

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

**PDGF-BB carried by endothelial Cell-derived extracellular vesicles reduces vascular smooth muscle cell apoptosis in diabetes**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1666503> since 2018-04-10T16:04:48Z

*Published version:*

DOI:10.2337/db17-0371

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

**PDGF-BB carried by endothelial cell-derived extracellular vesicles reduces vascular smooth muscle cell apoptosis in diabetes**

Gabriele Togliatto<sup>1</sup>, Patrizia Dentelli<sup>1</sup>, Arturo Rosso<sup>1</sup>, Giusy Lombardo<sup>1</sup>, Maddalena Gili<sup>1</sup>, Sara Gallo<sup>1</sup>, Chiara Gai<sup>1</sup>, Anna Solini<sup>2</sup>, Giovanni Camussi<sup>1\*</sup>, Maria Felice Brizzi<sup>1\*</sup>

<sup>1</sup>Department of Medical Sciences, University of Torino, Italy

<sup>2</sup>Department of Surgical, Medical, Molecular and Critical Area Pathology, University of Pisa, Italy

GT and PD equally contributed

**\*Address correspondence to:**

Maria Felice Brizzi and Giovanni Camussi, Department of Medical Sciences, University of Turin, Corso Dogliotti 14, 10126, Turin

mariafelice.brizzi@unito.it

giovanni.camussi@unito.it

**Abstract words:** 200

**Main text words:** 3906

**Number of tables/figures:** 8

**Running Title:** mbPDGF-BB in CD31EVs and vascular smooth muscle cell apoptosis

**Key words:** miR-296-5p, PDGF-BB, extracellular vesicles, diabetes, vascular smooth muscle cells.

## 1    **ABSTRACT**

2    Endothelial cell-derived extracellular vesicles (CD31EVs) are a new entity for therapeutic/prognostic  
3    purposes. The roles of CD31EVs as mediators of smooth muscle cell (VSMC) dysfunction in type 2  
4    diabetes (T2D) is investigated herein.

5    We demonstrated that, unlike non-diabetic, diabetic serum-derived-EVs (D-CD31EVs) boosted  
6    apoptosis resistance of VSMCs cultured in hyperglycaemic condition. Biochemical analysis revealed  
7    that this effect relies on changes in the balance between anti-apoptotic/pro-apoptotic signals: increase  
8    of bcl-2 and decrease of bak/bax. D-CD31EV cargo analysis demonstrated that D-CD31EVs are  
9    enriched in membrane-bound-platelet-derived-growth-factor-BB (mbPDGF-BB). Thus, we  
10    postulated that mbPDGF-BB transfer by D-CD31EVs could account for VSMC resistance to  
11    apoptosis. By depleting CD31EVs of PDGF-BB or blocking the PDGF-BB-receptor $\beta$  on VSMCs, we  
12    demonstrated that mbPDGF-BB contributes to D-CD31EV-mediated bak/bax and bcl-2 levels.  
13    Moreover, we found that bak expression is under the control of PDGF-BB-mediated miR-296-5p  
14    expression. In fact, while PDGF-BB-treatment recapitulated D-CD31EV-mediated anti-apoptotic  
15    program and VSMC resistance to apoptosis, PDGF-BB-depleted CD31EVs failed. D-CD31EVs also  
16    increased VSMC migration and recruitment to neovessels, by means of PDGF-BB. Finally, we found  
17    that VSMCs, from human atherosclerotic arteries of T2D individuals, express low bak/bax and high  
18    bcl-2 and miR-296-5p levels. This study identifies the mbPDGF-BB in D-CD31EVs as a relevant  
19    mediator of diabetes-associated VSMC resistance to apoptosis.

20

## 21 INTRODUCTION

22 Cardiovascular complications are a leading cause of morbidity and premature mortality in  
23 diabetes (1,2). Structural alterations to vessel walls result in intima-media thickening which marks  
24 individuals at high risk to develop acute cardiovascular events (3,4). Moreover, restenosis is still a  
25 major complication in the diabetic setting. A main-cause of re-occlusion is intimal hyperplasia which  
26 is due to the migration and/or excessive growth of vascular smooth muscle cells (VSMCs). A  
27 dysregulated balance between apoptosis and the proliferation of VSMCs seems to play a crucial role  
28 in intima-media thickening in diabetic individuals (5,6). Indeed, *in vitro* studies have suggested that  
29 high glucose (HG) induces the expression of bcl-2 family members and inhibits the apoptotic protein  
30 Inhibitor of Apoptosis Protein 1, (IAP-1) in VSMCs (7). In addition, Ruiz *et al.* (8) have demonstrated  
31 that VSMCs, recovered from diabetic patients, showed a resistance to apoptosis which was possibly  
32 due to bcl-2 over-expression. Although circulating high glucose concentration might *per se* induce  
33 VSMC dysfunction, additional events can contribute to this process *in vivo*.

34 Several studies have focused on extracellular RNA (exRNA) transporters, indicating that they  
35 may be present in biological fluids in the form of vesicles, which have been denoted microvesicles,  
36 exosomes, membrane particles and apoptotic bodies (9,10). Despite the lack of consensus on vesicle  
37 classification, the presence of overlapping characteristics and biological activity has evoked the use  
38 of the inclusive term; “extracellular vesicles” (EVs) (11,12). The paracrine/endocrine effects of EVs  
39 have recently gained significant attention (13,14). Indeed, EV biological activity has been linked to  
40 the transfer of bioactive molecules, including proteins and microRNA (miRs) (10-14). EVs are widely  
41 distributed in human body fluids, while circulating EV cargo usually reflects the cell of origin in its  
42 physiological and/or pathological condition (9-15). Indeed, the number and cargo of circulating EVs  
43 have been suggested as a means to predict the presence of disease and even the risk of developing  
44 disease (16,17).

45           Increased levels of circulating platelet- and endothelial cell- (EC) derived microparticles have  
46   been proposed as “biomarkers” of cell dysfunction (18,19). However, EVs might also deliver specific  
47   drivers of disease, as they behave as diffusible vectors of biological activity and participate in  
48   exchanging information. This study therefore investigates the role of EC-derived EVs as mediators  
49   of VSMC fate in type 2 diabetes (T2D).

50

## 51    **RESEARCH DESIGN AND METHODS**

52    Reagents and antibodies are reported in Supplemental Table 1.

53    **Patients and Controls.** 11 T2D and 6 non-diabetic individuals (controls), who had undergone carotid  
54    endoarterectomy surgery in our clinic, were included in the study. Clinical characteristics are  
55    reported in Supplemental Table 2. All diabetic individuals were under statin and metformin treatment.  
56    Ethical approval was obtained from Azienda Ospedaliero-Universitaria (AOU), Città della Salute e  
57    della Scienza di Torino, Italy. Informed consent was obtained from all individuals in accordance with  
58    the Declaration of Helsinki. We had no direct contact with the participants.

59    **Isolation of VSMCs from human atherosclerotic plaque specimens.** Human atherosclerotic  
60    plaque specimens were recovered from the above reported subjects (T2D: D; non-diabetic: ND) and  
61    processed as previously described (20). Vascular tissue was rinsed 3 times with phosphate-buffered  
62    saline (PBS) and intima was removed in order to furnish the VSMCs. Tunica media were finely cut  
63    into 2-3 mm pieces and subjected to enzymatic digestion using collagenase type I (0.1mg/ml) in a  
64    Dulbecco's Modified Eagle Medium (DMEM) for 1.5h at 37°C. Digestion media were collected and  
65    filtered through nylon mesh cell strainers (100µm) to remove the undigested explants. The resulting  
66    supernatants were centrifuged at 1200 rpm for 10 min and cells were plated at  $2.5 \times 10^4$  cells/cm<sup>2</sup> and  
67    cultured with Modified Eagle Medium (MEM) supplemented with 20% (v/v) foetal bovine serum  
68    (FBS), 1% penicillin-streptomycin. Fluorescence-activated cell sorting (FACS) analysis was  
69    performed on D-VSMCs and ND-VSMCs to characterize them, as indicated in (20), using antibodies  
70    directed to CD31 and alpha-smooth muscle actin ( $\alpha$ -SMA), directly or indirectly conjugated with  
71    fluorescein isothiocyanate (FITC) fluorochrome. FITC mouse non-immune isotypic IgG (BD  
72    Bioscience Pharmingen) was used as control.

73    **Cell cultures.** Primary macrovascular endothelial cells (ECs) and VSMCs were purchased from  
74    Lonza (Basel, Switzerland) and cultured as described by the manufacturer's instructions. VSMCs and

75 ECs were used at II-III cell-culture passage. To collect the EVs, ECs were starved under either low  
76 (LG, 5mmol/l) or high glucose (HG, 25 mmol/l) and 24h deprived of bovine calf serum (BCS). Cell  
77 viability was evaluated. siRNA technology was also performed in HG-cultured ECs using siRNA  
78 negative control or the Platelet-Derived Growth Factor (PDGF-BB) siRNA (Applied Biosystems)  
79 (21). EV isolation was obtained from HG-cultured ECs depleted of PDGF-BB. In selected  
80 experiments, VSMCs were cultured in LG or HG and then treated in the presence of ND-CD31EVs,  
81 D-CD31EVs or EC-derived-EVs ( $5 \times 10^3$  EVs/target cell), or stimulated with PDGF-BB. In selected  
82 experiments, HG-cultured VSMCs were pre-incubated with a blocking PDGF-Receptor- $\beta$  (PDGFR $\beta$ )  
83 antibody (5 $\mu$ g/ml). Details are reported in Supplemental Materials. All experiments were performed  
84 in accordance with European Guidelines and policies and approved by the Ethical Committee of the  
85 University of Turin.

#### 86 **Isolation and characterization of CD31EVs from sera of T2D and non-diabetic individuals.**

87 Human serum from all above T2D and non-diabetic individuals was obtained before surgery and after  
88 informed consent. EVs from each participant were obtained by centrifuging serum as previously  
89 described (22). The supernatant was subsequently submitted to differential ultracentrifugation at 10k  
90 and 100k g for 2h at 4°C. EV pellets were then re-suspended in DMEM and stored at -80°C. FACS  
91 analysis of D-EVs and ND-EVs was performed as indicated in (23), using anti-CD31-  
92 allophycocyanin (APC), anti-CD14-phycoerythrin (PE) and anti-CD42b-FITC antibodies. FITC, PE  
93 or APC mouse non-immune isotypic IgG (BD Bioscience Pharmingen) were used as controls. FACS  
94 analysis was performed using a Guava easyCyte<sup>TM</sup> Flow Cytometer (Millipore, Germany).  
95 Fluorochrome conjugated antibodies were added to a suspension of EVs ( $2.5 \times 10^6$  particles/100  $\mu$ l)  
96 for 15 min at 4°C. Surface marker expression is reported in the representative histograms as the  
97 percentage of expression $\pm$ SD. The CD31 microbead kit (Miltenyi Biotec, Auburn, CA, USA) was  
98 used to isolate CD31EVs from the sera of T2D (D-CD31EVs) and non-diabetic individuals (ND-  
99 CD31EVs) (24). Briefly, 0,5 ml of freshly-thawed plasma was incubated with 100  $\mu$ l of CD31

100 microbeads for 4h at 4°C. EVs captured on CD31 Ab-coated magnetic beads were recovered from  
101 the magnetic column (MS column) as described in the manufacturer's instructions. EV-bound beads  
102 were submitted to differential ultracentrifugation (Beckman Coulter Optima L-90K ultracentrifuge;  
103 Beckman Coulter, Fullerton, CA) for 3h at 4°C. CD31EVs were either used fresh or were stored at -  
104 80°C and then processed for transmission electron microscopy (TEM), biological effects, western  
105 blot and q-RT-PCR analysis. CD63 content in CD31EVs was analyzed by western blot. Details are  
106 reported in Supplemental Materials.

107 **Transmission electron microscopy.** TEM was performed on CD31EVs that had been isolated by  
108 ultracentrifugation and re-suspended in PBS, placed on 200 mesh nickel formvar carbon coated grids  
109 (Electron Microscopy Science, Hatfield, PA) and left to adhere for 20 min. Grids were then processed  
110 as previously described (25) and observed under a Jeol JEM 1010 electron microscope (Jeol, Tokyo,  
111 Japan). Details are reported in Supplemental Materials.

112 **Isolation of EC-derived EVs.** ECs were cultured in LG or HG DMEM without BCS for 24h, in order  
113 to collect the EVs from supernatants as previously described (21,22) and detailed in Supplemental  
114 Materials. EV number and size distribution analysis was performed using a NanoSight LM10  
115 (NanoSight Ltd, Minton Park UK). Results were displayed as number per ml and as a frequency size  
116 distribution graph, outputted to a spreadsheet. EC-derived-EVs (LG-EVs or HG-EVs) were processed  
117 for biological, western blot and q-RT-PCR analysis (21,22).

118 **Western blot analysis.** Cells and EVs were lysed and protein concentrations were obtained, as  
119 previously described (25). Protein levels were normalized to  $\alpha$ -SMA,  $\beta$ -actin, or CD63 content.  
120 Details are reported in Supplemental Materials.

121 **EV internalization.** The internalization of EVs was evaluated using confocal microscopy (LSM5-  
122 PASCAL; Zeiss, Oberkochen, Germany) as previously described (25). EV pellets were added to HG-



123 cultured VSMCs ( $2 \times 10^4$ ) pre-treated or not with a blocking PDGFR $\beta$  antibody. Z-stack confocal  
124 microscopy VSMC images were also obtained (25). Details are reported in Supplemental Materials.

125 **RNA isolation and quantitative real-time PCR (qRT-PCR) for miRs.** Total RNA was isolated  
126 from the VSMCs of atherosclerotic plaque specimens and from human VSMCs, that had been treated  
127 as indicated or left untreated, using the TRIzol reagent (Invitrogen) as previously described (26).  
128 RNA from cells and EVs was then reverse-transcribed using a TaqMan microRNA RT kit, specific  
129 for miR-24-3p, miR-221, miR-222 and miR-296-5p, or a Syber Green microRNA RT Kit specific for  
130 miR-21-5p, miR-29a and miR-145, as indicated. miR expression was normalized to the small nuclear  
131 RNA, RNU6B. Loss- and gain-of-function experiments were performed in VSMCs that had been  
132 transfected with the antago-miR control, the antago-miR-296-5p, pre-miR control or pre-miR-296-  
133 5p oligonucleotides (Applied Biosystem), according to manufacturer's instructions (26). Details are  
134 reported in Supplemental Materials.

135 **Luciferase miRNA target reporter assay.** The luciferase reporter assay was performed using a  
136 construct generated by subcloning the PCR products amplified from the full-length 3'UTR of human  
137 *BAK1* DNA into the Xba restriction site of the luciferase reporter vector pGL3 (Promega, Madison,  
138 WI, USA). The PCR products were obtained using the primers for *BAK1* and reported in detail in  
139 Supplemental Materials (26).

140 **Cell proliferation and apoptosis assay.** Proliferative activity was assayed as previously described  
141 (27). For the apoptosis assay, VSMCs were subjected to Muse Annexin V and the cell dead assay  
142 (Merck, Darmstadt, Germany) in accordance with manufacturer's instructions. Details are reported  
143 in Supplemental Materials.

144 **Tubule-like structure formation assay.** To analyze the EC/VSMC interaction, 24-well-plates were  
145 coated with growth factor-reduced Matrigel matrix (BD Biosciences) (28). Briefly, HG-cultured ECs  
146 and VSMCs were pre-treated, for 24h, with either D-CD31EVs, ND-CD31EVs or HG-EVs that had  
147 either been depleted, or not, of PDGF-BB.  $4.5 \times 10^4$  red labeled ECs (PKH26 vital dye) were placed

148 in HG medium on top of the polymerized matrix.  $2 \times 10^4$  green labeled VSMCs (PKH67 vital dye)  
149 were then added to ECs. Details are reported in Supplemental Materials.

150 **Scratch assay on VSMCs.** Scratch assays were performed on HG-cultured VSMCs, treated as  
151 indicated, to evaluate cell migration activity. VSMCs were seeded to a final density of 100,000 cells  
152 per well for 24h in order to allow cell adhesion and the formation of a confluent monolayer to occur.  
153 Details are reported in Supplemental Materials.

154 **ELISA Assays.** PDGF-BB concentration in D-CD31EVs were measured using a commercially  
155 available competitive enzyme immunoassay (ELISA) kit (R&D Systems, MN, USA), according to  
156 manufacturer's instructions. To evaluate mbPDGF-BB, intact or lysates D-CD31EVs ( $2.5 \times 10^8$   
157 particles) were compared. The same samples were pre-treated with trypsin (0.25%) for 1h (negative  
158 control). Details are reported in Supplemental Materials.

159 **Statistical analysis.** All data are presented as mean  $\pm$  SEM, unless otherwise reported. The  
160 D'Agostino–Pearson test was used to test normality. Data on *in vitro* angiogenesis, cell proliferation,  
161 ELISA, apoptosis and scratch assays, on qRT-PCR-miR expression, loss- and gain-of-function  
162 experiments, characterization of recovered EVs and, lastly, on the densitometric analysis for Western  
163 blots were analyzed using the Student *t* tests for 2-group comparison and using 1-way ANOVA,  
164 followed by Tukey's multiple comparison test, for  $\geq 3$  groups. All western blot experiments were  
165 performed in triplicate. The minimum sample size was four experiments performed in triplicate, thus  
166 ensuring 90% statistical power among experimental groups, and a probability level of 0.05, two-tailed  
167 hypothesis. The cut-off for statistical significance was set at  $P < 0.05$ . All statistical analyses were  
168 carried out using GraphPad Prism version 5.04 (Graph Pad Software, Inc).

169

## 170    **RESULTS**

### 171    **D-CD31EVs potentiate VSMCs resistance to apoptosis in high glucose (HG) conditions**

172    Circulating EVs are able to modulate cell fate (9,10,13). It was therefore decided to investigate  
173    whether and how circulating EVs, derived from ECs of T2D-individuals (D-EVs), may impact on  
174    VSMC fate. Guava analysis was used to demonstrate the presence of a high percentage of EVs of  
175    endothelial origin in the sera of non-diabetic (ND-EVs) and T2D individuals (Figure 1A). A  
176    significant reduction of EVs from T2D individuals was detected (Figure 1A). EC-derived EVs from  
177    sera were therefore isolated using CD31-coated magnetic beads (ND-CD31EVs and D-CD31EVs)  
178    and analyzed using transmission electron microscopy (Figure 1B) and western blot (Figure 1C).  
179    Functional studies were then performed to evaluate the biological relevance of D-CD31EVs in  
180    mediating VSMC dysfunction in hyperglycaemic condition (HG-culture condition). LG-conditioned  
181    VSMCs served as control. Consistent with data provided by Ruiz et al. (8), we found that, unlike LG,  
182    HG treatment was associated with a significant up-regulation of bcl-2 and down-regulation of bak/bax  
183    (Supplemental Figure S1A). By contrast the expression of another member of the bcl-2 protein  
184    family, bcl2l2, did not change (Supplemental Figure S1B). Moreover, HG treatment *per se*  
185    significantly decreased the number of apoptotic cells, without affecting VSMC proliferation  
186    (Supplemental Figure S1C-S1D). Functional studies were then performed on VSMCs cultured in HG  
187    conditions and treated with CD31EVs. As shown in Figure 1D, D-CD31EVs, unlike ND-CD31EVs,  
188    were able to further reduce both the number of apoptotic VSMCs and bak/bax content and increase  
189    bcl-2 level (Figure 1E).

### 190    **D-CD31EVs are enriched in PDGF-BB**

191    The transfer of proteins and/or genetic information into recipient cells is the main mechanism of EV  
192    action (9,10,13,14). CD31EV protein cargo and, in particular, the content of well-known VSMC  
193    proliferation/survival factor, PDGF-BB, were therefore analyzed in both CD31EVs and EVs

194 recovered from LG- and HG-treated ECs. Supplemental Figure S2A shows Nanosight analysis of  
195 EVs recovered from LG- or HG-cultured ECs (LG-EVs or HG-EVs). No differences in EV size and  
196 number between LG-EVs and HG-EVs were detected (data not shown). Conversely, PDGF-BB was  
197 found to be enriched only in D-CD31EVs (Figure 2A) and in HG-EVs (Supplemental Figure S2B).

#### 198 **Membrane bound-PDGF-BB drives D-CD31EV anti-apoptotic cues**

199 LG- and HG-cultured VSMCs were treated with PDGF-BB in order to investigate the contribution of  
200 PDGF-BB in mediating D-CD31EV biological effects. In fact, PDGF-BB further increased bcl-2  
201 content, while reducing the number of apoptotic cells and bak/bax content in HG-conditioned  
202 VSMCs. Conversely, these effects were not detected in VSMCs cultured in LG conditions (Figure  
203 2B-2C). siRNA technology was therefore harnessed to abrogate PDGF-BB expression in EVs (Figure  
204 2D-2E) and validate the role of PDGF-BB in regulating D-CD31EV survival signals in HG  
205 conditions. As expected, PDGF-BB-depleted EVs were no longer able to either increase bcl-2, or  
206 decrease bak/bax expression (Figure 2F) and the number of apoptotic cells (Figure 2G). To investigate  
207 whether different mechanisms might account for free PDGF-BB and D-CD31EV-PDGF-BB-induced  
208 effects, HG-cultured VSMCs were pre-treated with a blocking PDGFR $\beta$  antibody, stimulated with  
209 D-CD31EVs or free PDGF-BB, and analyzed for bak/bax and bcl-2 expression. As shown in Figure  
210 3A PDGFR $\beta$  blockade impacts on both free PDGF-BB and D-CD31EVs-mediated bak/bax and bcl-  
211 2 expression. To rule out the possibility that this effect depended on inhibition of PDGFR $\beta$ -mediated  
212 EV internalization, Z-stack analysis was performed. Figure 3B shows that PDGFR $\beta$  blockade did not  
213 hamper EV internalization. Of note, the ELISA assay led to the discovery that PDGF-BB was  
214 anchored to the membrane of D-CD31EVs (Figure 3C) indicating that EV-mbPDGF-BB, by binding  
215 to the PDGFR $\beta$ , might drive D-CD31EV biological effects.

#### 216 **D-CD31EVs induce VSMC migration and recruitment to neovessels via PDGF-BB-mediated** 217 **effects**

CD31EVs were also evaluated in an EC/VSMC co-culture assay. A reduced number of vessels was detected in HG-cultured ECs (data not shown) and D-CD31EVs were nevertheless able to promote VSMC recruitment to neo-formed tubule-like structures (Figure 4A-4B), unlike ND-CD31EVs. A scratch assay also highlighted increased VSMC motility upon D-CD31EV treatment (Figure 4C). As shown in Supplemental Figure S2C, these effects did not depend on differences in EV-VEGF content. Furthermore, the contribution of PDGF-BB to both processes was validated in experiments using PDGF-BB-depleted EVs; such EVs failed to induce either effects (Figure 4D-F).

### **T2D individual-derived VSMCs express high bcl-2 and low bak/bax content**

To validate the above results VSMCs isolated from either T2D (D) or non-diabetic (ND) human atherosclerotic plaque specimens were analyzed for bak/bax and bcl-2 expression. As shown in Figure 5A, the majority of the recovered cells express VSMC marker. Moreover, as previously reported by Ruiz et al. (8), it was confirmed that D-VSMCs expressed high bcl-2 levels. In addition, we found that D-VSMCs also expressed low bak/bax content (Figure 5B-5C).

### **miR-296-5p-post-transcriptionally controls bak level in response to HG and D-CD31EVs**

To gain further insight into the mechanisms regulating VSMC fate the expression of miRs potentially involved in this process was first analyzed in D-VSMCs and compared to ND-VSMCs. As shown in Figure 6A, no significant differences were detected in the expression of miR-21-5p, miR-24-3p, miR-145, miR-29a (not included in the panel: CT>38), miR-221 and miR-222 between D- and ND-VSMCs (29-30). Moreover, since bak, unlike bax, is one of miR-296-5p putative target genes (TarBase v7.0) (31) its expression was also analyzed. Indeed, high miR-296-5p levels was detected in D-VSMCs (Figure 6A). In order to validate the role of miR-296-5p in controlling bak expression, VSMCs cultured in LG- or HG-conditions were analyzed for miR-296-5p expression. As shown in Figure 6B an increased miR-296-5p expression was detected upon HG treatment. Moreover, the depletion of miR-296-5p in HG-cultured VSMCs (Supplemental Figure S3) led to the decrease of

242 bcl-2 levels, the increase of bak/bax content (Figure 6C) and a consistent increase in the number of  
243 apoptotic cells (Figure 6D). To confirm the role of miR-296-5p in bak post-transcriptional regulation,  
244 the full-length 3'UTR *BAK1* nucleotide sequence was analyzed for miR-296-5p blasting sequences  
245 revealing several base pairings (1138–1158bp) (Figure 7A). The luciferase assay was used to  
246 demonstrate that bak is indeed a direct miR-296-5p target (Figure 7B). This observation was further  
247 validated by gain-of-function experiments (Figure 7C-7D).

248 In order to investigate the contribution of PDGF-BB in mediating miR-296-5p expression, LG- and  
249 HG-cultured VSMCs were also treated with PDGF-BB. As shown in Figure 6E, PDGF-BB further  
250 increased miR-296-5p only in HG-cultured VSMCs. These results were validated by siRNA  
251 technology (Figure 6F), thus suggesting that VSMC survival may be under the control of miR-296-  
252 5p.

#### 253 **D-CD31EVs are almost depleted of miR-296-5p content**

254 To exclude the possibility that D-CD31EV-mediated effects could also depend on the delivery of  
255 miR-296-5p, the miR-296-5p content was also evaluated in CD31EVs and LG- or HG-cultured EC-  
256 derived EVs (LG-EVs or HG-EVs). Almost undetectable levels of miR-296-5p were found in D-  
257 CD31EVs (Supplemental Figure S2D) and in EVs from HG-treated ECs (Supplemental Figure S2E).  
258 Thus, the role of mbPDGF-BB-D-CD31EVs in mediating miR-296-5p-driven post-transcriptional  
259 regulation of bak and its down-stream events was further strengthened.

260

## 261    **DISCUSSION**

262            Atherosclerosis and its associated complications is a major cause of death worldwide (32).  
263    Diabetes accelerates atherosclerosis and restenosis after angioplasty (33,34). Indeed, increased  
264    VSMC migration and survival/proliferation are crucial for restenosis, particularly in diabetics (35-  
265    37). *In vitro* and *ex-vivo* studies have shown that the up-regulation of bcl-2/bcl-xl is crucial for VSMC  
266    resistance to apoptosis in diabetes (7,8,38,39). Moreover, Li H *et al.* (7) have reported that, while HG  
267    enhances the expression of bcl-2 family members in VSMCs, it reduced the expression of the IAP1.  
268    We herein demonstrate that VSMCs, exposed to HG concentrations, up-regulate bcl-2 and down-  
269    regulate bak/bax, without affecting VSMC proliferation. Moreover, we discovered that these effects  
270    are boosted by D-CD31EV treatment. A cell's survival or death depends on the integrity of the  
271    mitochondrial outer membrane (MOM) (40). In this regard, while the pro-apoptotic proteins, bak and  
272    bax, are involved in the permeabilization of MOM, the anti-apoptotic bcl-2 family members counteract  
273    such pro-apoptotic signals by preventing cytochrome *c* efflux (40). Our results therefore indicate that  
274    an additional shift in the balance, from apoptotic to anti-apoptotic signals, might be the main  
275    mechanism behind - D-CD31EVs-induced resistance to apoptosis in HG conditions, and suggest that  
276    hyperglycaemia-mediated cues preferentially translate into VSMC resistance towards apoptosis  
277    rather than VSMC proliferation.

278            The genetic material in EV cargo has sparked considerable interest. The role of miRs as  
279    mediators of epigenetic changes has been extensively reported, particularly in diabetes (41,42). The  
280    transfer of miRs into recipient cells has been described as a relevant mechanism of EV biological  
281    action (9-14). As a matter of fact, Gu *et al.* (43) recently reported that the transfer of miR-195 from  
282    EC-derived EVs to VSMCs regulates VSMC proliferation. Despite the ability of D-CD31EVs to  
283    induce functional changes in VSMCs, our results demonstrate that D-CD31EV-mediated VSMC  
284    dysfunction relies on a mechanism independent of the delivery of miRs. Indeed, EVs also transport  
285    and deliver proteins which can affect VSMC fate, including PDGF-BB. PDGF-BB is a growth factor

286 known to regulate VSMC outcomes (44,45). In this regard, PDGF-BB derived from platelet and EC  
287 is considered a relevant mediator of VSMC dysfunction and restenosis (46). As a matter of fact, we  
288 demonstrate that D-CD31EVs are enriched in PDGF-BB and that PDGF-BB enriched D-CD31EVs  
289 may contributes to VSMC dysfunction in diabetic setting. Several lines of evidence indicate that  
290 downstream signaling events, activated by PDGF-BB, trigger various biological processes, including  
291 VSMC migration and recruitment to neo-formed vessels (44,45). It is worth noting that PDGF-BB  
292 depleted EVs failed to induce both VSMC migration and recruitment to neovessels. This suggests  
293 that CD31EV-PDGF-BB cargo might play a crucial role in accelerating VSMC dysfunction and  
294 restenosis in T2D.

295 PDGF-BB synthesis and release can increase in response to various stimuli including intima damage  
296 (47). As shown herein, in diabetic setting, CD31EVs are a relevant circulating PDGF-BB reservoir  
297 and contribute to VSMC fate. We established that PDGF-BB is bound to the membrane of CD31EVs,  
298 and is required for their biological action but not for their internalization. As a proof of concept,  
299 PDGFR $\beta$  blockade completely hampers CD31EV-mediated bak/bax expression, without impeding  
300 their entry into the cell. This indicates that, along with free PDGF-BB, mbPDGF-BB enriched D-  
301 CD31EVs contribute to PDGF-BB paracrine effects (48). A co-operative action between CD31EVs  
302 and platelets-derived EVs could be postulated *in vivo*. In fact, EVs released from platelets accumulate  
303 in human atherosclerotic plaques and can induce major biological pathways by transferring their  
304 PDGF-BB content (49). In line with the results presented herein, it has been recently reported that  
305 EC- and platelet-derived EVs are enriched in PDGF-BB in patients with cardiovascular diseases (50).  
306 miRs are key regulators of gene expression, mainly at the post-transcriptional level (51). We herein  
307 demonstrate, both *ex vivo* and *in vitro*, that the hyperglycaemia milieu enhances miR-296-5p  
308 expression and modulates bak content in VSMCs, and that these effects are strictly dependent on D-  
309 CD31EV-PDGF-BB cargo, rather than EV-miR-296-5p delivery. In addition, a shift between anti-  
310 apoptotic to pro-apoptotic signals after PDGFR $\beta$  blockade (down-regulation of bcl-2 expression) was



311 observed. This suggests that PDGF-BB directly, or indirectly by changing the balance of cellular  
312 miRs, might transcriptionally or post-transcriptionally regulate its expression. Mutually, these events  
313 translate in VSMC resistance to apoptosis.

314 Emerging evidence suggests that EVs can serve as specific diagnostic/prognostic biomarkers  
315 since they can provide intercellular state information on a given disease condition (52). Increased  
316 levels of “small (submicroscopic) membranous particles” of endothelial origin, such as  
317 CD31<sup>+</sup>/annexin V<sup>+</sup> and CD31<sup>+</sup>/CD42<sup>-</sup>, have been detected in circulation in patients with coronary  
318 artery disease (CAD), suggesting that they may be an additional risk stratification factor (18,19). A  
319 significant reduction in CD31EVs (CD31<sup>high</sup>/CD42b<sup>low</sup>/CD14<sup>low</sup>) has been found in T2D individuals  
320 in the present study. The phenotype of the “small membrane particles”, which also includes apoptotic  
321 bodies, and the lack of exosome refining (18,19) in CAD patient studies could explain the discrepancy  
322 with our results. However, increasing amounts of evidence indicate that healthy subjects and diseased  
323 individuals release EVs with different cargo.

324 Our *ex vivo* and *in vitro* results, reinforce the notion that D-CD31EV cargo, rather than D-CD31EV  
325 number, is the crucial determinant of their biological activity.

326 The present study reports that hyperglycaemia *per se* induces epigenetic mechanisms in  
327 VSMCs by enriching the circulating CD31EV cargo with mbPDGF-BB which translates into VSMC  
328 resistance to apoptosis (Figure 8). We are also the first to demonstrate that EV-mbPDGF-BB-  
329 mediated miR-296-5p overexpression and the post-transcriptional regulation of bak is a relevant  
330 mechanism of D-CD31EV action. Overall, these results identify D-CD31EV-mbPDGF-BB as a novel  
331 driver of VSMC dysfunction in diabetic setting.

332

333

334 **ACKNOWLEDGEMENTS:** Dr. MF Brizzi is the guarantor of this work, had full access to all data  
335 and takes full responsibility for data integrity and the accuracy of data analysis.

336 **FUNDING:** This work was supported by grants obtained by grant from the Ministero dell'Università  
337 e della Ricerca Scientifica (MIUR) ex 60% to PD, and by grant No. 071215 from Unicyte to GC and  
338 MFB. Associazione Italiana per la Ricerca sul Cancro (AIRC) project IG 2015.17630 to MFB.

339

#### 340 **DUALITY OF INTEREST**

341 The authors declare that there is no duality of interest associated with this manuscript.

#### 342 **AUTHOR CONTRIBUTIONS**

343 GT: performed *ex vivo* and *in vitro* experiments, EC-EV-miRs and protein analysis; PD: performed  
344 in vitro angiogenesis assay and FACS analysis; GL: performed in vitro experiments and EV isolation;  
345 AR: generated constructs and performed transfections; MG: performed loss- and gain-of-function  
346 approaches; SG: performed Western blot analysis; CG: performed EV isolation and characterization;  
347 AS: contributed to data interpretation and revised the manuscript; GC: contributed to the study  
348 conception and revised the manuscript; MFB: performed the study conception design and wrote the  
349 manuscript.

350

351

## 352 REFERENCES

- 353 1. Emerging Risk Factors Collaboration. Diabetes mellitus, fasting blood glucose concentration,  
354 and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. *Lancet*  
355 2010;375:2215–2222
- 356 2. Fox CS, Golden SH, Anderson C, Bray GA, Burke LE, de Boer IH, Bray GA, Burke LE, de  
357 Boer IH, Deedwania P, Eckel RH, Ershow AG, Fradkin J, Inzucchi SE, Kosiborod M, Nelson  
358 RG, Patel MJ, Pignone M, Quinn L, Schauer PR, Selvin E, Vafiadis DK; American Heart  
359 Association Diabetes Committee of the Council on Lifestyle and Cardiometabolic Health,  
360 Council on Clinical Cardiology, Council on Cardiovascular and Stroke Nursing, Council on  
361 Cardiovascular Surgery and Anesthesia, Council on Quality of Care and Outcomes Research,  
362 and the American Diabetes Association. Update on prevention of cardiovascular disease in  
363 adults with type 2 diabetes mellitus in light of recent evidence: a scientific statement from the  
364 American Heart Association and the American Diabetes Association. *Circulation*  
365 2015;132:691–718
- 366 3. Terry JG, Tang R, Espeland MA, Davis DH, Vieira JL, Mercuri MF, Crouse JR 3rd. Carotid  
367 arterial structure in patients with documented coronary artery disease and disease-free control  
368 subjects. *Circulation* 2003;107:1146–1151
- 369 4. Schram MT, Henry RM, van Dijk RA, Kostense PJ, Dekker JM, Nijpels G, Heine RJ, Bouter  
370 LM, Westerhof N, Stehouwer CD. Increased central artery stiffness in impaired glucose  
371 metabolism and type 2 diabetes: the Hoorn Study. *Hypertension* 2004;43:176–81
- 372 5. Gomez D, Owens GK. Smooth muscle cell phenotypic switching in atherosclerosis.  
373 *Cardiovasc Res* 2012;95:156–164
- 374 6. Goel SA, Guo LW, Liu B, Kent KC. Mechanisms of post-intervention arterial remodelling.  
375 *Cardiovasc Res* 2012;96:363–371
- 376 7. Li H, Télémaque S, Miller RE, Marsh JD. High glucose inhibits apoptosis induced by serum  
377 deprivation in vascular smooth muscle cells via upregulation of Bcl-2 and Bcl-xl. *Diabetes*  
378 2005;54:540–545
- 379 8. Ruiz E, Gordillo-Moscoso A, Padilla E, Redondo S, Rodriguez E, Reguillo F, Briones AM,  
380 van Breemen C, Okon E, Tejerina T. Human vascular smooth muscle cells from diabetic  
381 patients are resistant to induced apoptosis due to high Bcl-2 expression. *Diabetes*  
382 2006;55:1243–1251
- 383 9. Simons M, Raposo G. Exosomes—vesicular carriers for intercellular communication. *Curr*  
384 *Opin Cell Biol* 2009;21:575–581
- 385 10. Mause SF, Weber C. Microparticles: protagonists of a novel communication network for  
386 intercellular information exchange. *Circ Res* 2010;107:1047–1057
- 387 11. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat*  
388 *Rev Immunol* 2009;9:581–593
- 389 12. Gould SJ, Raposo G. As we wait: coping with an imperfect nomenclature for extracellular  
390 vesicles. *J Extracell Vesicles* 2013;2
- 391 13. Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Exosomes/microvesicles as  
392 a mechanism of cell-to-cell communication. *Kidney Int* 2010;78:838–848
- 393 14. Ratajczak MZ, Ratajczak J. Horizontal transfer of RNA and proteins between cells by  
394 extracellular microvesicles: 14 years later. *Clin Transl Med* 2016;5:7
- 395 15. Yuana Y, Sturk A, Nieuwland R. Extracellular vesicles in physiological and pathological  
396 conditions. *Blood Rev* 2013;27:31–39

16. Chironi G, Simon A, Hugel B, Del Pino M, Gariepy J, Freyssinet JM, Tedgui A. Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects. *Arterioscler Thromb Vasc Biol* 2006;26:2775-2780
17. Ueba T, Nomura S, Inami N, Nishikawa T, Kajiwara M, Iwata R, Yamashita K. Plasma level of platelet-derived microparticles is associated with coronary heart disease risk score in healthy men. *J Atheroscler Thromb* 2010;17:342-349
18. Sinning JM, Losch J, Walenta K, Böhm M, Nickenig G, Werner N. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. *Eur Heart J* 2011;32:2034-2041
19. Jung KH, Chu K, Lee ST, Bahn JJ, Kim JH, Kim M, Lee SK, Roh JK. Risk of macrovascular complications in type 2 diabetes mellitus: endothelial microparticle profiles. *Cerebrovasc Dis* 2011;31:485-493
20. Brizzi MF, Formato L, Dentelli P, Rosso A, Pavan M, Garbarino G, Pegoraro M, Camussi G, Pegoraro L. Interleukin-3 stimulates migration and proliferation of vascular smooth muscle cells: a potential role in atherogenesis. *Circulation* 2001;103:549-554
21. Togliatto G, Dentelli P, Gili M, Gallo S, Deregibus C, Biglieri E, Iavello A, Santini E, Rossi C, Solini A, Camussi G, Brizzi MF. Obesity reduces the pro-angiogenic potential of adipose tissue stem cell-derived extracellular vesicles (EVs) by impairing miR-126 content: impact on clinical applications. *Int J Obes (Lond)* 2016;40:102-111
22. Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, Bruno S, Bussolati B, Camussi G. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood* 2007;110:2440-2448
23. Bruno S, Grange C, Collino F, Deregibus MC, Cantaluppi V, Biancone L, Tetta C, Camussi G. Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury. *PLoS One* 2012;7:e33115
24. Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Methods Mol Biol* 2015;1295:179-209
25. Lombardo G, Dentelli P, Togliatto G, Rosso A, Gili M, Gallo S, Deregibus MC, Camussi G, Brizzi MF. Activated stat5 trafficking via endothelial cell-derived extracellular vesicles controls IL-3 pro-angiogenic paracrine action. *Sci Rep* 2016;6:25689
26. Gallo S, Gili M, Lombardo G, Rossetti A, Rosso A, Dentelli P, Togliatto G, Deregibus MC, Taverna D, Camussi G, Brizzi MF. Stem Cell-Derived, microRNA-Carrying Extracellular Vesicles: A Novel Approach to Interfering with Mesangial Cell Collagen Production in a Hyperglycaemic Setting. *PLoS One* 2016;11:e0162417
27. Dentelli P, Barale C, Togliatto G, Trombetta A, Olgasi C, Gili M, Riganti C, Toppino M, Brizzi MF. A diabetic milieu promotes OCT4 and NANOG production in human visceral-derived adipose stem cells. *Diabetologia* 2013;56:173-184
28. Zeoli A, Dentelli P, Rosso A, Togliatto G, Trombetta A, Damiano L, di Celle PF, Pegoraro L, Altruda F, Brizzi MF. Interleukin-3 promotes expansion of hemopoietic-derived CD45+ angiogenic cells and their arterial commitment via STAT5 activation. *Blood* 2008;112:350-361
29. Shantikumar S, Caporali A, Emanuelli C. Role of microRNAs in diabetes and its cardiovascular complications. *Cardiovasc Res* 2012;93:583-593

30. Wei Y, Schober A, Weber C. Pathogenic arterial remodeling: the good and bad of microRNAs. *Am J Physiol Heart Circ Physiol*. 2013;304:H1050-1059.
31. Karginov FV, Hannon GJ. Remodeling of Ago2-mRNA interactions upon cellular stress reflects miRNA complementarity and correlates with altered translation rates. *Genes Dev* 2013;27:1624-1632
32. GBD 2013 Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2015;385:117-171
33. Kornowski R, Mintz GS, Kent KM, Pichard AD, Satler LF, Bucher TA, Hong MK, Popma JJ, Leon MB. Increased restenosis in diabetes mellitus after coronary interventions is due to exaggerated intimal hyperplasia. A serial intravascular ultrasound study. *Circulation* 1997;95:1366-1369
34. Brooks MM, Jones RH, Bach RG, Chaitman BR, Kern MJ, Orszulak TA, Follmann D, Sopko G, Blackstone EH, Califf RM. Predictors of mortality and mortality from cardiac causes in the bypass angioplasty revascularization investigation (BARI) randomized trial and registry. For the BARI Investigators. *Circulation* 2000;101:2682-2689
35. Henry RM, Kostense PJ, Dekker JM, Nijpels G, Heine RJ, Kamp O, Bouter LM, Stehouwer CD. Carotid arterial remodeling: a maladaptive phenomenon in type 2 diabetes but not in impaired glucose metabolism: the Hoorn study. *Stroke* 2004;35:671-676
36. Watson PA, Nesterova A, Burant CF, Klemm DJ, Reusch JE. Diabetes-related changes in cAMP response element-binding protein content enhance smooth muscle cell proliferation and migration. *J Biol Chem* 2001;276:46142-46150
37. Yasunari K, Kohno M, Kano H, Yokokawa K, Minami M, Yoshikawa J. Antioxidants improve impaired insulin-mediated glucose uptake and prevent migration and proliferation of cultured rabbit coronary smooth muscle cells induced by high glucose. *Circulation* 1999;99:1370-1378
38. Hall JL, Matter CM, Wang X, Gibbons GH. Hyperglycemia inhibits vascular smooth muscle cell apoptosis through a protein kinase C-dependent pathway. *Circ Res* 2000;87:574-580
39. Sakuma H, Yamamoto M, Okumura M, Kojima T, Maruyama T, Yasuda K. High glucose inhibits apoptosis in human coronary artery smooth muscle cells by increasing bcl-xL and bfl-1/A1. *Am J Physiol Cell Physiol* 2002;283:C422-C428
40. Karch J, Molkentin JD. Regulated necrotic cell death: the passive aggressive side of Bax and Bak. *Circ Res* 2015;116:1800-1809
41. Guay C, Regazzi R. Circulating microRNAs as novel biomarkers for diabetes mellitus. *Nat Rev Endocrinol* 2013;9:513-521
42. Togliatto G, Dentelli P, Brizzi MF. Skewed Epigenetics: An Alternative Therapeutic Option for Diabetes Complications. *J Diabetes Res* 2015;2015:373708
43. Gu J, Zhang H, Ji B, Jiang H, Zhao T, Jiang R, Zhang Z, Tan S, Ahmed A, Gu Y. Vesicle miR-195 derived from Endothelial Cells Inhibits Expression of Serotonin Transporter in Vessel Smooth Muscle Cells. *Sci Rep*. 2017;7:43546
44. Grotendorst CR, Chang T, Seppa HEJ, Kleinman HK, Martin GR. Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. *J Cell Physiol* 1982;113:261-266

45. Bilato C, Pauly RR, Melillo G, Monticone R, Gorelick-Feldman D, Gluzband YA, Sollott SJ, Ziman B, Lakatta EG, Crow MT. Intracellular signaling pathways required for rat vascular smooth muscle cell migration. Interactions between basic fibroblast growth factor and platelet-derived growth factor. *J Clin Invest* 1995;96:1905-1915
46. McNamara CA, Sarembock IJ, Bachhuber BG, Stouffer GA, Ragosta M, Barry W, Gimple LW, Powers ER, Owens GK. Thrombin and vascular smooth muscle cell proliferation: implications for atherosclerosis and restenosis. *Semin Thromb Hemost* 1996;22:139-144
47. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev* 2008;22:1276-1312
48. Raines EW. PDGF and cardiovascular disease. *Cytokine Growth Factor Rev* 2004;15:237-254
49. Weber A, Köppen HO, Schrör K. Platelet-derived microparticles stimulate coronary artery smooth muscle cell mitogenesis by a PDGF-independent mechanism. *Thromb Res* 2000;98:461-466
50. Goetzl EJ, Schwartz JB, Mustapic M, Lobach IV, Daneman R, Abner EL, Jicha GA. Altered cargo proteins of human plasma endothelial cell-derived exosomes in atherosclerotic cerebrovascular disease. *FASEB J* 2017;31:3689-3694
51. Tüfekci KU, Meuwissen RL, Genç S. The role of microRNAs in biological processes. *Methods Mol Biol* 2014;1107:15-31
52. Barile L, Vassalli G. Exosomes: Therapy delivery tools and biomarkers of diseases. *Pharmacol Ther* 2017;pii:S0163-7258(17)30034-7
53. Pant S, Hilton H, Burczynski ME. The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities. *Biochem Pharmacol* 2012;83:1484-1494

## 511 **FIGURE LEGENDS**

512 **Figure 1. D-CD31EVs increase VSMC survival.** (A) Representative FACS analysis of EVs  
513 recovered from sera of T2D (D, n=11) and ND individuals (ND, n=6); CD42b-FITC, CD14-PE and  
514 CD31-APC were analyzed. All data are reported in the histograms (mean of percentage $\pm$ SD) ( $p<0.01$ ,  
515 D-EVs vs ND-EVs for all markers). Isotype controls were included. (B) Representative transmission  
516 electron microscopy (TEM) imaging of D- and ND-CD31EVs negatively stained with NanoVan.  
517 JEOL Jem 1010 electron microscope was used (black bars= 100 nm). (C) CD31EVs were lysed and  
518 evaluated for CD31 content (CD31+), normalized to CD63. CD31EV negative (CD31-) fraction was  
519 used as the negative control. The results are representative of all samples (D, n=11; ND, n=6)  
520 ( $p<0.001$ , CD31+ vs CD31- fraction of T2D and non-diabetic individuals). (D) Apoptosis assay was  
521 applied to HG-cultured VSMCs, treated as indicated (percentage $\pm$ SEM of total apoptotic cells, n=6).  
522 Doxorubicin (1 $\mu$ mol/l) was used as positive control (c+) ( $p<0.001$ , all experimental conditions vs  
523 control (c+);  $p<0.001$ , D-CD31EVs vs ND-CD31EVs and none). (E) Cell extracts from HG-cultured  
524 VSMCs, treated with D-CD31EVs or ND-CD31EVs, were analyzed for bak/bax and bcl-2 content,  
525 normalized to  $\alpha$ -SMA ( $p<0.001$ , D-CD31EVs vs ND-CD31EVs and none for bak;  $p<0.05$ , D-  
526 CD31EVs vs ND-CD31EVs and none for bax and bcl-2, n=6).

527 **Figure 2. D-CD31EVs enriched in PDGF-BB induce anti-apoptotic signals (A).** Negative and  
528 positive fractions of CD31EVs were analyzed by western blot for PDGF-BB, normalized to CD63.  
529 The results are representative of all samples (D, n=11; ND, n=6) ( $p<0.001$ , CD31EVs+ vs CD31EVs-  
530 of D and ND;  $p<0.001$ , D-CD31EVs+ vs ND-CD31EVs+ for PDGF-BB). (B) Cell extracts from LG-  
531 and HG-cultured VSMCs untreated or treated with PDGF-BB (10 ng/ml), were analyzed for bak/bax  
532 and bcl-2 content, normalized to  $\alpha$ -SMA (PDGF-BB vs none in HG-cultured VSMCs,  $p<0.001$  for  
533 bak and bax,  $p=0.04$  for bcl-2, n=5). (C) Apoptosis assay was applied to VSMCs, treated as above  
534 (percentage $\pm$ SEM of total apoptotic cells, n=6). Doxorubicin (1 $\mu$ mol/l) served as positive control  
535 (c+) (LG-cultured VSMCs,  $p=0.05$ , all experimental conditions vs positive control (c+); HG-cultured

VSMCs,  $p=0.001$ , all experimental conditions vs positive control (c+),  $p=0.008$ , PDGF-BB vs none).  
**(D)** PDGF-BB content was evaluated in HG-cultured ECs transfected or not for 48h with siRNA empty vector, used as control (control siRNA), or with PDGF-BB siRNA, and normalized to  $\beta$ -actin ( $p=0.007$ , PDGF-BB siRNA vs none and  $p=0.003$ , PDGF-BB siRNA vs control siRNA). **(E)** PDGF-BB content was evaluated in EVs recovered from HG-cultured ECs, treated as above, and normalized to CD63 ( $p<0.001$ , PDGF-BB siRNA vs control siRNA and none). **(F)** Cell extracts from HG-cultured VSMCs, treated as indicated, were analyzed for bak/bax and bcl-2 content, normalized to  $\alpha$ -SMA ( $p<0.01$ , HG-EVs PDGF-BB siRNA vs HG-EVs control and none;  $n=6$ ). **(G)** Apoptosis assay was performed in HG-cultured VSMCs, treated as indicated (percentage $\pm$ SEM of total apoptotic cells,  $n=6$ ). Doxorubicin (1 $\mu$ mol/l) served as positive control (c+) ( $p<0.001$ , all experimental conditions vs positive control (c+);  $p=0.02$ , HG-EVs control siRNA vs none;  $p=0.009$ , HG-EVs PDGF-BB siRNA vs HG-EVs control siRNA).

**Figure 3. PDGFR $\beta$  blockade interferes with free PDGF-BB- and D-CD31EV-mediated effects.**

**(A)** HG-cultured VSMCs, pre-incubated or not with a blocking PDGFR $\beta$  antibody (5 $\mu$ g/ml), were untreated or treated with PDGF-BB (10 ng/ml) or with D-CD31EVs for 24h. Cell extracts were analyzed for bak/bax and bcl-2 content, normalized to  $\alpha$ -SMA ( $p<0.001$ , PDGF-BB and D-CD31EVs vs PDGF-BB and D-CD31EVs, pre-treated with anti-PDGFR $\beta$  antibody) ( $n=4$ ). **(B)** VSMC-D-CD31EV up-take. VSMCs, pre-incubated or not with the blocking PDGFR $\beta$  antibody, were evaluated for the uptake of PKH26-labeled D-CD31EVs and analyzed. DAPI was used as nuclear marker. Representative sections (first-middle-last) of images (Z-stack) obtained on a confocal microscope are reported. Four different experiments performed in triplicate ( $n=4$ ). Scale bars indicate 10  $\mu$ m. **(C)** To evaluate mbPDGF-BB, intact or lysates D-CD31EVs ( $2.5 \times 10^8$  particles), untreated or treated with trypsin (0,25%) for 1h, were measured using a competitive enzyme immunoassay (ELISA) kit (\*\*  $p<0.01$ , D-CD31EVs intact and lysates vs D-CD31EVs +trypsin) ( $n=3$ ).



560 **Figure 4. D-CD31EVs increase VSMC migration and recruitment to tubule-like structures. (A-**  
 561 **B)** An *in vitro* angiogenesis assay was performed using pre-labeled ECs (red) and VSMCs (green)  
 562 co-cultured in HG medium with or without the indicated CD31EVs for 6h (scale bars=20μm, 40X  
 563 magnification). Data are reported in the histogram as number±SEM of VSMCs per number of tubular  
 564 structures ( $p<0.001$ , ND-CD31EVs vs none and D-CD31EVs;  $p<0.05$ , D-CD31EVs vs none, n=5).  
 565 **(C)** VSMC migration assay was performed in HG conditions and the indicated treatment. (n=5, 20X  
 566 magnification) ( $p=0.04$ , D-CD31EVs vs none;  $p=0.01$ , D-CD31EVs vs ND-CD31EVs). **(D-E)** An *in*  
 567 *vitro* angiogenesis assay was performed, as above, using HG-EVs control siRNA or HG-EVs depleted  
 568 of PDGF-BB (PDGF-BB siRNA) (scale bars=20μm, 40X magnification). Data are reported in the  
 569 histogram as number±SEM of VSMCs per number of tubular structures ( $p<0.001$ , HG-EVs PDGF-  
 570 BB siRNA vs HG-EVs control siRNA and none, n=5). **(F)** VSMC migration assay was performed in  
 571 HG conditions under the indicated treatment (n=5, 20X magnification) ( $p=0.009$ , HG-EVs PDGF-  
 572 BB siRNA vs HG-EVs control siRNA). Representative images were acquired on a confocal  
 573 microscope.

574 **Figure 5. VSMCs from T2D individuals express high levels of bcl-2 and low bak/bax content.**  
 575 **(A)** Representative FACS analysis of CD31 and alpha-smooth muscle cell ( $\alpha$ -SMA) surface markers  
 576 expressed by VSMCs recovered from T2D (D, n=11) and non-diabetic (ND) individuals (ND, n=6).  
 577 All data are reported in the Table (mean percentage±SD). Isotype control was included. **(B)** bak/bax  
 578 and bcl-2 content was analyzed on all ND- or D-VSMC samples, normalized to  $\alpha$ -SMA content. The  
 579 statistical analysis of all samples (D, n=11; ND, n=6) is reported in **(C)** ( $p<0.01$ , D vs ND for bak,  
 580  $p<0.001$ , D vs ND for bax,  $p<0.05$ , D vs ND for bcl-2).

581 **Figure 6. VSMC miR-296-5p expression is increased in hyperglycaemic condition and boosted**  
 582 **by PDGF-BB (A)** The indicated miRs were evaluated by qRT-PCR in VSMCs recovered from T2D  
 583 (D) and ND human atherosclerotic plaque specimens. Data normalized to RNU6B are representative  
 584 of all samples (D, n=11; ND, n=6) ( $p=0.02$ , D vs ND for miR-296-5p). **(B)** miR-296-5p was evaluated

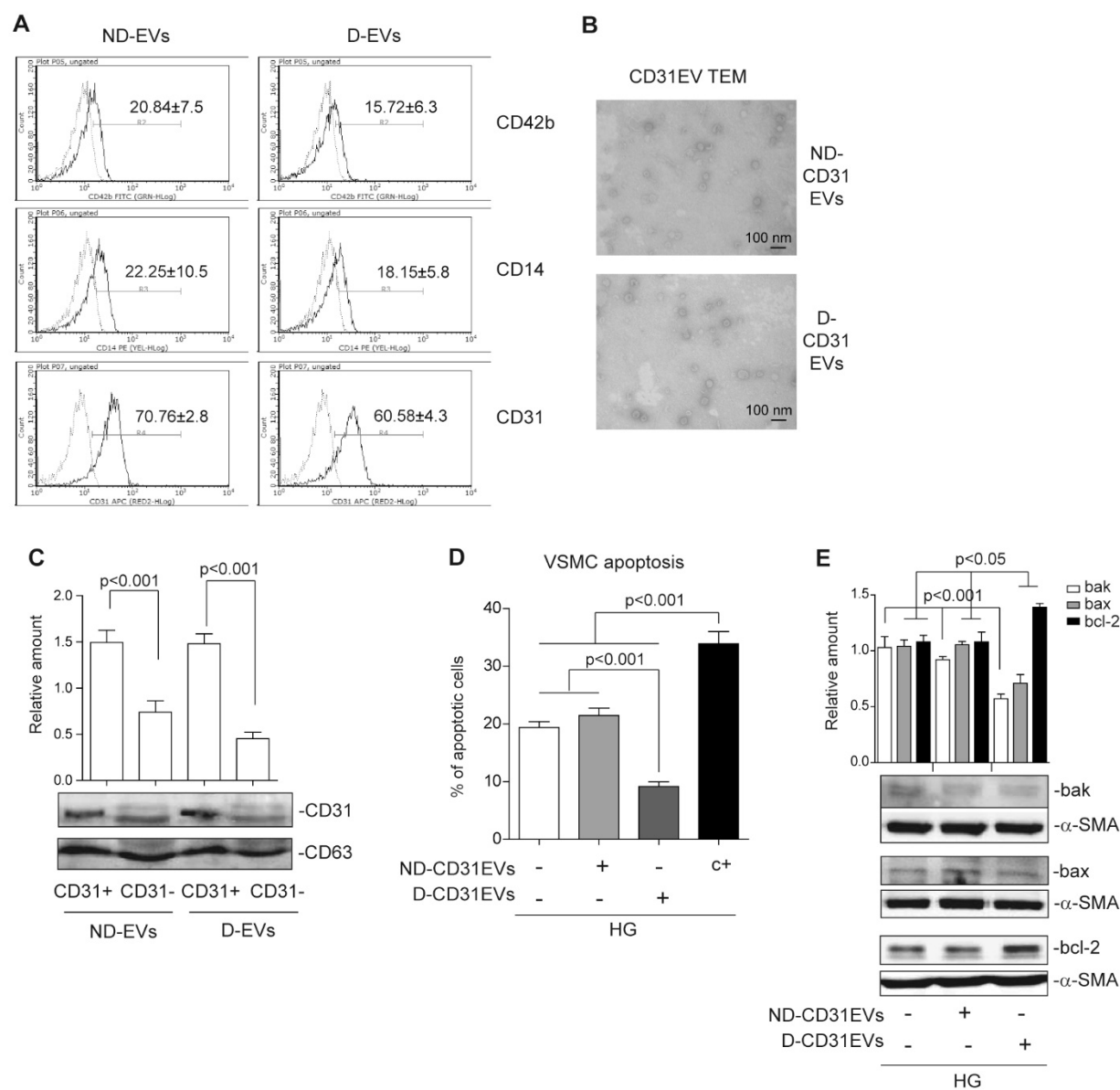
585 by qRT-PCR on LG- or HG-treated VSMCs and normalized to RNU6B ( $p=0.03$ , HG- vs LG-treated  
586 VSMCs,  $n=6$ ). **(C)** Loss-of-function experiments were performed on LG- and HG-cultured VSMCs  
587 for 48h, using antago-miR control or antago-miR-296-5p oligonucleotides. After 48h cells were lysed  
588 and analyzed for bak/bax and bcl-2 content, normalized to  $\alpha$ -SMA ( $p<0.001$ , LG-antago-miR control  
589 and LG-anti-miR-296-5p vs HG-antago-miR control for bak/bax and bcl-2;  $p<0.001$ , HG-antago-  
590 miR control vs HG-antago-miR-296-5p for bak and bcl-2;  $p<0.05$ , HG-antago-miR control vs HG-  
591 antago-miR-296-5p for bax,  $n=3$ ). **(D)** Apoptosis assay was performed on VSMCs cultured and  
592 treated as in (C). Doxorubicin ( $1\mu\text{mol/l}$ ) served as positive control (c+). Data are expressed as  
593 percentage $\pm$ SEM ( $n=5$ ) of total apoptotic cells ( $p<0.001$ , LG-antago-miR control vs HG- antago-miR  
594 control, HG- antago-miR control vs HG-antago-miR-296-5p;  $p<0.001$ , all experimental conditions  
595 vs control, c+). **(E)** miR-296-5p expression, normalized to RNU6B, was evaluated by qRT-PCR in  
596 LG and HG-cultured VSMCs both with and without PDGF-BB ( $10\text{ ng/ml}$ ) ( $p=0.002$ , PDGF-BB vs  
597 none in HG-treated VSMCs,  $n=6$ ). **(F)** miR-296-5p expression, normalized to RNU6B, was evaluated  
598 by qRT-PCR in HG-cultured VSMCs, treated as indicated ( $p=0.007$ , HG-EVs control siRNA vs  
599 none;  $p=0.002$ , HG-EVs PDGF-BB siRNA vs HG-EVs control siRNA,  $n=6$ ).

600 **Figure 7. miR-296-5p post-transcriptionally regulates bak expression.** **(A)** Blast analysis of hsa-  
601 miR-296-5p sequence and *BAK1* 3'UTR full-length shows a base pairing from 1138 to 1158 bp. **(B)**  
602 pGL3 empty vector and pGL3-3'UTR *BAK1* luciferase constructs were transfected into LG- and HG-  
603 cultured VSMCs. Relative luciferase activity is reported ( $p<0.001$  HG vs LG in pGL3-3'UTR *BAK1*-  
604 transfected cells,  $n=5$ ). **(C)** pGL3 and pGL3-3'UTR *BAK1* constructs were transfected into LG-  
605 cultured VSMCs previously transfected with pre-miR control or with pre-miR-296-5p. Relative  
606 luciferase activity is reported ( $p<0.001$  pre-miR-296-5p vs pre-miR control in pGL3-3'UTR *BAK1*  
607 transfected cells,  $n=5$ ). **(D)** Bak content was analyzed on cell extracts from VSMCs overexpressing  
608 miR-296-5p (pre-miR-296-5p), not transfected or transfected with pGL3 or pGL3-3'UTR *BAK1*

609 constructs and normalized to  $\alpha$ -SMA ( $p<0.001$  VSMCs/pre-miR-296-5p none and pGL3 vs  
610 VSMCs/pre-miR-296-5p+pGL3-3'UTR *BAK1*, n=5).

611 **Figure 8. Schematic representation of HG and D-CD31EV mechanism of action.** ND-CD31EVs  
612 do not affect VSMC fate due to their low mbPDGF-BB content (left panel). In the diabetic setting,  
613 D-CD31EVs enriched in mbPDGF-BB content affect VSMC fate by promoting resistance to  
614 apoptosis (right panel).

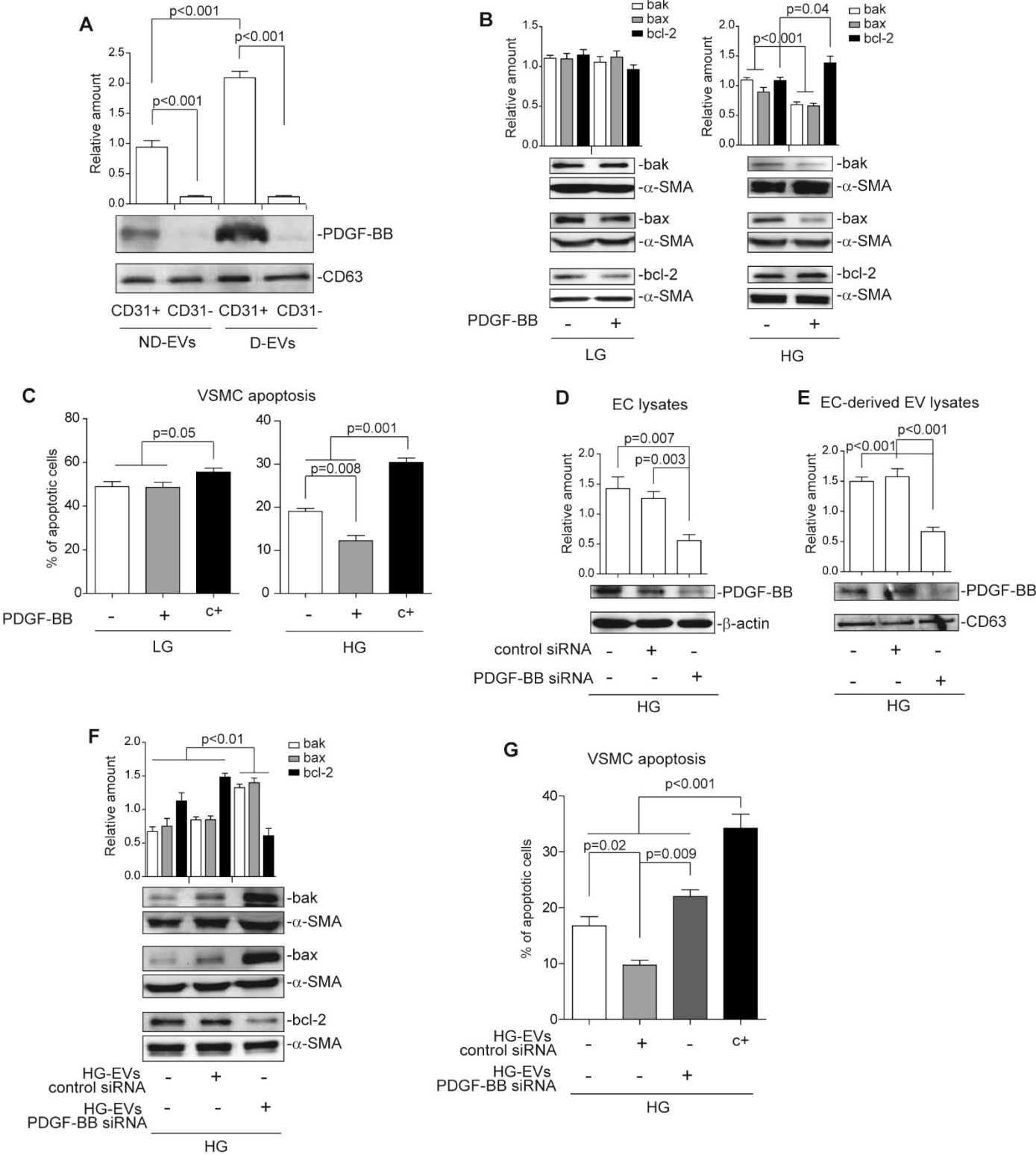
Figure 1



615

616

Figure 2



617

618

619

Figure 3

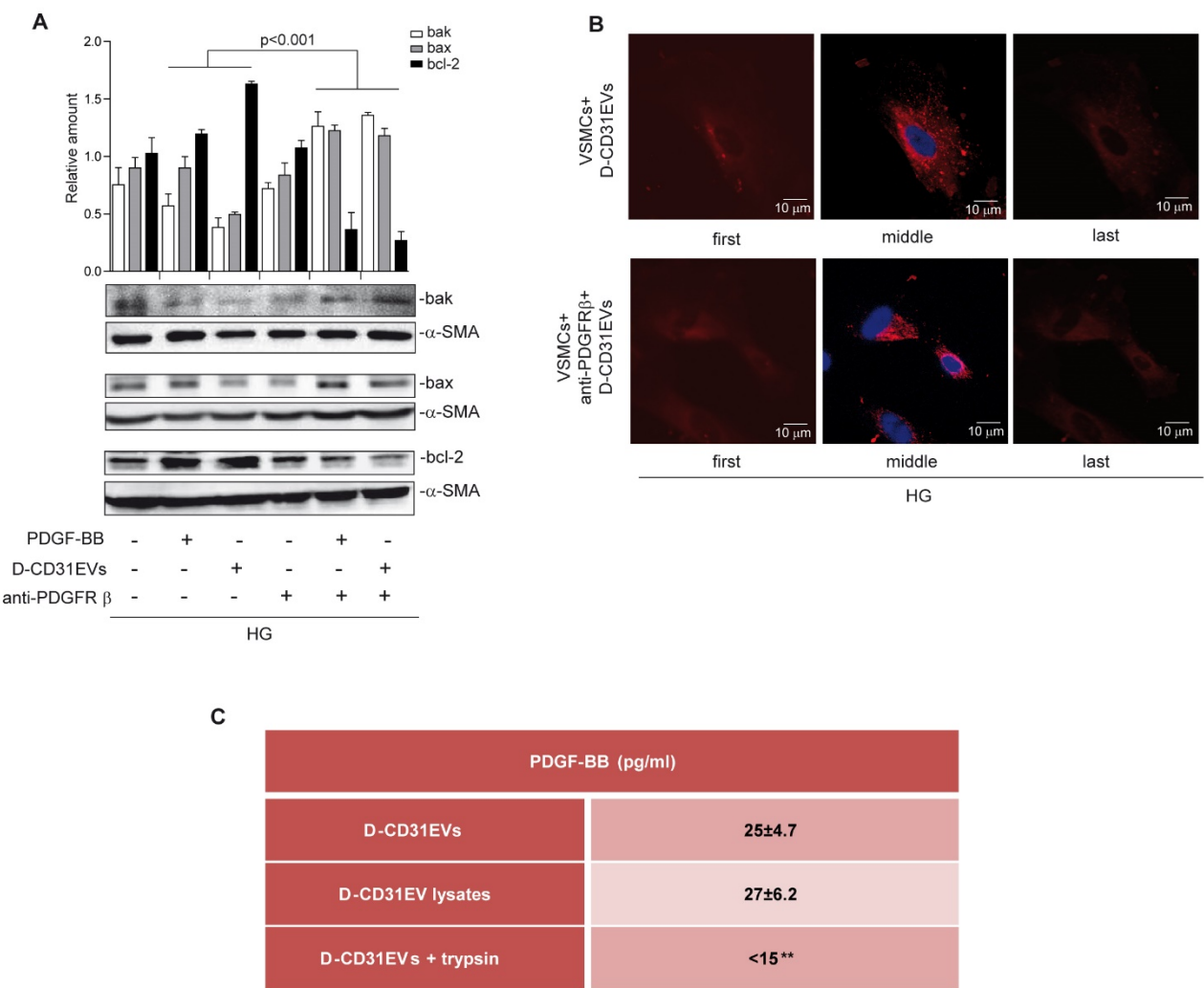


Figure 4

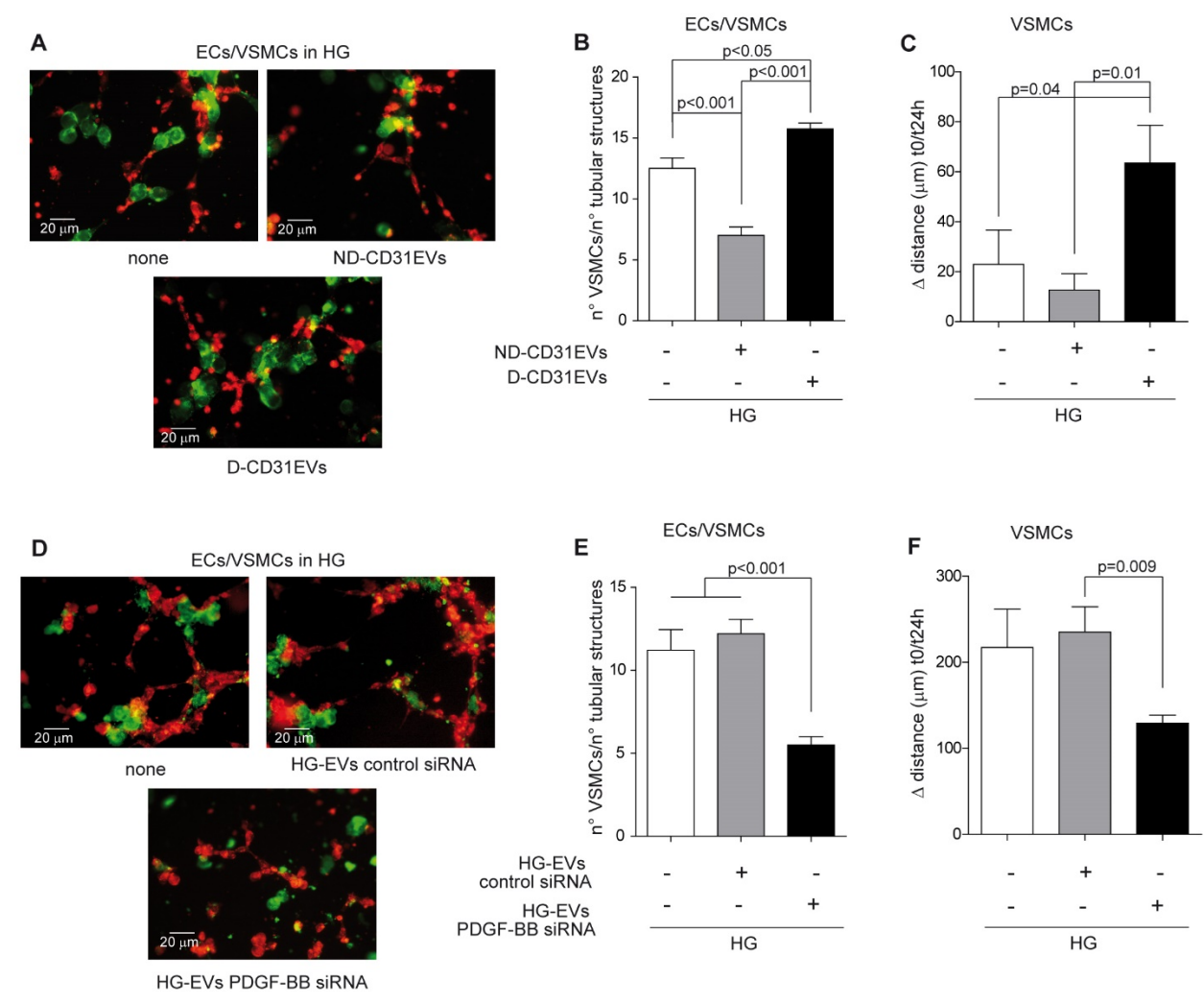


Figure 5

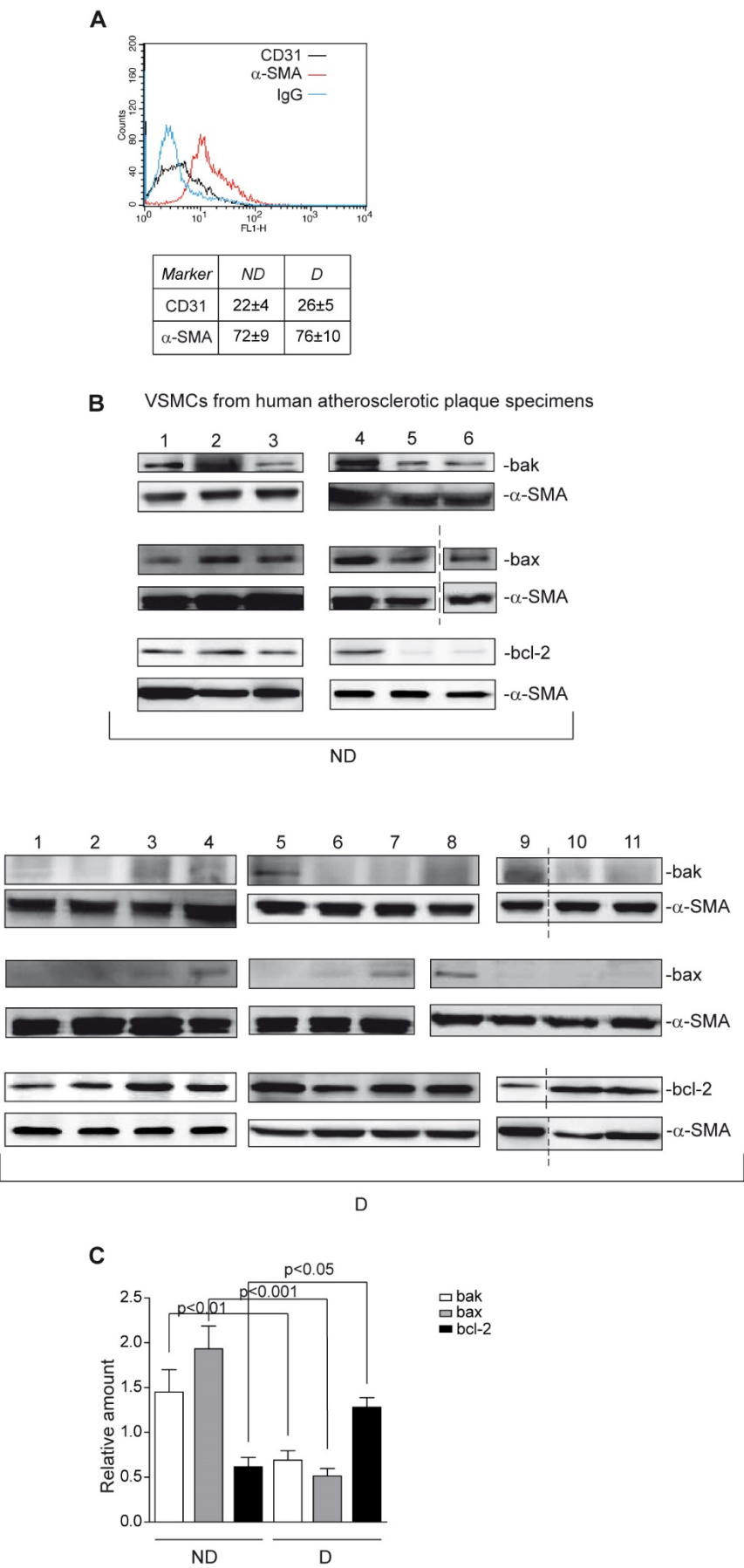




Figure 6

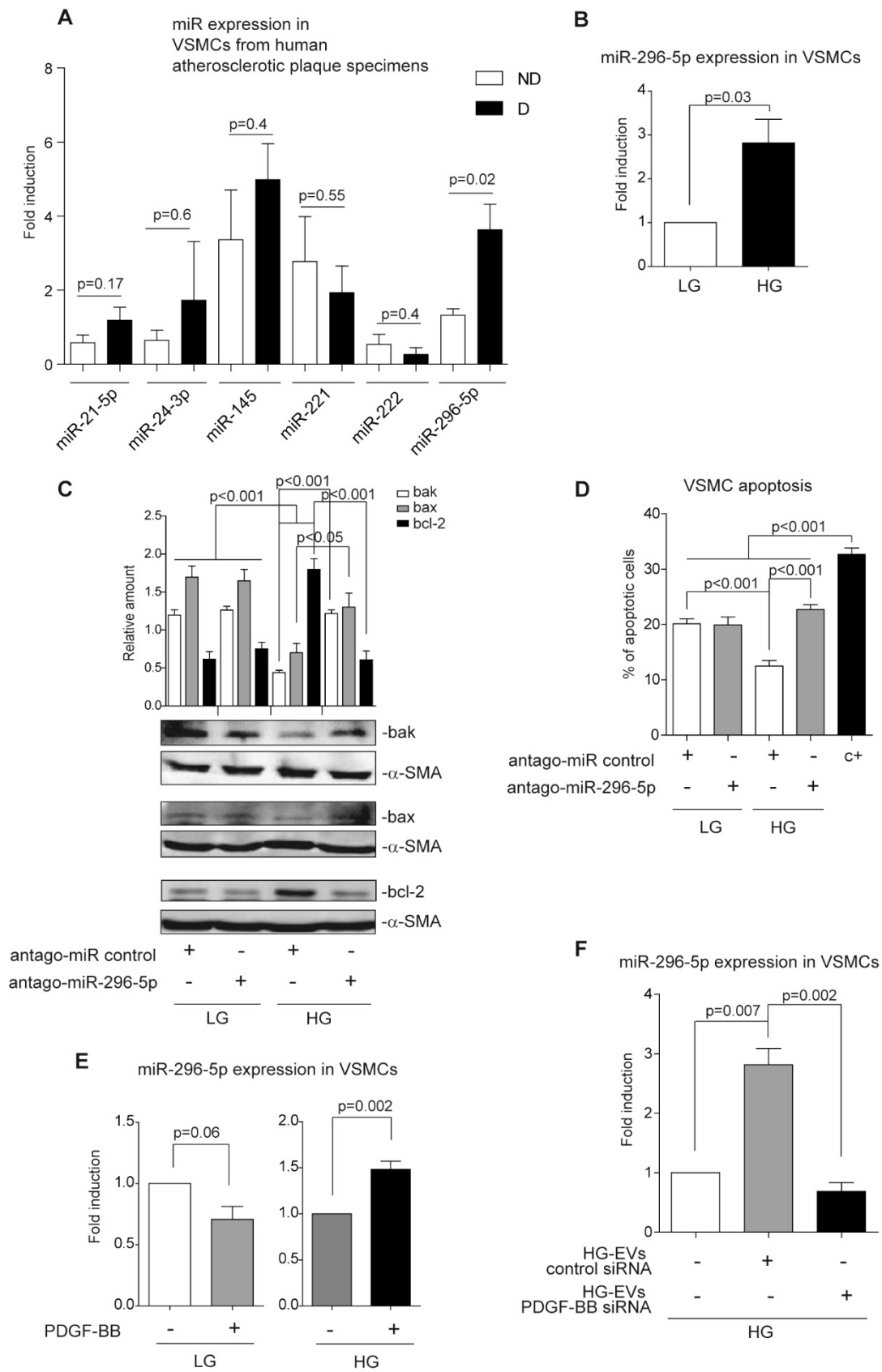


Figure 7

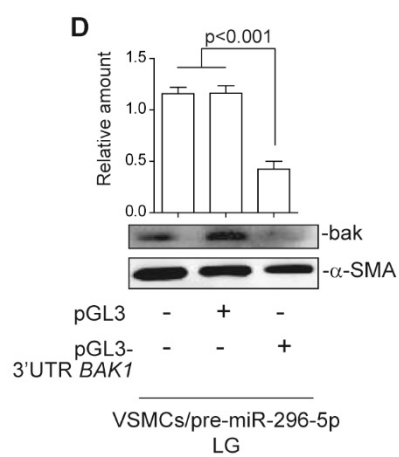
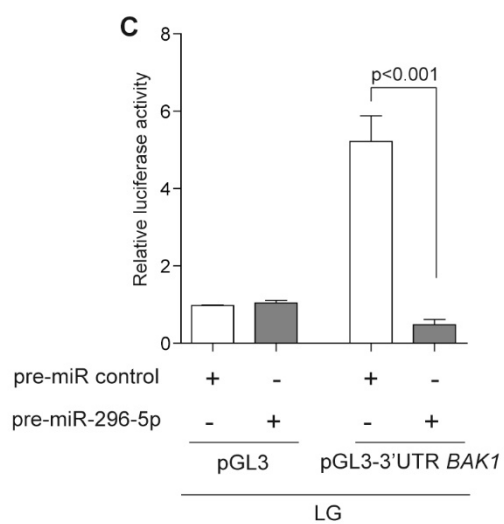
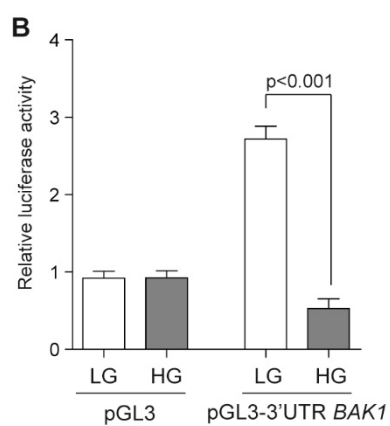
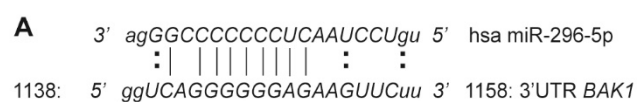


Figure 8

