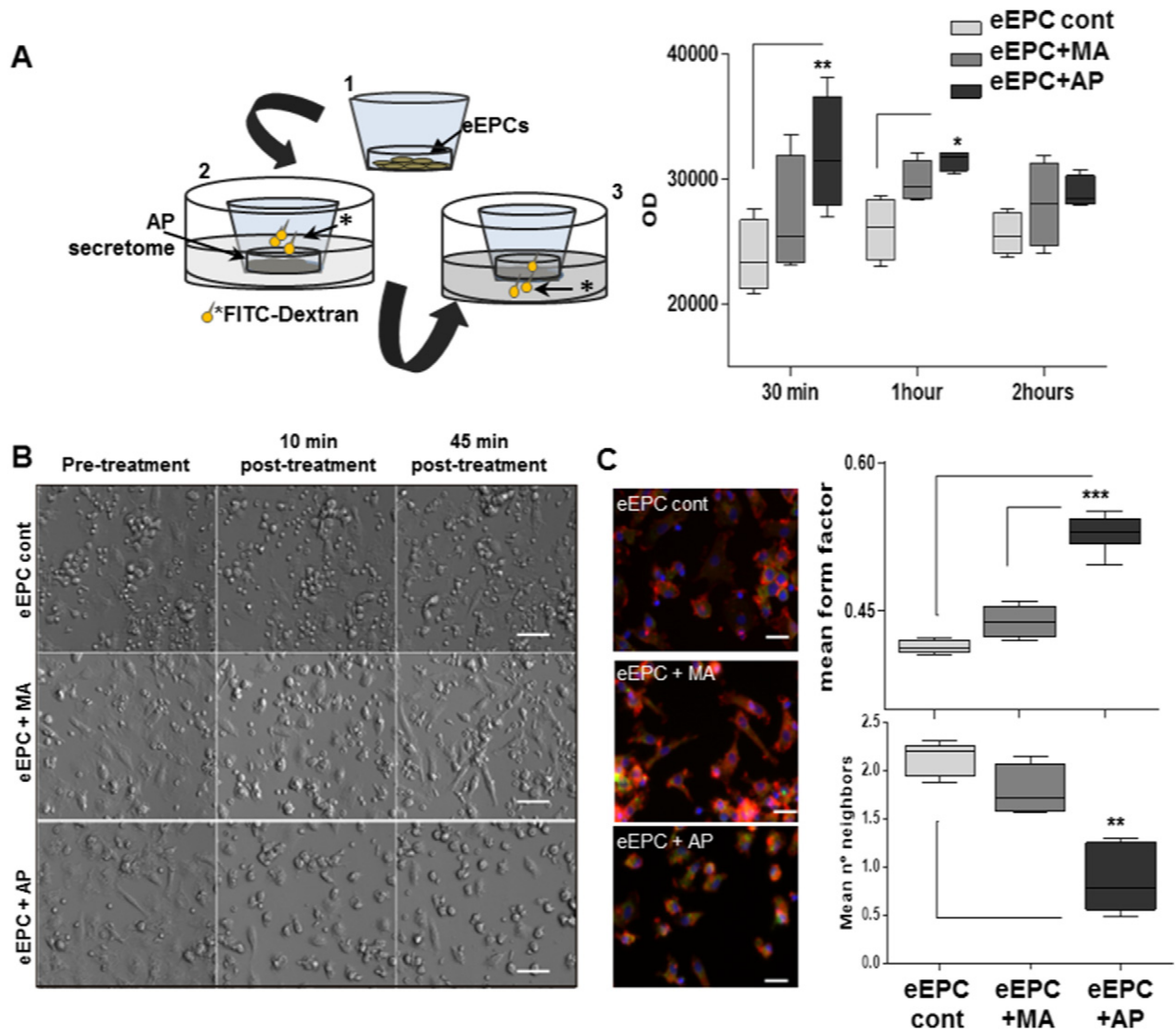

Fig. 2. AP secretome affects eEPCs permeability and induces eEPCs contraction and morphology changes. A) Permeability changes were assessed by measuring the levels of FITC-Dextran passing through the eEPC monolayer in a transwell system (left panel). Permeability increased significantly in eEPCs after 30 min and 1 h incubation with the AP secretome, decreasing after 2 h (n:6). B) Time-lapse microscopy showed that the AP secretome induced an immediate contraction of healthy eEPCs (10 min) and such effect remained over time, compared to eEPC cont or eEPC + MA treated cells (n:9). C) Quantification of shape and morphological parameters after immunofluorescent labeling with F-actin (red) and a-tubulin (green) confirmed a reduction of the area occupied by eEPC + AP treated cells and also a reduction of cell-cell contacts (n:9). Compared to eEPC cont: *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 3B). These adhesions had also a bigger area (*P < 0.01; Fig. 3C) and a more intense vinculin staining (***P < 0.001; Fig. 3D) compared to non-treated cells. Previous reports indicated that a dynamic adhesion to the extracellular substrate is necessary for cell migration and an increased number of dynamic focal contacts have been correlated with increased cell migration [29,30]. Our results indicate that the AP secretome modifies the adhesive properties and cytoskeletal morphology of eEPCs increasing their mobilization.

3.5. AP specific released factors

In order to identify protein factors that could be responsible for the differential effect of the AP secretome, we performed a proteomic analysis comparing the secretome of carotid atherosclerotic arteries (AP, n:7) and non-atherosclerotic mammary arteries (MA, n:7). In total, we identified 539 proteins, 242 of them detected only in the AP secretome while 94 there were identified only in the MA fraction. The rest of proteins were common for both secretomes (Supplementary Table ST1).

We then focused our analysis in the proteins secreted specifically by APs. According to the analysis performed with IPA software (Table 1), the proteins identified were significantly related to several cell functions: movement (52 molecules), proliferation (76), chemotaxis (19), angiogenesis (24) and vasculogenesis (21). 19 proteins were specifically related with the movement of ECs. Proteins released by APs also correlated to peripheral vascular disease (21), acute coronary syndrome (15), infarction (17), or vascular lesion (9).

3.6. AP secretome induces protein expression changes on healthy eEPCs

We finally applied a quantitative proteomic approach [24,25] to evaluate whether the AP secreted factors can promote functional and intracellular changes in healthy eEPCs. Thus, we compared the proteins expressed by eEPCs from healthy donors (eEPC cont), healthy eEPCs incubated 24 h with the AP secretome (eEPC + AP) and by eEPCs isolated from atherosclerotic patients (eEPC pat).

From the total 2043 proteins identified, we quantified the expression changes of 1179 proteins (detected in at least 2 out of 3 replicates). In eEPC + AP treated cells, 10 proteins were down-regulated and 20 up-regulated compared to non-treated cells (eEPC cont), while the eEPC pat showed 32 down-regulated and 68 up-regulated proteins *versus* the eEPC cont (Fig. 4A). A hierarchical cluster including all differential protein patterns and some protein examples is shown in Fig. 4B.

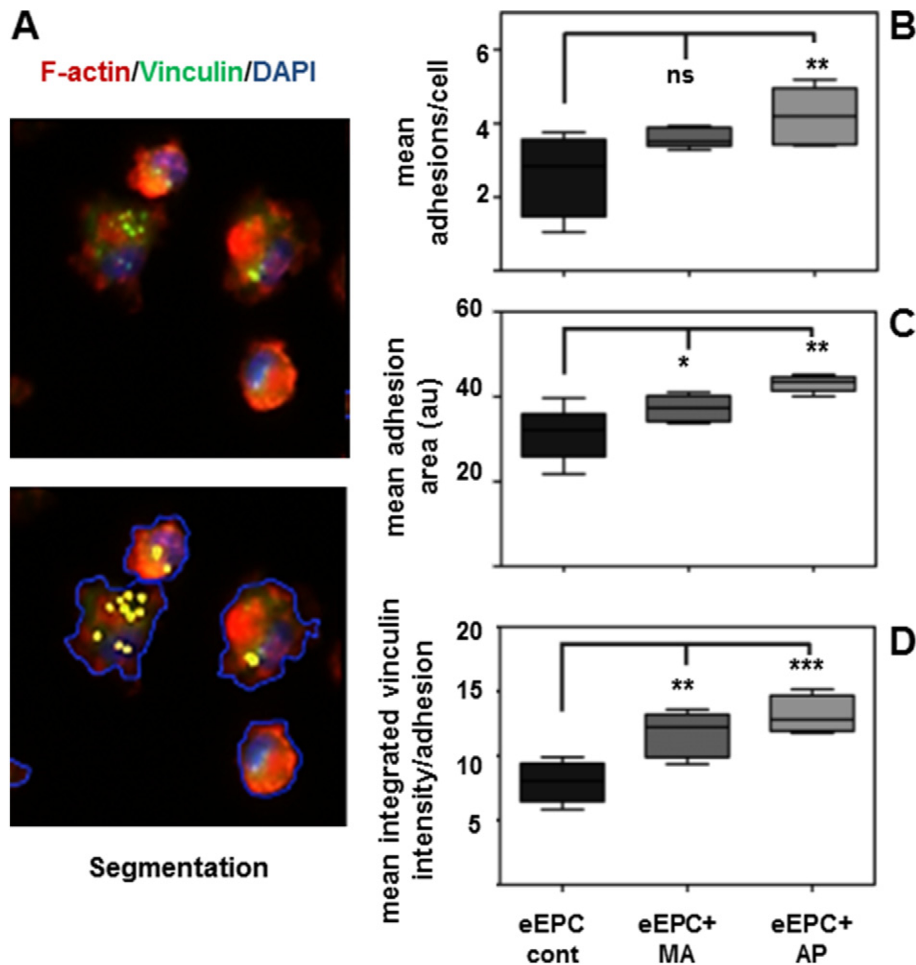

Fig. 3. AP secretome increases eEPCs migratory adhesions. A) eEPCs were immuno-stained with vinculin and F-actin after incubation with either the MA (eEPC + MA) or the AP secretome (eEPC + AP), or left untreated, and adhesion was measured by quantification of vinculin levels. An example of the segmentation procedure is shown. B) The number of adhesions per cell increased significantly only in eEPC + AP treated cells compared to controls. C) These adhesions had a bigger area and D) higher intensity of vinculin compared to eEPC cont and eEPC + MA treated cells (n:9). Compared to controls: ns (not significant changes): *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Supplementary Tables S2 and ST3 contain complete information concerning protein identification and quantification data.

Eight of the protein changes found in the healthy eEPCs in response to the AP secretome were also detected in the eEPC pat: HMOX1, CD9, VKORC1 and KCD12 were up-regulated while UBE2I, PARP1, MCTS1 and AIMP1 appeared down-regulated. These proteins could represent initial changes that remained over time, maintained in the eEPCs of chronical patients. The analysis by WB of CD9 and HMOX1 expression in healthy eEPCs after 1 h and 24 h of stimulation with the AP secretome as well as in the eEPC pat, confirmed the proteomic results (Fig. 4C).

Further analysis with the IPA software indicated that the differential proteins detected in the eEPC + AP treated cells have been mostly involved in the cellular functions: cell proliferation (18 molecules), migration (8 molecules), cell death (10), vasculogenesis (4) or wound-healing (2). The set was also enriched in proteins with roles in vascular-related diseases, including peripheral vascular disease (5), acute coronary syndrome (5), stroke (3) and stabilization of atherosclerotic lesion (1) (Fig. 5A and Supplementary Table ST4). Interestingly, most of the down-regulated proteins were associated to proliferation and also apoptosis/cell death (AIMP1, UBE2I, PARP1, MCTS1), while the up-regulated ones were mainly associated to cell migration but also to vascular-related diseases.

Regarding the proteins up-regulated in eEPC pat, IPA analysis indicated that these proteins are mostly involved in cell proliferation (32 molecules), cell migration (20) or movement (22), vasculogenesis (13), invasion (17) or adhesion of ECs (4). These proteins were related to peripheral vascular disease (9), acute coronary syndrome (5), stroke (4), accumulation of cholesterol (2) and atherosclerotic lesions (8) (Fig. 5C and Table ST4).

4. Discussion

Diverse experimental and clinical studies have corroborated that bone marrow-derived or peripheral blood-derived eEPCs significantly contribute to neovasculogenesis and angiogenesis after tissue ischemia [31]. On the other hand, other studies indicate that in humans, cardiovascular risk factors impair number and function of eEPCs, potentially restricting the therapeutic potential of progenitor cells. To overcome such limitations, eEPCs could either be extensively amplified *in vitro* or be genetically modified to improve their vasculogenic properties [32]. In this regard, understanding how these cells are mobilized in response to the atherosclerotic damage and the mechanisms regulating such recruitment becomes essential in order to enable their use in vascular medicine. Taking this into account, we decided to evaluate the direct effect of the factors released by atherosclerotic but non-thrombosed, carotid arteries, termed here as the AP secretome, over healthy eEPCs *ex vivo*, in order to evaluate how these cells become activated and therefore how to modulate them in an atherosclerotic but still not thrombotic situation.

We have shown, for the first time, that the *ex vivo* incubation of healthy eEPCs with the AP secretome, induces in these cells several changes resembling the initial response to the atherosclerotic damage.

Table 1
Functional classification of proteins released only by atherosclerotic carotid arteries (AP secretome), compared to the non-atherosclerotic mammary arteries secretome. Classification was made by the IPA software which, based on biomedical literature and integrated databases, allows to determine the most probably pathways or functions in which the proteins of interest are involved. The table includes the main categories containing related functions or diseases, number of molecules per category identified in the AP secretome, the p-values assigned, and gene names.

Categories	Diseases or functions annotation	Molecules	*p*-Value	Molecules
Cellular Movement	Cell movement	52	4,40E-15	APOE, ARPC2, C5, CNN2, COL4A1, CRP, CRYAB, CST3, CTGF, CTSB, CXCL1, CXCL5, CXCL8, CYR61, GPI, GRN, HLAA, HNRNPK, HRG, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IL6, KRT2, LAMB1, LCP1, LPA, LRP1, LSP1, MGP, MIF, MMP2, NME1, NOV, ORM1, PPIB, PROS1, S100A4, S100A9, SAA1, SERPINA3, SERPINC1, SERPINE1, SERPINF1, SPP1STAB1, TAGLN2, THBS2, TIMP1, TIMP2, WARS
	Migration of cells	48	2,74E-14	APOE, ARPC2, C5, CNN2, COL4A1, CRP, CRYAB, CTGF, CTSB, CXCL1, CXCL5, CXCL8, CYR61, GRN, HLAA, HNRNPK, HRG, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IL6, KRT2, LAMB1, LCP1, LPA, LRP1, LSP1, MGP, MIF, MMP2, NOV, ORM1, PPIB, PROS1, S100A4, S100A9, SAA1, SERPINA3, SERPINC1, SERPINE1, SERPINF1, SPP1, STAB1, THBS2, TIMP1, TIMP2, WARS
	Invasion of cells	28	4,18E-09	ACTA2, ARHGDIA, CD14, CTGF, CTSB, CTSL, CXCL8, CYR61, GPI, GRN, HTRA1, IL6, KRT8, LRP1, MIF, MMP2, NME1, NOV, PLS1, S100A10, S100A9, SERPINE1, SERPINF2, SPP1, TAGLN2, THBS2, TIMP1, TIMP2
	Chemotaxis of cells	19	1,65E-08	C5, CXCL1, CXCL5, CXCL8, CYR61, IL6, LCP1, LPA, MIF, MMP2, NOV, PPIB, S100A4, S100A9, SAA1, SERPINA3, SERPINE1, SPP1, THBS2
Cardiovascular System Development and Function, Cellular Movement	Cell movement of endothelial cells	19	8,96E-10	APOE, CNN2, COL4A1, CRYAB, CXCL1, CXCL8, CYR61, HRG, IGFBP3, MGP, ORM1, PROS1, SAA1, SERPINE1, SERPINF1, STAB1, THBS2, TIMP1, TIMP2
	Cell movement of microvascular endothelial cells	6	7,72E-07	CXCL1, CXCL8, ORM1, THBS2, TIMP1, TIMP2
	Migration of endothelial cells	18	1,71E-09	APOE, CNN2, COL4A1, CRYAB, CXCL1, CXCL8, CYR61, HRG, IGFBP3, MGP, ORM1, PROS1, SAA1, SERPINE1, SERPINF1, STAB1, TIMP1, TIMP2
Cardiovascular System Development and Function, Organismal Development	Angiogenesis	24	1,09E-08	APOE, COL4A1, CRYAB, CXCL1, CXCL8, CYR61, HRG, IGFBP3, IGFBP4, IL6, MGP, MMP2, NOV, ORM1, PROS1, PTX3, SAA1, SERPINC1, SERPINE1, SERPINF1, STAB1, THBS2, TIMP2, WARS
	Vasculogenesis	21	2,23E-08	APOE, COL4A1, CRYAB, CXCL1, CXCL8, CYR61, HRG, IGFBP3, IGFBP4, IL6, MGP, MMP2, ORM1, PROS1, PTX3, SAA1, SERPINC1, SERPINE1, SERPINF1, STAB1, TIMP2
Cellular Growth and Proliferation	Proliferation of cells	76	7,85E-14	ACTG1, AHSG, APOD, APOE, C5, CAPN1, CAPZA1, CCT2, CD14, CD163, COL4A1, CORO1B, CTGF, CTSB, CTSD, CTSL, CXCL1, CXCL5, CXCL8, CYR61, DCTN2, EMILIN2, FBP1, FRZB, FTL, GLUL, GPI, GPNMB, GRN, HLA-DRB1, HNRNPK, HRG, HSP90AA1, IFI30, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IL6, KRT2, LAMB1, LCP1, LMNB1, LPA, LRP1, MGP, MIF, MMP2, NME1, NOV, PEBP1, PLS3, PRG4, PROS1, PTX3, PXDN, S100A11, S100A4, S100A9, SAA1, SERPINC1, SERPINE1, SERPINF1, SERPINF2, SPP1, TAGLN2, THBS2, TIMP1, TIMP2, TMSB10/TMSB4X, TNFRSF11B, TRAP1, TTR, TXN, WARS, WISP2
Cell Death and Survival	Cell death	59	1,06E-10	AKR1B1, APOB, APOC3, APOE, APOL1, ARHGDIA, B2M, C5, C8A, C8B, C8G, C9, CANX, CCT2, CFB, CFHR3, CFI, CFL1, CRP, CRYAB, CST3, CTGF, CTSB, CTSD, CXCL1, CXCL8, CYR61, DCTN2, EMILIN2, FRZB, GPI, GRN, HLAA, HLAB, HLADRB1, HNRNPK, HRG, HSP90AA1, HSP90B1, IGFBP3, IL6, LRP, MIF, NME1, RNASE1, S100A11, S100A9, SAA1, SERPINC1, SERPINE1, SERPINF1, SPP1, TAGLN2, TIMP1, TIMP2, TNFRSF11B, TRAP1, TXN, TYMP
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	Transport of cholesterol	12	3,79E-12	APOA2, APOA4, APOB, APOC1, APOC3, APOE, CANX, IL6, NPC2, PLTP, PON1, SAA1
	Transport of lipid	15	9,43E-12	APOA2, APOA4, APOB, APOC1, APOC3, APOE, CANX, CD14, CFHR4, IL6, LRP1, NPC2, PLTP, PON1, SAA1
Inflammatory Response	Inflammatory response	24	1,10E-11	AHSG, APOE, C5, CD14, CPB2, CRP, CXCL1, CXCL5, CXCL8, IL6, KLKB1, LCP1, LGALS3BP, LPA, MIF, ORM1, PPIB, PROS1, PTX3, S100A9, SAA1, SERPINA3, SERPINE1, SPP1
Cardiovascular Disease	Peripheral vascular disease	21	9,26E-08	APOB, APOE, B2M, CD163, COL4A1, CST3, CTSB, F13A1, F9, HLADRB1, IFI30, IGFBP5, KCTD12, LCP1, LPA, PLTP, PROS1, RRBP1, S100A9, SERPINC1, SPP1
	Vascular lesion	9	3,84E-07	ACTA2, COL3A1, CRP, CST3, CTSL, MMP2, RRBP1, TIMP1, TIMP2
	Infarction	17	3,22E-09	APOB, APOE, ATP5J, C5, CD14, CD163, CRP, F13A1, HTRA1, IL6, LPA, LRP1, PON1, PROS1, PTX3, SERPINC1, SERPINE1
	Acute phase reaction	5	9,61E-08	AHSG, APCS, CD14, CRP, IL6
Cardiovascular Disease, Organismal Injury and Abnormalities, Skeletal and Muscular Disorders	Acute coronary syndrome	15	1,45E-07	APOB, APOE, ATP5J, C5, CD14, CD163, CRP, F13A1, IL6, LPA, LRP1, PON1, PTX3, SERPINC1, SERPINE1
	Myocardial infarction	14	1,59E-07	APOE, ATP5J, C5, CD14, CD163, CRP, F13A1, IL6, LPA, LRP1, PON1, PTX3, SERPINC1, SERPINE1
Cardiovascular Disease, Hematological Disease	Thrombosis	16	2,95E-13	C5, CFB, CFHR3, CFI, COL4A1, CRP, CTGF, F13B, F9, HRG, IL6, MMP2, PON1, PROS1, SERPINC1, SERPINE1

In our study, eEPCs proliferation increased after 48 h of incubation with the AP and the mammary artery (MA) secretome, probably in response to common released factors, but only the AP secretome induced an increase of cell migration and extravasation through the transwell membranes just a few hours after first contact. Such increase was accompanied by an increase of cell permeability right after the incubation only with the AP supernatant, detected by a higher FITC-dextran transference from the inner to the outer compartment. This effect could be caused by changes in cell morphology, since cells rapidly contracted in response to the AP secretome, as observed in our time-lapse microscopy assay. In addition, a reduction in the number of cell-cell contacts was seen in the eEPC + AP treated cells, as well as a reduction of the area occupied by them, compared with the control or the MA treated cells. All these data indicate that the AP secretome stimulated the activation of a motility-prone phenotype of eEPCs, without reducing the number of cells, as seen in the proliferation assay. We evaluated if

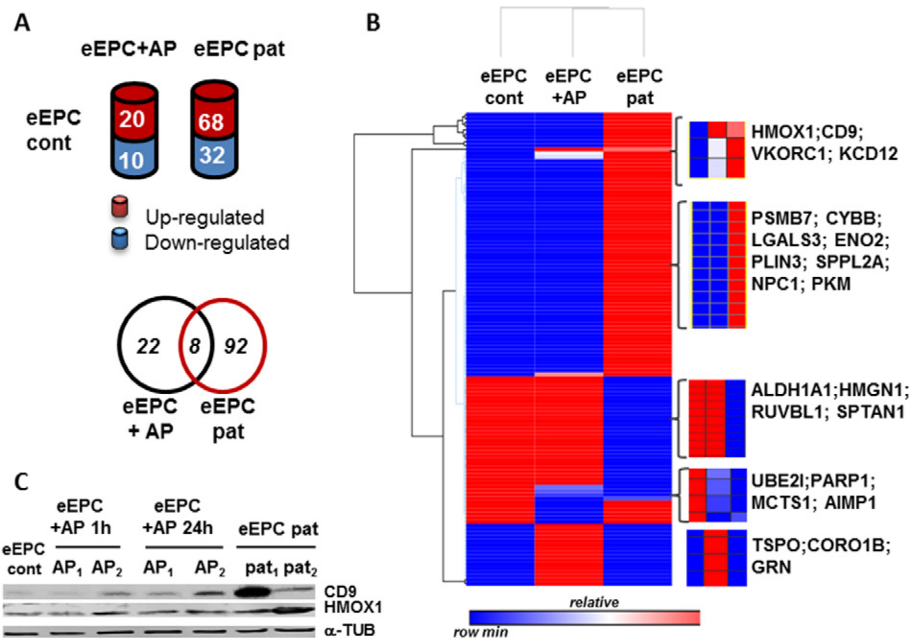

Fig. 4. Differential protein expression of eEPCs in response to AP secretome. MS-based quantitative analysis revealed a differential expression pattern in eEPCs after 24 h incubation *ex vivo* with the AP secretome (eEPC + AP) compared to basal cells (eEPC cont) or eEPCs isolated from the same AP patient donors (eEPC pat). Three different sets of samples eEPC cont, eEPC + AP and eEPC pat were compared, and each of these samples were run in triplicates. A) A graphical description of the protein changes seen in at least two of the three sets of cells compared, and the overlapping proteins between comparisons, is shown. B) The hierarchical cluster includes the differential expression patterns detected in at least two out of the three sets of samples. As an example, some of the protein changes have been zoomed, including proteins altered in both, eEPC + AP treated cells and eEPC pat, compared to eEPC cont. C) The results obtained by WB for HMOX1 and CD9 in eEPCs of two different patients and eEPC + AP treated cells (after 1 h and 24 h treatment) validated the proteomic data.

such mobilization was accompanied by changes in cell adhesion, since adhesive properties are thought to affect cell motility, either controlling the movement-restraining forces at the rear edge of the cells or providing mechanical anchorage for contractile and dynamic forces. Interestingly, the adhesion strength of eEPC + AP treated cells increased, together with the levels of vinculin, a well-known molecule involved in focal adhesions that was also up-regulated in eEPC pat. The higher mobilization observed despite an increased adhesion could be explained by the attributed role for vinculin as a mechano-regulating protein, able to enhance the transmission and generation of contractile forces [33,34]. Vinculin has been found in FAs of subcellular areas with increased tensile forces, and these forces appear to act on vinculin itself [29]. FAs have also been shown to control directional mobility [30]. The use of animal models would help to clarify whether all these effects seen of the AP secretome over the eEPCs could correlate with an increased mobilization towards damaged tissues, as previously described [1–3].

Next, we focused on trying to identify factors that could promote the eEPCs changes detected. Although the exact composition of the AP secretome remains unclear, with many potential metabolites or proteins that might be released as single factors by diverse cells types (endothelial cells, smooth muscle cells, etc.) or even transported into microvesicles/exosomes as suggested [35], in the current work we focused on evaluating the role of proteins differentially released by atherosclerotic carotids as potential modulators of the eEPCs mobilization. Previous analyses have already compared the atherosclerotic carotid arteries secretome with non-atherosclerotic ones such as the mammary arteries [15,17,36]. Nevertheless, we decided to perform the assay with some modifications and the technologies available. Thus, according to the IPA software, which allows determining the most likely pathways or functions in which the proteins of interest are involved based on biomedical literature and integrated databases, many of the proteins identified only in the AP secretome participate in the regulation of cell proliferation, migration, and other processes involved during inflammatory conditions. Among the identified proteins we found typical secreted proteins but other non-classical ones that could indeed be linked to microvesicles. Our data confirmed previous results [15,17,36], but it also brought new proteins such as MIF (macrophage migration inhibitory factor) that were identified here as specifically secreted by APs. Not surprisingly, we found 19 chemoattractant proteins such as IL6, MMP2, or LPA that could promote the migration of eEPCs [37]. MIF itself has been reported to promote the mobilization and recruitment of eEPCs under ischemic conditions and such influence is dependent of the degree of ischemia [38]. Further analysis will help to clarify whether these proteins could affect the eEPCs mobility individually or in combination with other atherosclerotic factors.

Finally, we have also demonstrated that the AP secretome promotes changes in healthy eEPCs at a protein level, and more interestingly, affecting the expression of proteins related to the migratory and proliferative properties of these cells, but also to the development and function of the cardiovascular system. Thus, the AP secretome induced the up-regulation of several proteins involved in cell motility, like coronin-1B (CORO1B) or integrin alphaX/CD11c (ITGAX). CORO1B regulates lamellipodia formation and cell migration by regulating actin polymerization through the interaction with the Arp2/3 complex [39]. ITGAX belongs to the family of β2 integrins, which have been found to be expressed in eEPCs [40]. β2 integrins are thought to promote the homing of progenitor cells to ischemic tissues and to contribute to neovascularization after ischemia. In addition, they mediate the adhesion of eEPCs to mature ECs, *via* ICAM-1 and fibrinogen, and are also implicated in the chemokine-induced trans-endothelial migration of eEPCs [40].

Interestingly, from the 30 proteins differentially expressed in eEPCs after only 24 h incubation with the AP secretome, 8 of them were also found in the eEPCs isolated from the AP patient donors (eEPC pat). Our results suggest two facts: First, that some of the protein changes detected in the eEPC + AP treated cells participate in the cell activation but are not necessarily seen in a more chronical situation represented here by the eEPC pat. In agreement with this, previous studies analysing human arterial endothelial cells have detected a differential expression pattern at both, genomic and transcriptomic levels at different stages of human atherosclerosis [41]. Alternatively, the eight common proteins seen in eEPC + AP treated cells and eEPC patients could represent initial changes of protein expression that could remain and evolve to a

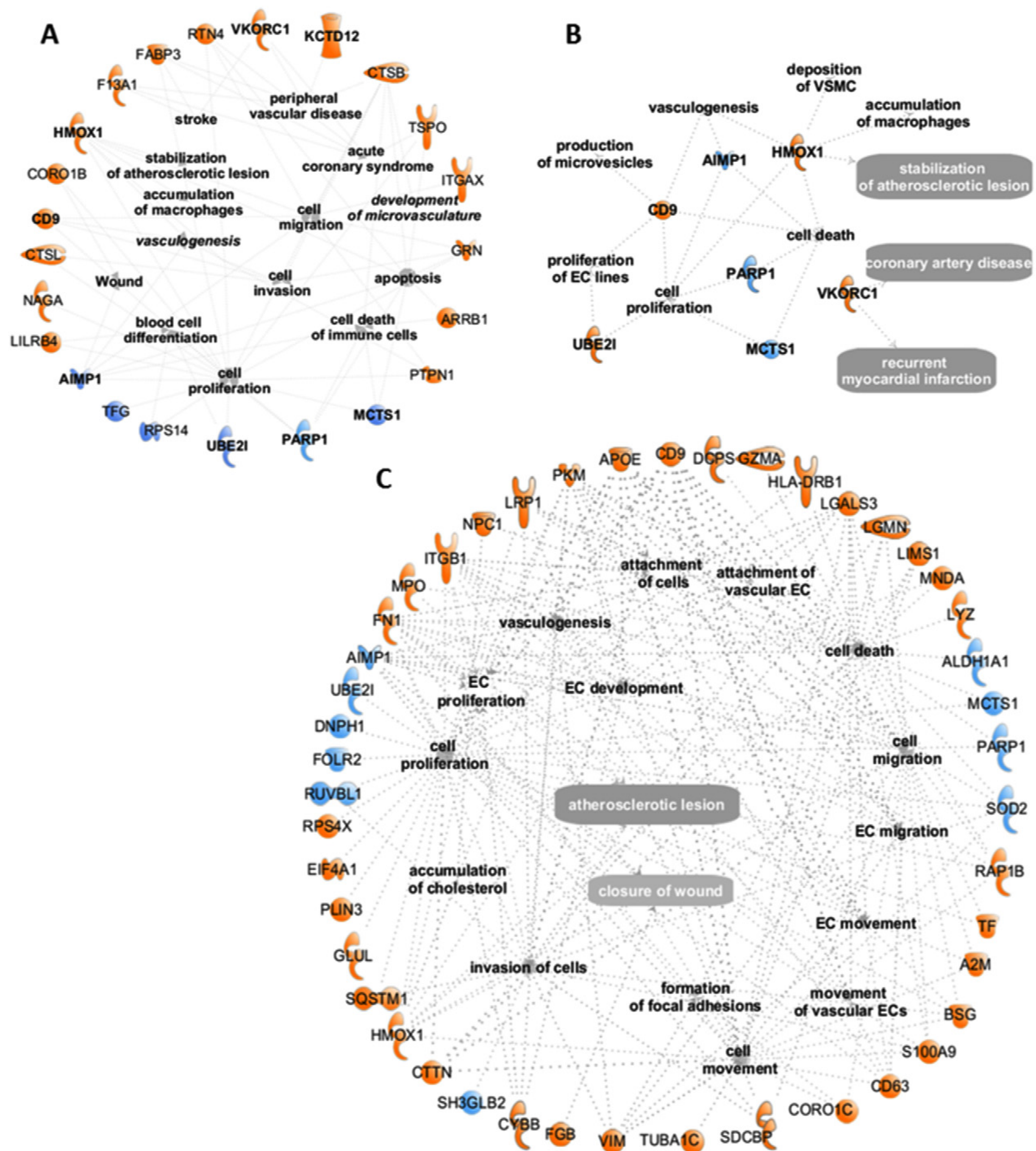

Fig. 5. Graphical correlations between differentially expressed proteins and functions. Major functions described for A) proteins with expression changes in healthy eEPCs after 24 h incubation with the AP secretome (eEPC + AP); B) Proteins differentially expressed in both eEPC + AP treated cells and eEPC pat; C) Protein changes detected only in the eEPCs of atherosclerotic patients (eEPC pat). Compared to basal conditions (eEPC cont): $P < 0.05$. Pathways were obtained with the Ingenuity Software (IPA).

chronical state if maintaining the eEPCs in longer contact with the atherosclerotic environment. Indeed, the eEPCs isolated from atherosclerotic patients presented a differential protein expression pattern regarding eEPCs from healthy donors, in agreement with similar analyses [42,43]. Although future research needs to be done to confirm such hypothesis, the common proteins identified have been previously correlated with the atherosclerotic disease and/or with the EPC remodeling properties. For example, the down-regulation of the ARS-interacting multifunctional protein 1 (AIMP1/p43), detected in both the eEPC + AP and the eEPC patients, has been previously associated to vasculogenesis and cell proliferation, but also with an increase of endothelial cells migration [44]. From the up-regulated proteins, CD9, HMOX1, VKORC1 and KCD12, we corroborated by WB the increased expression of the transmembrane protein CD9 and the HMOX1, after 1 h and 24 h of incubation with the AP secretome, but also in the eEPCs of atherosclerotic patients. CD9, member of the tetraspanin family, has been localized in the smooth muscle vascular cells (SMVC) of atherosclerotic aorta and coronary arteries but not in non-atherosclerotic arteries [45]. CD9 has been implicated in the regulation of differentiation, proliferation, cell adhesion and motility [40] usually by means of forming complexes with other tretraspanins and/or with specific β1 integrins. CD9 also regulates the contraction of SMVCs and actin reorganization *via* RhoA [46]. Although the exact role of CD9 has not been clarified, it is thought that promotes all the functions mentioned above acting as a pro-angiogenic factor [47].

Heme oxygenase-1 (HMOX1) catalyzes the oxidative degradation of cellular heme to liberate free iron, carbon monoxide (CO) and biliverdin. This protein appears to be up-regulated under pathophysiological conditions associated to oxidative stress, where it exerts cytoprotective functions through the action of its released products biliverdin and CO [4]. HMOX1 has been correlated with many vasoprotective functions, cell migration and proliferation. Its up-regulation seems to promote an increase in circulating eEPCs, and appears to promote the mobilization and re-endothelialization linked to these cells [4]. Thus, we assume that the up-regulation of HMOX1 in response to the AP secretome as well as in the eEPCs pat might have an atheroprotective role.

The vitamin K epoxide reductase complex subunit 1(VKORC1) is a vitamin-K dependent protein, known to participate in the regulation of the coagulation cascade and in maintaining blood flow and integrity of the vasculature [48]. Certain VKORC1 alleles are associated with arterial vascular diseases (stroke, coronary heart disease, and aortic dissection), mainly when arteries are complicated with calcification-related atherosclerosis. According to our results, VKORC1 could be taken as an initial marker of atherosclerosis since it was up-regulated just after 24 h of incubation of healthy eEPCs with the AP secretome and such up-regulation remained in the eEPCs from atherosclerotic patients.

Overall, our results show that the exposure of eEPCs to the AP secretome orchestrates a series of changes in protein expression that could reflect the initial response to such an inflammatory environment, promoting the activation and mobilization of these cells, and potentially their vascular remodeling role assigned. These findings bring the opportunity for future research to explore the precise role of the differential proteins identified in eEPCs activation by atherosclerotic factors, and therefore to validate if such proteins could be taken as molecular targets to modulate and potentiate the functionality of EPCs. Additionally, further analyses including the blockade or depletion of specific atherosclerotic factors could help to determine which of them promote indeed the effects described.

5. Conclusion

The experimental approach described has provided a more complete picture, integrated in Fig. 6, of the initial activation of eEPCs in response to the inflammatory environment created by atherosclerotic plaques, by confirming that eEPCs become mobilized in response to a combination of atherosclerotic factors released into the biological system. In addition, several of the proteins differentially expressed in eEPCs after being in contact with the AP secretome could have a role in the initial activation of eEPCs remodeling properties, and others like HMOX1, CD9 or VKORC1 could be taken as indicators of initial response to atherosclerosis. Thus, the *ex vivo* model proposed here should be taken as an efficient

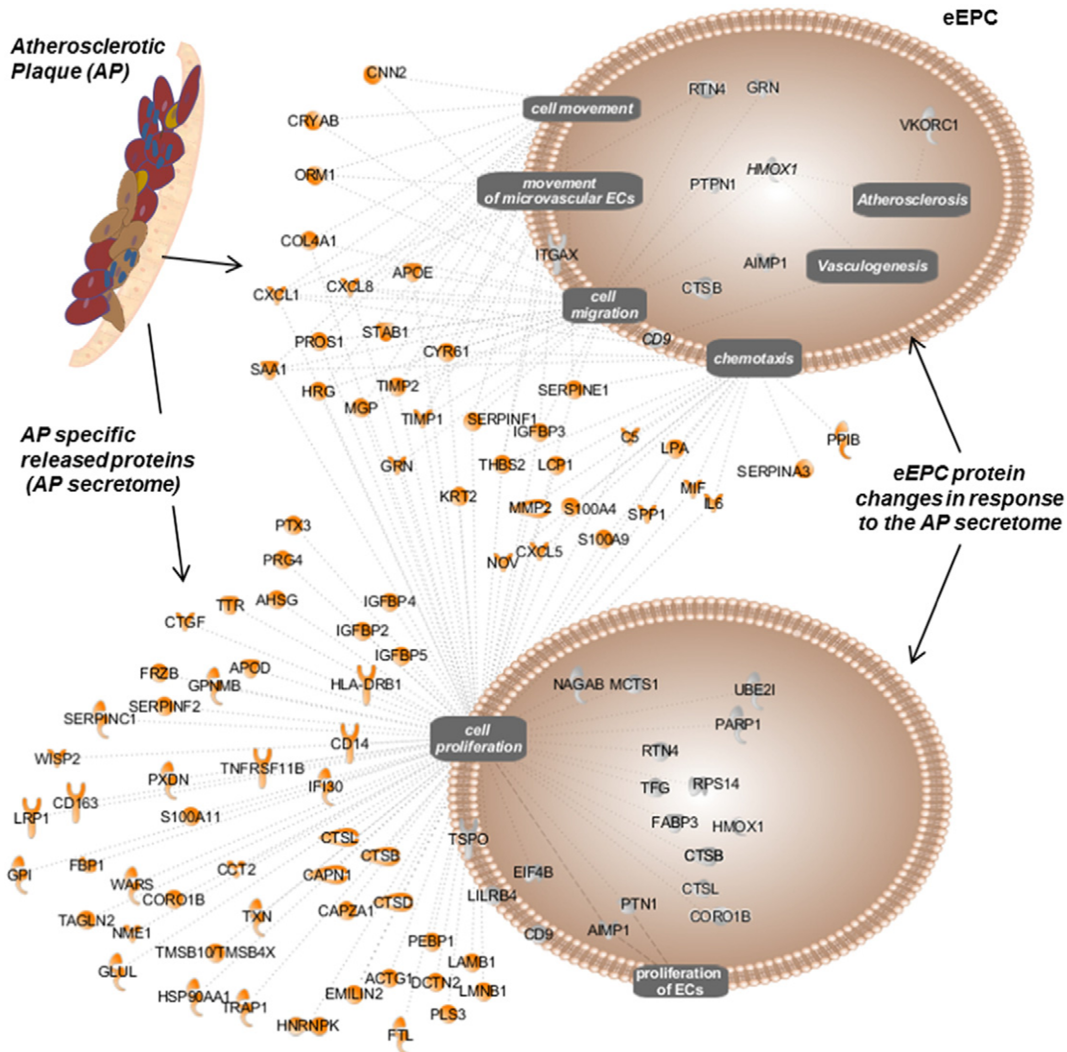

Fig. 6. Functional and protein expression changes promoted by the AP secretome in healthy eEPCs *ex vivo*. The current figure shows a schematic overview of the initial contact of the eEPCs with the proteins released specifically by atherosclerotic carotid arteries (in orange), and the cellular proteins changes detected after 24 h of AP stimulation (grey molecules). Both sets of proteins mainly correlated with cell movement, cell migration and chemotaxis (upper cell) as well as proliferation (lower cell). Graphs and molecular functions were obtained with the IPA Software.

and alternative strategy to elucidate the molecular mechanisms involved in eEPCs activation, opening new venues of study for eEPCs biology and their implication in vascular repair.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2017.02.001.

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Conflict of interest

Not declared.

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