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# **Aortic valve interstitial cells act as vasculogenic progenitor cells during human calcific aortic valve disease**

**Running title: Gendron et al. Aortic valve interstitial cells and vasculogenesis**

Nicolas Gendron<sup>a\*</sup>, Mickael Rosa<sup>b\*</sup>, Adeline Blandinieres<sup>a</sup>, Yoann Sottejeau<sup>b</sup>, Elisa Rossi<sup>a</sup>, Eric Van Belle<sup>b</sup>, Salim Idelcadi<sup>c</sup>, Séverine Lecourt<sup>a</sup>, André Vincentelli<sup>b</sup>, Audrey Cras<sup>d</sup>, Ramadan Jashari<sup>e</sup>, Richard Chocron<sup>f</sup>, Yaël Baudouin<sup>g</sup>, Thibault Pamart<sup>b</sup>, Ivan Bièche<sup>h</sup>, Nathalie Nevo<sup>a</sup>, Bernard Cholley<sup>c</sup>, Jeanne Rancic<sup>a</sup>, Bart Staels<sup>b</sup>, Pascale Gaussem<sup>a</sup>, Annabelle Dupont<sup>b</sup>, Alain Carpentier<sup>i</sup>, Sophie Susen<sup>b</sup> and David M. Smadja<sup>a</sup>

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<sup>a</sup>Université de Paris, Innovative Therapies in Haemostasis, INSERM, F-75006 Paris, France, Hematology department and Biosurgical Research Lab (Carpentier Foundation), AH-HP, Georges Pompidou European Hospital, F-75015 Paris, France.

<sup>b</sup>Univ. Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U1011 - EGID, F-59000 Lille, France.

<sup>c</sup>Université de Paris, Innovative Therapies in Haemostasis, INSERM, F-75006 Paris, France, Department of Anesthesia and Intensive Care and Biosurgical Research Lab (Carpentier Foundation), AH-HP, Georges Pompidou European Hospital, F-75015 Paris, France.

<sup>d</sup>Université de Paris, Innovative Therapies in Haemostasis, INSERM, F-75006 Paris, France, Cell therapy Department, AH-HP, Saint Louis Hospital, F-75010 Paris, France.

<sup>e</sup>European Homograft Bank, Clinic Saint Jean, Brussels, Belgium.

<sup>f</sup>Université de Paris, PARCC, INSERM, F-75015 Paris, France, Emergency Medicine Department, AH-HP, Georges Pompidou European Hospital, F-75015 Paris, France.

<sup>g</sup>Hematology Department, AP-HP, Hôpital Bichat-Claude Bernard, F-75018 Paris, France.

<sup>h</sup>Department of Genetics, Pharmacogenomics Unit, Institut Curie, Paris, France.

<sup>i</sup>Université de Paris, Biosurgical Research Lab (Carpentier Foundation), AH-HP, Georges Pompidou European Hospital, F-75015 Paris, France.

\* These two authors contributed equally to the work

## **Address for correspondence:**

Prof. David M. Smadja, European Georges Pompidou Hospital, Hematology Department, Biosurgical

Research Lab (Carpentier Foundation) and UMR-S 1140; 56 rue Leblanc, 75015 Paris, France,

Tel: +33 1 56 09 39 33; fax: +33 1 56 09 33 93 - E-mail: david.smadja@aphp.fr

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## 1 **Abstract**

2 **Objective.** The study's aim was to analyze the capacity of human valve interstitial cells (VICs) to participate in  
3 aortic valve angiogenesis.

4 **Approach and results.** VICs were isolated from human aortic valves obtained after surgery for calcific aortic  
5 valve disease (CAVD) and from normal aortic valves unsuitable for grafting (control VICs: VIC<sub>c</sub>). We examined  
6 VIC *in vitro* and *in vivo* potential to differentiate in endothelial and perivascular lineages. VIC paracrine effect  
7 was also examined on human endothelial colony forming cells (ECFCs). A pathological VIC (VIC<sub>p</sub>)  
8 mesenchymal-like phenotype was confirmed by CD90<sup>+</sup>/CD73<sup>+</sup>/CD44<sup>+</sup> expression and multipotent-like  
9 differentiation ability. When VIC<sub>p</sub> were co-cultured with ECFCs, they formed microvessels by differentiating  
10 into perivascular cells both *in vivo* and *in vitro*. VIC<sub>p</sub> and VIC<sub>c</sub> conditioned media (CM) were compared using  
11 serial ELISA regarding quantification of endothelial and/or vasculogenic factors. Higher expression of VEGF-  
12 A was observed at the protein level in VIC<sub>p</sub>-CM and confirmed at the mRNA level in VIC<sub>p</sub> compared to VIC<sub>c</sub>.  
13 CM from VIC<sub>p</sub> induced *in vitro* a significant increase in ECFC proliferation, migration and sprouting compared  
14 with CM from VIC<sub>c</sub>. These effects were inhibited by blocking VEGF-A with blocking antibody or siRNA  
15 approach, confirming VIC<sub>p</sub> involvement in vasculogenesis by a VEGF-A dependent mechanism.

16 **Conclusions.** We provide here the first proof of a vasculogenic potential of human VICs isolated from CAVD  
17 patients. These results point to a novel function of VIC<sub>p</sub> in valve vascularization during CAVD, with a  
18 perivascular differentiation ability and a VEGF-A paracrine effect. Targeting perivascular differentiation and/or  
19 VEGF-A to slow CAVD progression warrants further investigation.

**1 Abbreviations**

- 2**  $\alpha$ SMA:  $\alpha$ -smooth muscle actin
- 3** CAVD: calcific aortic valve disease
- 4** CM: conditioned media
- 5** ECFCs: endothelial colony forming cells
- 6** mab VEGFA: monoclonal blocking antibody against VEGF-A
- 7** MSCs: mesenchymal stem-cells
- 8** NG2: neural glial antigen-2
- 9** VECs: valve endothelial cells
- 10** VICs: valve interstitial cells
- 11** VIC<sub>c</sub>: control VIC
- 12** VIC<sub>p</sub>: pathological VIC

## 1 Introduction

2

3 Calcific aortic valve stenosis (CAVD) is a major public health issue and affects 2 to 7% of adults over the age  
4 of 65. Its prevalence will double within the next 50 years due to population aging<sup>1</sup>. CAVD pathogenesis is  
5 unclear and is described as a progressive fibro-calcifying process with calcific lesion formation associated with  
6 inflammatory cells accumulation, fibrosis and neovascularization of the aortic valve<sup>2,3,4</sup>. There is currently no  
7 effective pharmacological treatment to prevent or slow disease progression.

8 Normal aortic valve displays a layered architectural pattern of cells, including valve endothelial cells (VECs) at  
9 the blood-contacting surface and valve interstitial cells (VICs), which are the main cells found in the 3 layers of  
10 the valve. VICs are responsible for tissue homeostasis and valve function. They account for a dynamic,  
11 heterogeneous cell population, negative for CD31 and CD68, with a fibroblast-like phenotype and the capacity  
12 to differentiate into osteoblast-like cells and to promote calcification<sup>5</sup>. A normal valve is avascular and the  
13 pathophysiology of aberrant angiogenesis in CAVD is still unknown. We and others have described several  
14 mesenchymal-type stem cells able to act as post-natal vasculogenic cells. While bone marrow mesenchymal  
15 stem cells (MSCs)<sup>6</sup> and circulating fibrocytes<sup>7</sup> are able to behave as perivascular cells and to support  
16 vasculogenesis *via* the release of angiogenic factors<sup>8</sup>, two mesenchymal subtypes isolated from CD133 cells  
17 have been described as giving rise to endothelial cells. They are respectively hemangioma stem cells<sup>9,10</sup> isolated  
18 from infantile tumors and very small embryonic-like stem cells isolated from human bone marrow<sup>11,12,13</sup>. Both  
19 cell types are able to differentiate into endothelial and perivascular cells.

20 VICs have been described as mesenchymal cells with a highly plastic phenotype<sup>5</sup>. In 2016, Arevalos *et al.*  
21 showed that porcine VICs exhibited a pericyte-like behavior as they stabilized early VEC vasculogenic  
22 networks, expressing  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and neural glial antigen-2 (NG2). However, cellular  
23 mechanisms involved in this vasculogenesis process remain unknown, and particularly in the CAVD context.  
24 The aims of this study were 1) to investigate the ability of VICs from CAVD patients to differentiate into  
25 endothelial and perivascular lineages and 2), to analyze the paracrine effects of VICs on human endothelial  
26 progenitor cells and the subsequent signaling pathways controlling VIC effects on the formation of new vessels  
27 in CAVD.

## 1 **Material and methods**

### 2 **Study population**

3 Study patients were recruited at the cardiovascular surgery department of the CHU in Lille, France. The study  
4 protocol, in compliance with the ethical guidelines of the Declaration of Helsinki, was approved by the local  
5 ethics committee (ATHERAO study, CPP2008\_13/0839). Informed consent was obtained from each patient  
6 included. A total of 22 consecutive patients who underwent aortic valve replacement for severe tricuspid CAVD  
7 (according to AHA guidelines<sup>14</sup>) were enrolled.

8 Human aortic valves were obtained immediately after surgery. As controls, non-calcified human aortic valves  
9 were collected from prospective donor hearts considered unsuitable for transplantation by the European  
10 Homograft Bank (Clinique Saint-Jean, Brussels, Belgium; n=14) that were rapidly excised and washed in  
11 Polyphosphate Buffer Saline (PBS) 1X (Online supplemental Figure 1).

12 In keeping with the typical characteristics of patients presenting with CAVD, median age of patients was 71.5  
13 years (ranging from 49 to 84) and 63.4% were males (Online supplemental Table 1). Control aortic valves were  
14 collected from 14 autopsy hearts from patients with a median age of 52 years (ranging from 32 to 72) with 50%  
15 males.

16

### 17 **Aortic VIC isolation and culture**

18 Pathological VICs (VIC<sub>p</sub>) and control VICs (VIC<sub>c</sub>) were obtained after type-I collagenase digestion of the aortic  
19 valve, as previously described<sup>15</sup>, from patients after surgery and after autopsy for controls, respectively. VICs  
20 were seeded on fibronectin-coated plates (0.4 µg/cm<sup>2</sup>, Millipore, Billerica, MA, USA), cultured in Smooth  
21 Muscle Growth Medium-2 (SMGM-2, Lonza, Walkersville, MD, USA) supplemented with 20% FBS (fetal  
22 bovine serum, Hyclone, Logan, UT, USA) and used between passages 2-6. The absence of contamination by  
23 endothelial cells and/or macrophages was ensured after digestion by flow cytometry with fluorochrome-  
24 conjugated antibodies CD31 (BD Biosciences, San Jose, CA, USA) and CD68 (BD Biosciences). All assays  
25 were performed at 37°C, on 95% sterile air, and 5% CO<sub>2</sub> in a saturated humidified incubator.

26

## **1 ECFC and MSC isolation and culture**

2 Human umbilical cord blood samples from healthy, full-term newborns were obtained through a partnership  
3 with the Cord Blood Bank of St Louis Hospital (Paris, France), which is authorized by the French Regulatory  
4 Authority (authorization no. PPC51) and participates in scientific research. Cord blood endothelial progenitor  
5 cells (ECFCs) were isolated from the adherent mononuclear cells fraction as previously described<sup>16,17,18</sup>. ECFCs  
6 were expanded on fibronectin-coated plates using Endothelial Growth Medium-2 (EGM-2, Lonza)  
7 supplemented with 5% FBS. MSCs were isolated from the mononuclear cell fraction of human adult bone  
8 marrow as previously described and according to International Society for Cellular Therapy standard criteria for  
9 MSC isolation and characterization<sup>19</sup>. The medium was replaced every 2 or 3 days until confluence. Angiogenic  
10 assays have been described elsewhere<sup>12,15,20</sup> and are detailed in the supplementary file.

11

## **12 Statistical analysis**

13 Data are shown as mean  $\pm$  SEM. Assessment of parametric model assumptions was performed using Levene's  
14 test of equal variances and Shapiro's test of normal distribution. Data sets failed normality and equal variance  
15 tests and were analyzed using the Mann-Whitney nonparametric test or Kruskal-Wallis test followed by the  
16 Dunn multiple comparisons test. All statistical analyses were performed using GraphPad Prism 5 software  
17 (GraphPad Software Inc., San Diego, CA, USA) and the StatView software package (SAS, Cary, NC, USA).  
18 Differences were considered significant at  $p < 0.05$  and shown as \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

## 1 **Results**

### 2 **VICs isolated from CAVD patients exhibit a mesenchymal phenotype**

3 Human VIC<sub>p</sub> in monolayer cultures showed the elongated, spindle-shaped morphology characteristic of  
4 mesenchymal cells in culture<sup>21</sup> (Figure 1A). ECFCs served as positive controls for endothelial and vasculogenic  
5 cells. VIC<sub>p</sub> were found negative for endothelial (CD144, Tie-2 and CD31) and progenitor markers (CD133 and  
6 CD34), positive for CD90, CD73 and CD44 at the same level as MSCs (Figure 1B) and positive for integrins  
7  $\alpha_v$ ,  $\beta_1$  and  $\beta_3$ . Furthermore, mesenchymal, endothelial and hematopoietic marker gene expression was quantified  
8 by RT-qPCR (Figure 1C). Cultured VIC<sub>p</sub> expressed a high level of mesenchymal markers such as THY1 (CD90),  
9 CD44 and fibroblast markers such as VIM, S100A4 and TWIST1, at similar levels as MSCs. Moreover, VIC<sub>p</sub>  
10 expressed low levels of endothelial markers such as FLT1 and KDR, contrary to ECFCs. All isolated and  
11 cultured MSCs, VIC<sub>p</sub> and ECFCs expressed extremely low levels of hematopoietic markers such as PTPRC  
12 (CD45) and fat cell markers such as LEP and ADIPOQ. We then explored the multipotent differentiation ability  
13 of VIC<sub>p</sub>. In an osteogenic medium, VIC<sub>p</sub> underwent osteogenic differentiation firstly revealed by ALP activity  
14 and then by calcium deposits as detected by Alizarin Red staining (Figure 1D). Human VIC<sub>p</sub> were cultured on  
15 fibronectin-coated tissue culture plastic because our objectives were to explore the angiogenic properties and  
16 vasculogenic differentiation of VIC<sub>p</sub>. Calcification assays were evaluated in different matrix-coated tissue  
17 conditions in the absence of coating, gelatin or fibronectin (Online supplemental Figure 2). When VIC<sub>p</sub> were  
18 cultured in control or osteogenic cell differentiation medium, no significant differences in Alizarin Red staining  
19 were observed between the different matrices evaluated ( $p>0.05$  for each). We also explored calcium deposition  
20 by VIC<sub>p</sub> cultured alone or with ECFCs in control medium or in osteogenic cell differentiation medium (Online  
21 supplemental Figure 3). No significant differences in Alizarin Red staining were observed between VIC<sub>p</sub> alone  
22 or cocultured with ECFCs ( $p>0.05$  for each).

23

### 24 **VICs from CAVD patients differentiate into perivascular cells but not into endothelial lineages**

25 In order to assess whether human VIC<sub>p</sub> were able to differentiate into endothelial and/or perivascular lineages,  
26 VIC<sub>p</sub> alone, ECFCs alone, or VIC<sub>p</sub>+ECFCs were suspended in Matrigel and injected into nude mice as previously  
27 described<sup>12,22</sup> (Figure 2A). Control matrigels used MSCs+ECFCs. After 10 days, explanted Matrigel plug



1 sections showed numerous vascular channels filled with red blood cells in the Matrigel explants containing  
2 VIC<sub>p</sub>+ECFCs, contrary to those containing VIC<sub>p</sub> or ECFCs alone, that did not form vessels (Figure 2B).  
3 Interestingly, matrigel explants containing VIC<sub>p</sub>+ECFCs showed similar microvessel density ( $0.012 \pm 0.002$   
4 vessels/ $\mu\text{m}^2$ ) than control explants MSCs+ECFCs ( $0.017 \pm 0.003$  vessels/ $\mu\text{m}^2$ ,  $p=0.23$ , Figure 2B, right panel).  
5 In these explants, immunofluorescence analysis for  $\alpha\text{SMA}$  and human-specific CD31 showed perivascular cells  
6 positive for  $\alpha\text{SMA}$  surrounding human CD31 blood vessels. On the contrary, explanted Matrigel containing  
7 ECFCs alone contained CD31-positive cells but no  $\alpha\text{SMA}$ -positive cells. These results suggest that no  $\alpha\text{SMA}$ -  
8 positive cells migrated from the mouse into the matrigel plugs during the experiment.

9 Matrigel explants with VIC<sub>p</sub> alone did not show either any vascularization (Figure 2C) in contrast to what we  
10 previously observed with hemangioma stem cells<sup>10</sup> or with very small embryonic-like stem cells<sup>11</sup>. To confirm  
11 this finding, we investigated the ability of VIC<sub>p</sub> to differentiate *in vitro* into endothelial lineages under culture  
12 in the presence of VEGF-B, as previously described<sup>11,23</sup>. After 14 days, no induction of VE-cadherin expression  
13 was found, thus confirming *in vivo* data (Online supplemental Figure 4).

14 The ability of VIC<sub>p</sub> to differentiate into perivascular  $\alpha\text{SMA}$  cells was then explored *in vitro* with coculture  
15 experiments. In order to investigate expression changes in perivascular markers in coculture 50:50, we  
16 investigated the expression of perivascular markers on VIC<sub>p</sub> alone and after coculture with ECFCs (Figure 3A).  
17 After confirmation that ECFCs did not express any significant level of the pericyte marker NG2 (Online  
18 supplemental Figure 5), we cocultured VIC<sub>p</sub> for 10 days with ECFCs and demonstrated a significant increase in  
19 the mRNA expression of NG2 compared to ECFCs alone (Figure 3B). Moreover, VIC<sub>p</sub> acquired a perivascular  
20 phenotype confirmed by the induction of  $\alpha\text{SMA}$ , NG2 and calponin protein in confocal analysis (Figure 3C).  
21 VIC<sub>p</sub> without direct contact with ECFCs did not express higher levels of these proteins after 10 days as evaluated  
22 by fluorescence intensity quantification (Online supplemental Figure 6). Because ECFCs are known to be  
23 involved in perivascular differentiation through paracrine PDGF-BB signaling<sup>24</sup>, we investigated PDGF-BB  
24 involvement in VIC<sub>p</sub>/ECFCs coculture to explore its potential modulation in VIC differentiation into  
25 perivascular cells. We did not observe any significant difference in PDGFB mRNA levels between ECFCs alone  
26 and ECFCs cocultured with VIC<sub>p</sub> for 5 days (Online supplemental Figure 7).

27 **VEGF-A upregulation in pathologic VICs increases vasculogenesis**

1 Given that a normal valve is avascular and becomes vascularized in CAVD<sup>2,25</sup>, we compared the vasculogenic  
2 effects of conditioned media (CM) from VIC<sub>c</sub> isolated from normal aortic valves unsuitable for grafting and  
3 VIC<sub>p</sub>. First, CM from VIC<sub>p</sub> induced *in vitro* a significant increase in ECFC proliferation compared to CM from  
4 VIC<sub>c</sub> (Figure 4A). Second, we evaluated the effects of CM from VICs on ECFC migration in a modified Boyden  
5 chamber assay that consisted in quantifying the cells bound on the outer membrane surface. CM from VIC<sub>p</sub>  
6 induced a significant increase in ECFC migration compared to CM from VIC<sub>c</sub> (Figure 4B, Boyden chamber).  
7 Then, to evaluate the ability of VIC<sub>p</sub> to induce ECFC sprouting, we used a three-dimensional fibrin gel assay  
8 model with ECFCs seeded onto Cytodex® 3 microcarrier beads and embedded in fibrin gel (Figure 4C). This  
9 model is classically used with a fibroblastic feeder on top of the fibrin gel<sup>26</sup>. For the present experiment, MSCs,  
10 VIC<sub>p</sub> and VIC<sub>c</sub> were used as feeders to induce endothelial sprouting, evaluated by the capillary length and the  
11 number of branches per bead. *In vitro*, ECFC sprouting in fibrin gel was stimulated by VIC<sub>p</sub> and MSCs, but not  
12 by VIC<sub>c</sub> (Figure 4D, E and F), confirming the proangiogenic phenotype of VIC<sub>p</sub> isolated from calcified valves.  
13 To further investigate the proangiogenic paracrine effects of CM from VICs, levels of angiogenic factors were  
14 screened (Table 1). CM from both VIC<sub>c</sub> and VIC<sub>p</sub> contained similar amounts of Ang-1, Ang-2, sVEGFR-1,  
15 PlGF, endothelin-1, sEPCR, sENG, TSP1 and IL-6. No detectable amounts of sVEGFR-2 and VEGF-B were  
16 found. Interestingly, a 15-fold higher VEGF-A level was found in CM from VIC<sub>p</sub> compared to VIC<sub>c</sub> (Table 1),  
17 further confirmed at the mRNA level (Figure 5A). In order to assess whether VEGF-A plays a role in VIC<sub>p</sub>  
18 angiogenic functions and in adult postnatal vasculogenesis, an inhibition strategy targeting VEGF-A expression  
19 in VIC<sub>p</sub> was set up using monoclonal blocking antibodies (mAb VEGF-A) and RNA interference (siVEGFA).  
20 ECFCs were exposed for 72h to CM from VIC<sub>p</sub> incubated with and without mAb VEGF-A. A significant  
21 decrease in ECFC proliferation and migration was reported when CM from VIC<sub>p</sub> were preincubated with  
22 blocking mAb VEGF-A (Figure 5B and 5C, p<0.05). We further used CM from VIC<sub>p</sub> transfected with specific  
23 VEGFA siRNA that generated 80% gene expression inhibition 2 days after transfection (Online supplemental  
24 Figure 8). CM were collected from VICs silenced for VEGFA or transfected with scrambled-siRNA and  
25 incubated for 3 days with ECFCs. A significant decrease in ECFC proliferation and migration was observed  
26 with CM from VIC<sub>p</sub> transfected with siVEGFA, confirming VEGF-A involvement in VIC<sub>p</sub>-induced endothelial  
27 proliferation and migration (Figure 5D and 5E). Furthermore, using the three-dimensional fibrin gel assay model  
28 with ECFCs seeded onto Cytodex® 3 microcarrier beads, VEGFA inhibition with siRNA in VIC<sub>p</sub> used as a

1 feeder significantly decreased ECFC sprouting network formation (Figure 6A, 6B and 6C). Taken together,  
2 these results indicate that VEGF-A is overexpressed in VIC<sub>p</sub> from CAVD patients and promotes vasculogenesis.

3

#### 4 **VEGF-A modulation in VIC<sub>p</sub> does not alter their osteogenic or perivascular differentiation**

5 Firstly, we investigated VEGF-A involvement in VIC<sub>p</sub> osteogenic and perivascular differentiation. Calcification  
6 assays were performed in the presence of mAb VEGF-A. No significant differences in Alizarin Red staining  
7 were observed when VIC<sub>p</sub> were cultured in the presence or absence of mAb VEGF-A ( $p > 0.05$  for each, Figure  
8 7A). RUNX2 has been described as driving VIC<sub>p</sub> osteogenic potential during CAVD<sup>27</sup> and VEGFA gene  
9 expression<sup>28</sup>. We explored whether RUNX2 could modulate VEGFA expression in VIC<sub>p</sub> and found that  
10 suppressing RUNX2 with siRNA did not modify VEGFA mRNA expression at any time ( $p > 0.05$  for each,  
11 Online supplemental 9). We then investigated whether mAb VEGF-A could interfere with perivascular  
12 differentiation during VIC<sub>p</sub>+ECFCs coculture (Figure 7B) and we did not observe any significant difference  
13 between  $\alpha$ SMA and calponin expression ( $p > 0.05$  for each condition, Figure 7C and D). Cellular mechanisms  
14 involved in vasculogenesis process associated with CAVD remain unknown. After analysis of VEGFA gene  
15 promoter, we identified putative binding sites for HIF1a, LXRA:RXRA and SREBP (Figure 8A). At the mRNA  
16 level, VIC<sub>p</sub> significantly overexpressed HIF1a compared to VIC<sub>c</sub> ( $p = 0.0159$ , Figure 8B) whereas there was no  
17 statistical differences in LXRA:RXRA nor in SREBP expression (Figure 8C, D, E). Interestingly, a significant  
18 correlation existed between HIF1a mRNA and VEGFA mRNA gene expression in VICs ( $p = 0.0006$ ,  $r^2 = 0.9119$ ,  
19 Figure 8F).

20

## 1 Discussion

2

3 We show that VIC<sub>p</sub> isolated from CAVD patients are vasculogenic cells, given their ability to differentiate into  
4 perivascular cells and to secrete VEGF-A. To our knowledge, we provide here the first exploration of the  
5 vasculogenic potential of VICs whether they were isolated from pathological or control human valves. In  
6 CAVD, VIC<sub>p</sub> showed an increased ability to promote vasculogenesis by a paracrine effect related to a burst of  
7 VEGF-A secretion. Indeed, inhibition of VEGF-A in VIC<sub>p</sub> through mAb or siRNA blocking strategies decreased  
8 their vasculogenic ability. This suggests that VEGF-A could directly contribute to newly-formed aortic valve  
9 vessels in CAVD.

10 Two studies previously proposed this triple association between VEGF-A, angiogenesis and CAVD. First,  
11 Rajamannan *et al.* observed that valve calcification was not a random passive process. Thus, using  
12 immunohistochemistry, they found that osteoblasts in calcified rheumatic valves were associated with  
13 proliferating myofibroblasts and neoangiogenic markers such as VEGF-A<sup>2</sup>. Second, by isolating mast cells and  
14 myofibroblasts from CAVD, Syväranta *et al.* demonstrated that both cells could secrete VEGF-A, in particular  
15 myofibroblasts when cultured with mast cells on hypoxia, inflammation and tobacco<sup>4</sup>. Furthermore, Porras *et*  
16 *al.* used biomaterial environment models of CAVD<sup>30</sup> based on extracellular matrix variation to show that  
17 glycosaminoglycan modifications, in particular increasing hyaluronic acid, induced major secretion of VEGF-  
18 A by resident VICs but not PDGF or inflammatory cytokines. Moreover, global changes of the miRNA  
19 expression profile in CAVD and controls were recently identified, including miR-21-5p microRNA, which is  
20 potentially involved in angiogenic and VEGF-related disorders<sup>34,35,36</sup>. Our findings provide evidence that  
21 blocking VEGF-A could have a pivotal role in maintaining normal valve functions without interfering with  
22 osteogenic potential. However further investigations are needed to find appropriate strategies to block VEGF-A  
23 secretion in VIC during CAVD.

24 Mainly using *in vitro* models, VICs have been shown to be progenitor cells with major plasticity properties<sup>5</sup>.  
25 Recently, Arevalos *et al.* showed a pericyte-like behavior of porcine VIC in stabilizing early VEC vasculogenic  
26 networks with  $\alpha$ -SMA and NG2 expression<sup>37</sup>. Herein we demonstrate for the first time in human aortic valves  
27 that VICs cultured with endothelial cells can undergo perivascular differentiation and may stabilize aberrant  
28 neoangiogenesis during CAVD. The present study also reveals the incapacity of human VIC<sub>p</sub> isolated from

1 CAVD to differentiate into endothelial cells, in contrast to other plastic mesenchymal subtypes. MSCs have  
2 been shown to support angiogenesis<sup>6</sup> through the release of angiogenic factors, in particular VEGF-A<sup>8</sup>, and  
3 enhance angiogenesis by differentiation into perivascular cells but are incapable of endothelial differentiation<sup>22</sup>.  
4 In the present study, we demonstrated that VICs are CD34<sup>-</sup>CD133<sup>-</sup> and show an mRNA profile comparable to  
5 MSCs and/or fibrocytes. VIC<sub>p</sub> are not able to differentiate into cells with the endothelial phenotype *in vivo* or  
6 when cultured with VEGF-B *in vitro*. However, we cannot exclude a potential endothelial differentiation by  
7 other pathways. According to these results, absence of endothelial differentiation excludes VICs them from stem  
8 cells and makes them a valve progenitor already committed in non-endothelial lineages. Specific contribution  
9 of these cells to valve plasticity during both homeostasis and response to injury is incompletely understood. As  
10 mentioned by Liu *et al.*, VICs have a progenitor phenotype<sup>38</sup>, especially during valve formation in the embryo  
11 and injured/diseased valves. Additionally, c-Kit<sup>+</sup> progenitor cells have been observed in healthy and pathological  
12 valves<sup>39</sup>, and bone marrow-derived stem cells migrate to human and mouse cardiac valves and differentiate into  
13 fibroblast-like cells<sup>40,41</sup>. Our results are in agreement with the hypothesis that VICs are bone marrow-  
14 mesenchymal derived cell valve residents<sup>40,43,44</sup>. Other progenitor cells isolated from the heart are involved in  
15 cardiac repair by enhancing angiogenesis in a paracrine manner, such as c-Kit<sup>+</sup> cardiac stem cells and  
16 MSCs<sup>44,45,46</sup>.

17 Most research on CAVD is drawn from animal cells (porcine or sheep). The strength of our study is to have  
18 studied isolated and cultured human cells from calcified tricuspid aortic valves and non-calcific valves collected  
19 from prospective donor hearts considered as high-quality control material<sup>5</sup>. Some limitations of this study should  
20 be mentioned. First, all the CAVD patients included presented the severe form of the disease, aortic valve  
21 stenosis, with heavily calcified leaflets. Our findings may therefore not be representative of the earlier processes  
22 of CAVD. Second, we used ECFCs as endothelial cells for all the experiment and not aortic VECs covering  
23 valve leaflets. ECFCs are progenitor cells committed in endothelial lineage. In CAVD, ECFCs from bone  
24 marrow may be mobilized and recruited to participate in VEC regeneration<sup>47</sup> and/or aberrant angiogenesis  
25 related to VEGF-A. We found that ECFCs induced VIC-perivascular differentiation but further study are  
26 warranted to confirm this interaction with mature endothelial cells (or VECs). Furthermore, VEGF-A secretion  
27 may be responsible of one of the early events in CAVD: disruption of the endothelial layer on the aortic side of  
28 the leaflets<sup>48</sup>. VECs have not been used in this study since they have been described as giving rise to VICs in

1 the pulmonary and mitral valves<sup>49,50</sup>. Since we wanted to study the differentiation potential of VICs in CAVD,  
2 we did not want to induce any bias with VEC-differentiation interference in these pathways. Lastly, it would be  
3 interesting to correlate plasma VEGF-A levels in patients with CAVD and valve function, calcification and  
4 angiogenesis. Indeed, immunohistochemical studies of explanted stenotic aortic valves showed a correlation  
5 between VEGF-A levels and neovessels in calcified areas<sup>4,51</sup>. In the present study, we observed in VICs a  
6 significant correlation between VEGFA and HIF1a expressions. As HIF1a shows putative binding sites for  
7 VEGFA gene promoter, further study should explore the interaction between HIFa and VEGFA during CAVD  
8 onset. As normal aortic valves are avascular and non-calcific valves are rarely-used control materials, we  
9 acknowledge that vasculogenic experiments with VIC<sub>c</sub> were not performed. VIC<sub>c</sub> enabled us to identify changes  
10 in the paracrine secretion of VIC<sub>p</sub> in CAVD. These control valves are clearly validated as a high-quality control  
11 material<sup>5</sup> and this is a strong argument to validate our results. Moreover, results from these experiments were  
12 validated in VIC<sub>p</sub> since VEGF-A inhibition was clearly associated to a modified phenotype and abolished  
13 functional differences between VIC<sub>p</sub> and VIC<sub>c</sub>. XXXX rajouter un mot sur l'absence de vrai modèle *in vivo*

#### 14 **Conclusion**

15 Our study demonstrates that VIC<sub>p</sub> isolated from CAVD patients have higher vasculogenic potential compared  
16 to controls and that angiogenesis in CAVD is an active process driven by the native cells in the aortic valve  
17 through VIC differentiation into perivascular cells. We highlight the perivascular potential of human VICs and  
18 their ability to modify their secretome during disease through increased VEGF-A secretion. Further studies are  
19 needed to decipher the clinical relevance of the vasculogenic properties of VICs during CAVD development.

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## **15 Disclosures**

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11

**1 Highlights:**

- 2** • Human VICs from calcified aortic valves gain increased VEGF-A secretion potential allowing ECFC  
**3** migration, proliferation and sprouting.
- 4** • Human VICs from calcified aortic valves are able to differentiate *in vivo* and *in vitro* into perivascular cells,  
**5** independently from VEGF-A.
- 6** • Blocking VIC vasculogenic potential could be an innovative pharmacological approach to prevent or slow  
**7** CAVD progression.



1 **Tables**

2 **Table 1. VIC<sub>p</sub> show increased VEGF-A secretion compared to VIC<sub>c</sub>.**

3 Conditioned media from VIC<sub>p</sub> contained a significant higher level of VEGF-A compared to VIC<sub>c</sub>. Results are

4 mean ± SD. n=4 to 6 independent experiments each condition performed in triplicate. Statistical tests used:

5 Mann-Whitney test.

6

<b>Protein</b>	<b>VIC<sub>c</sub></b>	<b>VIC<sub>p</sub></b>	<b>VIC<sub>c</sub> vs VIC<sub>p</sub> (<i>p</i>-value)</b>
<b>Angiopoietin-1 - pg/10<sup>5</sup> cells</b>	68.42 ±124.7	175.0 ±106.3	<i>p</i> =0.065
<b>Angiopoietin-2 - pg/10<sup>5</sup> cells</b>	411.1 ±495.8	116.6 ±118.4	<i>p</i> =0.32
<b>VEGF-A - pg/10<sup>5</sup> cells</b>	37.59 ±22.3	575.0 ±83.0	<b><i>p</i>=0.008</b>
<b>VEGF-B - pg/10<sup>5</sup> cells</b>	0	0	<i>NA</i>
<b>PlGF - pg/10<sup>3</sup> cells</b>	2.88±2.8	0.90±1.2	<i>p</i> =0.40
<b>IL-6 - ng/10<sup>5</sup> cells</b>	0.34 ±0.5	6.55 ±14.8	<i>p</i> =0.25
<b>Thrombospondin-1 - ng/10<sup>5</sup> cells</b>	26.0±24.4	43.9±26.9	<i>p</i> =0.39
<b>soluble VEGFR-1 - pg/10<sup>5</sup> cells</b>	4.02±4.2	8.89±5.1	<i>p</i> =0.25
<b>soluble VEGFR-2 - pg/10<sup>5</sup> cells</b>	0.0	0.0	<i>NA</i>
<b>soluble EPCR - ng/10<sup>5</sup> cells</b>	48.2±28.37	63.0±51.5	<i>p</i> =0.88
<b>Endothelin-1 - pg/10<sup>5</sup> cells</b>	1.18±0.8	0.51±0.2	<i>p</i> =0.57
<b>soluble endoglin - ng/10<sup>5</sup> cells</b>	0.29±0.3	0.16±0.1	<i>p</i> =1.0

7

## 1 **Figure Legends**

2

### 3 **Figure 1. Mesenchymal-like phenotype of pathological VICs.**

4 A) Phase-contrast image of VIC<sub>p</sub> in monolayer culture presenting a spindle morphology at moderate confluent.

5 Scale bar = 400 μm.

6 B) Isolated aortic VIC<sub>p</sub> were stained with antibodies for CD133, CD34, CD90, CD73, CD44, Tie-2, CD31,  
7 CD144 (red) and the corresponding control antibodies (blue). Staining was quantified by acoustic flow  
8 cytometry. MSCs and VIC<sub>p</sub> were negative for CD144, Tie-2 and CD31 (endothelial markers) under culture  
9 conditions. MSCs and VIC<sub>p</sub> were negative for CD133 and CD34 (progenitor markers) but were positive for  
10 CD90 and CD73 (mesenchymal markers) under culture conditions. ECFCs, MSCs and VIC<sub>p</sub> were positive at  
11 different degrees of intensity for selected integrins ( $\alpha_v$ ,  $\beta_1$  and  $\beta_3$ ). n=3 for each condition and each experiment  
12 was performed with MSCs and ECFCs used as positive controls.

13 C) ColA1, S100A4, VIM1, TWIST1, THY1, CD44, CDH2, ZEB2, SNAI2, FLT1, KDR, PTPRC, LEP and  
14 ADIPOQ mRNA quantification in MSCs, VIC<sub>p</sub> and ECFCs. mRNA levels were normalized to TBP mRNA  
15 levels and to the sample with the lowest quantifiable level (i.e. 1 on the left ordinate, corresponding to a Ct value  
16 of 35). Values above 100 represent strong gene expression. Mean  $\pm$  SEM values of 3 independent experiments  
17 using VIC<sub>p</sub>, MSCs and ECFCs from different donors. \* for significant differences with p<0.05. Statistical tests  
18 used: Kruskal-Wallis test.

19 D) Osteogenic MSC and VIC<sub>p</sub> differentiation revealed by alkaline phosphatase (ALP) activity and confirmed  
20 by the presence of calcium deposits. n=3 for each condition. Scale bars = 400 μm.

21

### 22 **Figure 2. Pathological VICs are able to differentiate into perivascular cells.**

23 A) MSCs and VIC<sub>p</sub> with ECFCs ( $1.5 \times 10^6$  of each cell, 1:1) were co-injected and  $3 \times 10^6$  VIC<sub>p</sub> were injected into  
24 an athymic mouse model of Matrigel plug (schematic). Macroscopic view of explanted Matrigel plugs at day  
25 ten after euthanasia of mice.

26 B) Hematoxylin and eosin (H&E) staining showed luminal structures containing erythrocytes in implants  
27 receiving MSCs+ECFCs or VIC<sub>p</sub>+ECFCs after 10 days, but not in implants receiving VIC<sub>p</sub> or ECFCs alone. On  
28 the right panel, implants containing VIC<sub>p</sub>+ECFCs show a similar number of functional vessels compared to

1 those containing MSCs+ECFCs. Quantification of microvessel density was performed by counting red blood  
2 cell-filled vessels in implants. There was no functional vessels in the Matrigel implants of VIC<sub>p</sub> or ECFCs alone.  
3 Data for microvessel density are means ± SEM (n=5 mice per group). ns for non-significant differences  
4 considered with p>0.05. Statistical tests used: Kruskal-Wallis test.

5 C) Vascular network formation using VIC<sub>p</sub> or MSC injection with ECFCs promotes mature vessel formation in  
6 Matrigel implants observed using confocal analysis (magnification x40). We display vessel formation where  
7 mural cells (MSCs or differentiated VIC<sub>p</sub>) are stained for α-SMA (green), human endothelial cells (ECFCs) by  
8 human CD31 (red) and nuclei stained by DAPI. The zoom (arrows) display the morphology of the vessel with  
9 mural cells surrounding endothelial cells. VIC<sub>p</sub> alone are not able to form vessel structures.

10  
11 **Figure 3. Pathological VICs (VIC<sub>p</sub>) differentiate into perivascular cells when cocultured with cord blood**  
12 **endothelial progenitor cells (ECFCs).**

13 A) MSCs, VIC<sub>p</sub>, or VIC<sub>p</sub>+ECFCs and MSCs+ECFCs cocultured for 5 days and then subjected to  
14 immunoseparation based on CD31 expression (schematic).

15 B) The CD31 negative fraction is analyzed using real-time RT-qPCR for the relative expression of neural glial  
16 antigen-2 (NG2) and results are compared with MSCs or VIC<sub>p</sub> cultured alone. Results represent the mean ± SEM  
17 of 4 independent experiments using VIC<sub>p</sub> and MSCs from different donors. \* and \*\* for significant differences  
18 with p<0.05, p<0.01. Statistical tests used: Mann-Whitney test.

19 C) VIC<sub>p</sub> cocultured with ECFCs (labeled using von Willebrand factor in red) acquire a perivascular phenotype  
20 confirmed by induction of αSMA, NG2 and calponin proteins (green).

21  
22 **Figure 4. Angiogenic potential of VIC<sub>p</sub> on ECFCs.**

23 A) Proliferation of ECFCs cultured in CM from VIC<sub>c</sub> (VIC<sub>c</sub>-CM) or from VIC<sub>p</sub> (VIC<sub>p</sub>-CM) evaluated after three  
24 days by measuring cellular phosphatase activity. Results represent the mean ± SEM of 3 independent  
25 experiments using ECFCs and CM from different donors. Statistical tests used: Mann-Whitney test.

26 B) Migration of ECFCs in VIC<sub>c</sub>-CM or VIC<sub>p</sub>-CM evaluated after five hours in a modified Boyden chamber and  
27 quantification of migrated cells bound on the membrane surface. Results represent the mean ± SEM of 4  
28 independent experiments using ECFCs and CM from different donors. Statistical tests used: Mann-Whitney test.

1 C) Three-dimensional fibrin gel assays were performed with ECFCs seeded onto Cytodex® 3 microcarrier beads  
2 and embedded in fibrin gel. MSCs or VIC<sub>p</sub> or VIC<sub>c</sub> were plated on the top of the gel as feeders in the presence  
3 of EGM-2 without VEGF-A. MSCs were used as positive controls. Number of sprouts and cumulative tube  
4 length per bead were measured after 5 days of culture and then stained with Alexa Fluor-488- conjugated  
5 phalloidin and TO-PRO-3 using confocal laser scanning.  
6 D) Confocal laser scanning images of ECFC sprouting for each feeder condition.  
7 E) Quantification of ECFC capillary length per bead for each feeder. VIC<sub>p</sub> and MSCs induced a significant  
8 increase in ECFC capillary length compared with VIC<sub>c</sub>. Statistical tests used: Kruskal-Wallis test.  
9 F) Quantification of ECFC branches per bead for each feeder. VIC<sub>p</sub> and MSCs induced a significant increase in  
10 ECFC branches compared with VIC<sub>c</sub>. OD for optical density. Results represent the mean ± SEM of 4 independent  
11 experiments using ECFCs and CM from different donors. \*, \*\* and \*\*\* for significant differences with p<0.05,  
12 p<0.01 and p<0.001, respectively. Statistical tests used: Kruskal-Wallis test.

13  
14 **Figure 5. Blocking mAb and siRNA against VEGF-A abolished VIC<sub>p</sub>-induced ECFC proliferation and**  
15 **migration.**

16 A) Using real-time RT-qPCR, a significant increased mRNA relative expression of VEGFA was found in VIC<sub>p</sub>  
17 in contrast to VIC<sub>c</sub>. Results represent the mean ± SEM of 5 independent experiments using VIC<sub>p</sub> and VIC<sub>c</sub> from  
18 different donors. Statistical tests used: Mann-Whitney test.  
19 B) mAb VEGF-A abolished ECFC proliferation induced by VIC<sub>p</sub> conditioned media.  
20 C) mAb VEGF-A abolished ECFC migration induced by VIC<sub>p</sub> conditioned media.  
21 D) siVEGFA in VIC<sub>p</sub> abolished proliferation and E) migration of ECFCs induced by VIC<sub>p</sub> conditioned media.  
22 OD for optical density. Data are means ± SEM (n=4 CM per group). \* and \*\*\* for significant differences with  
23 p<0.05 and p<0.001, respectively. Statistical tests used: Kruskal-Wallis test.

24

25

26

27 **Figure 6. Silencing VEGFA abolished VIC<sub>p</sub>-induced ECFC sprouting.**

1 A) Confocal laser scanning images of ECFC sprouting with feeder conditions using VIC<sub>p</sub> transfected with  
2 siVEGFA (VIC<sub>p</sub>-siVEGFA) or scrambled (VIC<sub>p</sub>-Scr).

3 B) Quantification of ECFC capillary length per bead for each feeder evaluated. VIC<sub>p</sub>-siVEGFA induced a  
4 significant decrease in ECFC capillary length in contrast to VIC<sub>p</sub>-Scr. \*\*\* for significant differences with  
5  $p < 0.001$ . Statistical tests used: Mann-Whitney test.

6 C) Quantification of ECFC branches per bead for each feeder evaluated. VIC<sub>p</sub>-siVEGFA induced a significant  
7 decrease in ECFC branches compared with VIC<sub>p</sub>-Scr. Results represent the mean  $\pm$  SEM of 4 independent  
8 experiments using ECFCs and CM from different. \*\*\* for significant differences with  $p < 0.001$ . Statistical tests  
9 used: Mann-Whitney test.

10

11

12 **Figure 7. VEGF-A modulation in VIC<sub>p</sub> did not alter their osteogenic or perivascular differentiation.**

13 A) Calcium deposit by VIC<sub>p</sub> was evaluated using Alizarin Red staining in the presence or absence of mAb  
14 VEGF-A. No significant differences in Alizarin Red staining were observed when VIC<sub>p</sub> were cultured in the  
15 presence or in the absence of mAb VEGF-A ( $p > 0.05$  for each). Results represent the mean  $\pm$  SEM of 4  
16 independent experiments using VIC<sub>p</sub> from different donors. Statistical tests used: Mann-Whitney test.

17 B) Fluorescence intensity of  $\alpha$ SMA and Calponin (green) when VIC<sub>p</sub> are cocultured with ECFCs (labeled using  
18 von Willebrand factor in red) in the presence or absence of mAb VEGF-A.

19 C) Quantification of fluorescence intensity of  $\alpha$ SMA with ImageJ software. No significant differences between  
20  $\alpha$ SMA expressions were observed in the presence or in the absence of mAb VEGF-A ( $p > 0.05$  for each). Results  
21 represent the mean  $\pm$  SEM of 3 independent experiments using VIC<sub>p</sub> and ECFCs from different donors.  
22 Statistical tests used: Mann-Whitney test.

23 D) Quantification of fluorescence intensity of calponin with ImageJ software. No significant differences between  
24 calponin expressions were observed in the presence or in the absence of mAb VEGF-A ( $p > 0.05$  for each). OD  
25 for optical density. Results represent the mean  $\pm$  SEM of 3 independent experiments using VIC<sub>p</sub> and ECFCs  
26 from different donors. Statistical tests used: Mann-Whitney test.

27

28 **Figure 8. HIF1a expression is involved in VEGF-A upregulation in VIC<sub>p</sub>**

- 1 A) Promoter analysis performed using ConTra v3 web server to predict transcription factor binding sites of  
2 VEGFA promoter. ConTra v3 web server identifies putative binding sites for HIF1a, LXRA:RXRA and  
3 SREBP.
- 4 B) Using real-time RT-qPCR, a significant increased mRNA relative expression of HIF1A is found in VIC<sub>p</sub> in  
5 contrast to VIC<sub>c</sub>. Results represent the mean ± SEM of 5 independent experiments using VIC<sub>p</sub> and VIC<sub>c</sub>  
6 from different donors. \* and \*\*\* for significant differences with p<0.05. Statistical tests used: Mann-  
7 Whitney test.
- 8 C) Using real-time RT-qPCR, no significant difference in mRNA relative expression of SREBP exists between  
9 VIC<sub>p</sub> and VIC<sub>c</sub> (p>0.05). Results represent the mean ± SEM of 5 independent experiments using VIC<sub>p</sub> and  
10 VIC<sub>c</sub> from different donors. Statistical tests used: Mann-Whitney test.
- 11 D) Using real-time RT-qPCR, no significant difference in mRNA relative expression of LXRA exists between  
12 VIC<sub>p</sub> and VIC<sub>c</sub> (p>0.05) Results represent the mean ± SEM of 5 independent experiments using VIC<sub>p</sub> and  
13 VIC<sub>c</sub> from different donors. Statistical tests used: Mann-Whitney test
- 14 E) Using real-time RT-qPCR, no significant difference in mRNA relative expression of RXRA exists between  
15 VIC<sub>p</sub> and VIC<sub>c</sub> (p>0.05). Results represent the mean ± SEM of 5 independent experiments using VIC<sub>p</sub> and  
16 VIC<sub>c</sub> from different donors.
- 17 F) Correlation between mRNA relative expression of HIF1A and mRNA relative expression VEGF-A in  
18 calcified areas of aortic valves (p=0.0006, r<sup>2</sup>=0.9119). Results represent the mean ± SEM of 5 independent  
19 experiments using VIC<sub>p</sub> and VIC<sub>c</sub> from different donors. Statistical tests used: Pearson's correlation  
20 coefficient.

**Figure 1**

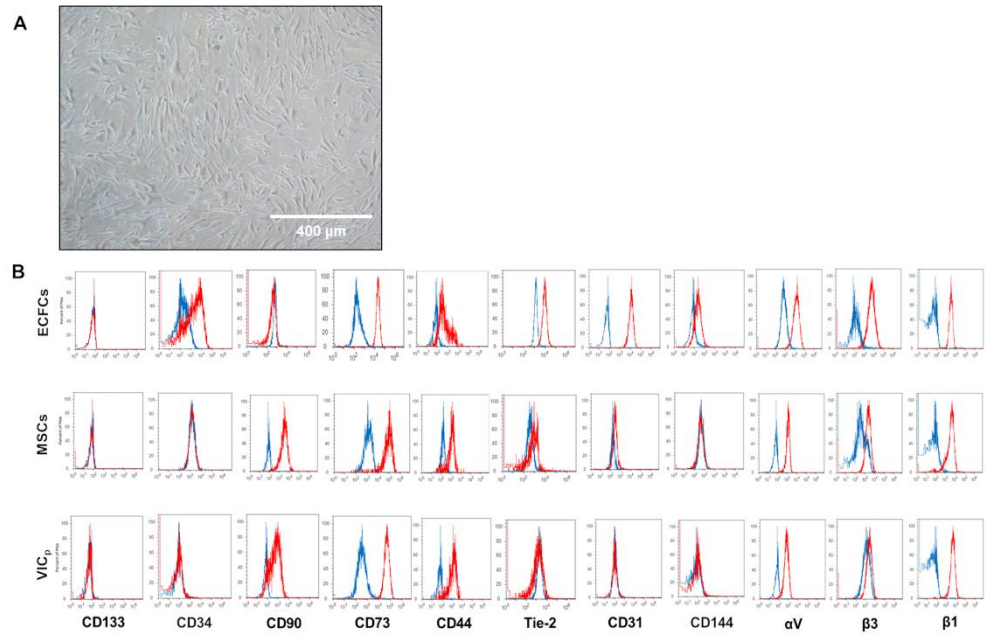
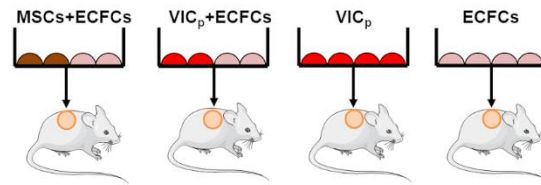
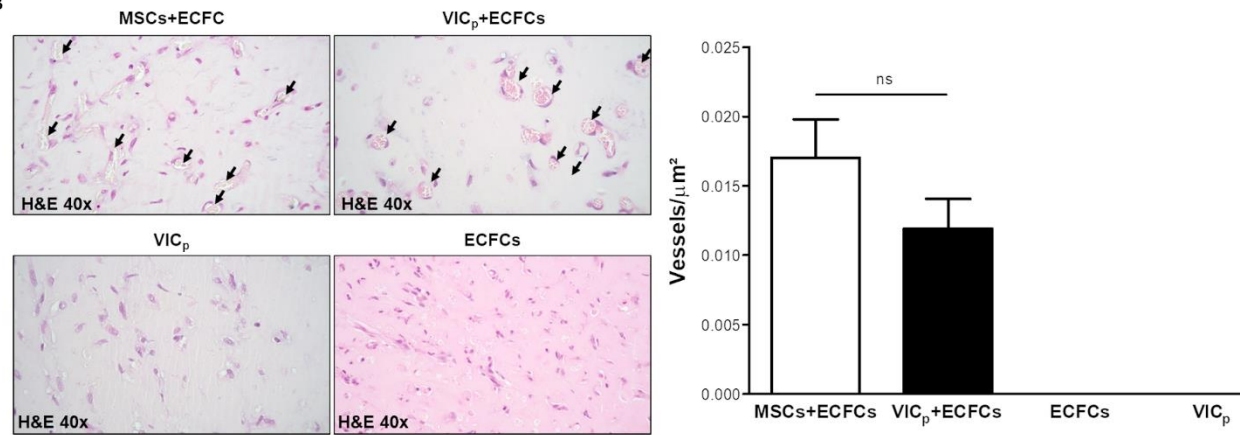


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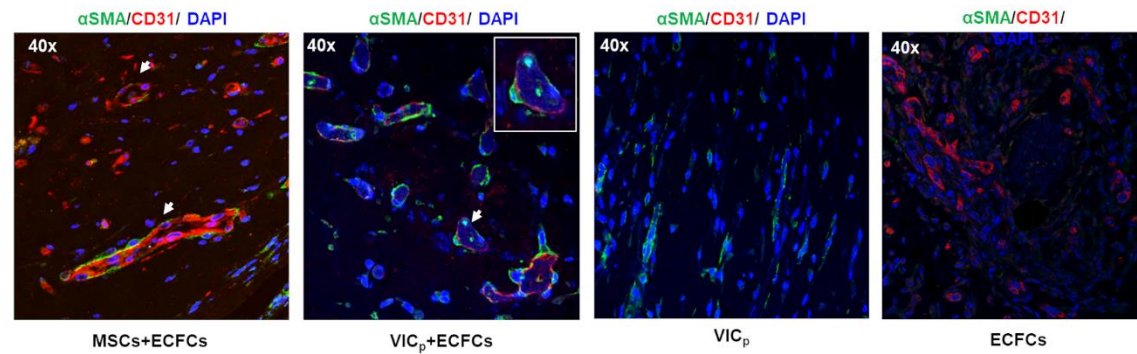
A



B



C





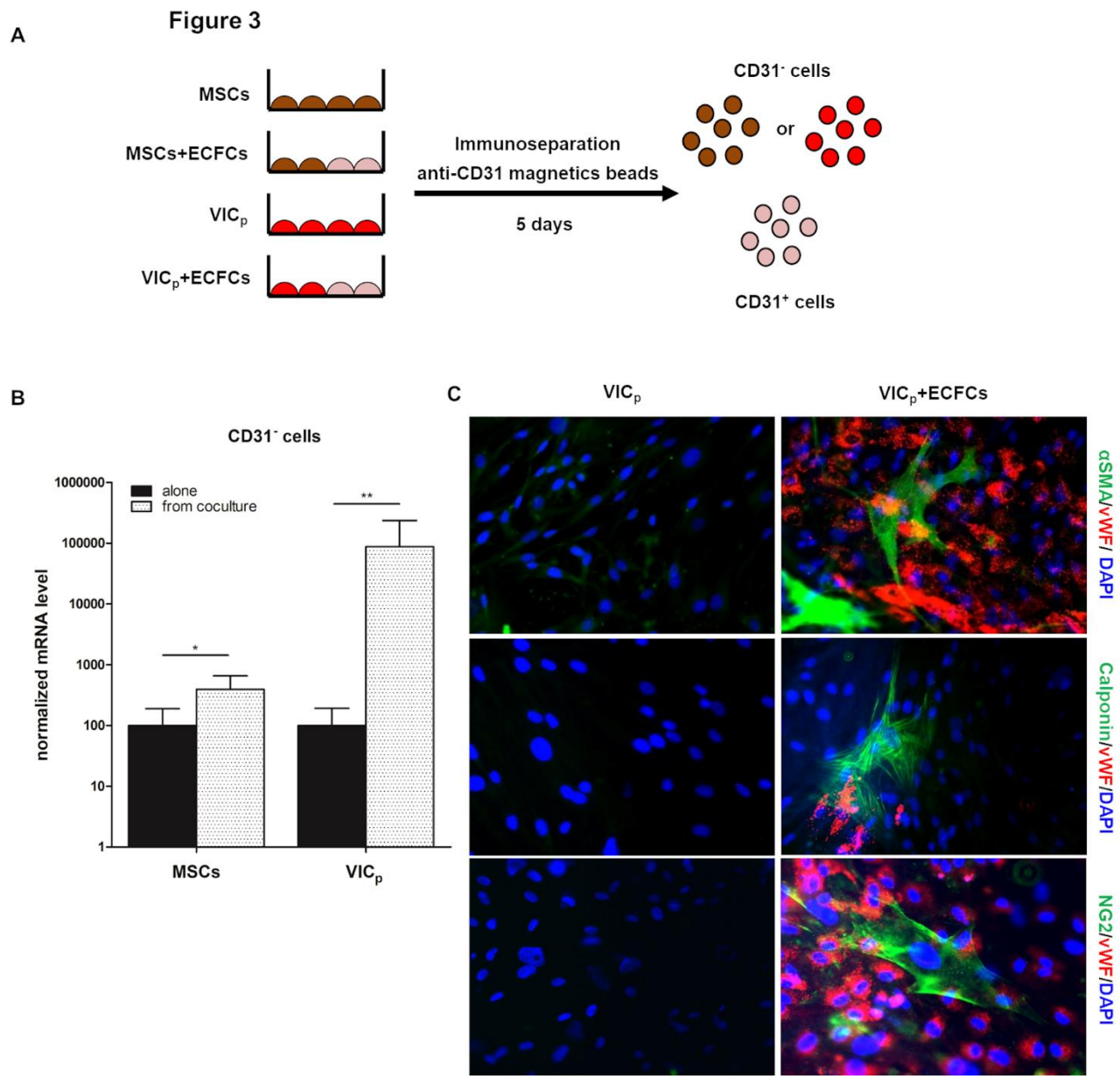
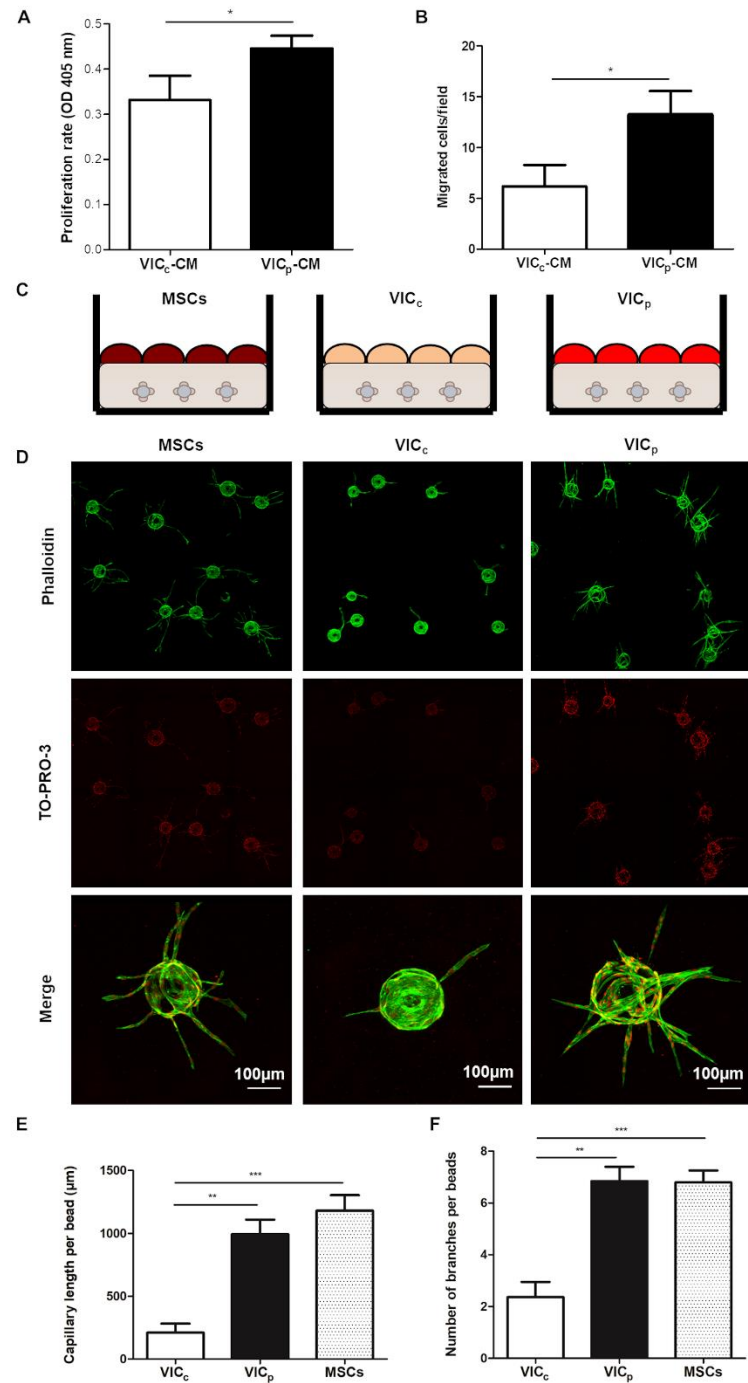


Figure 4



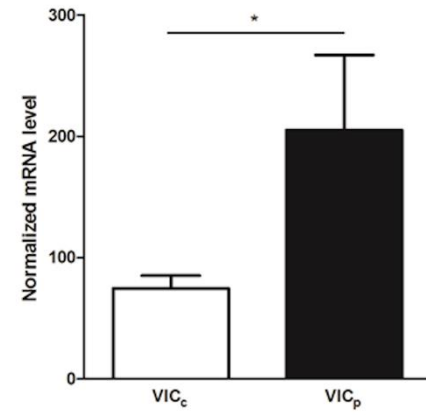
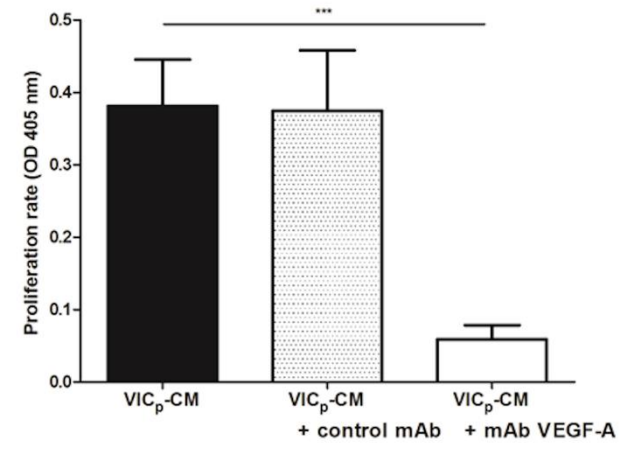
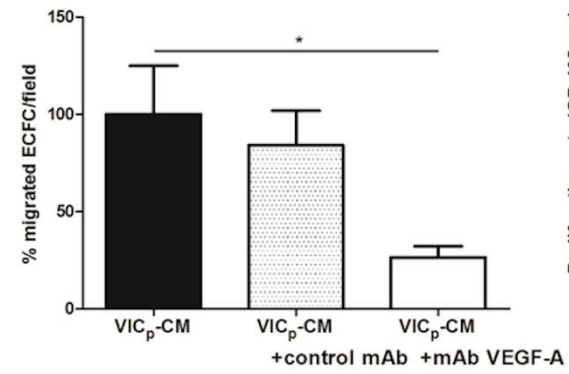
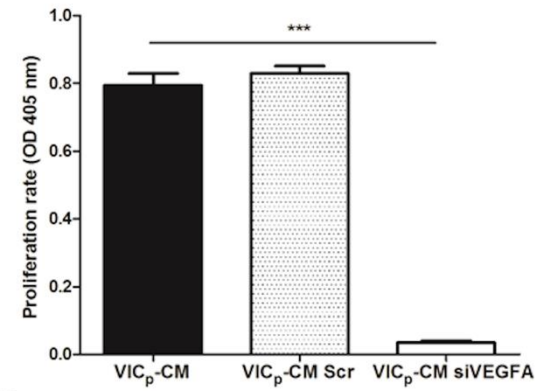
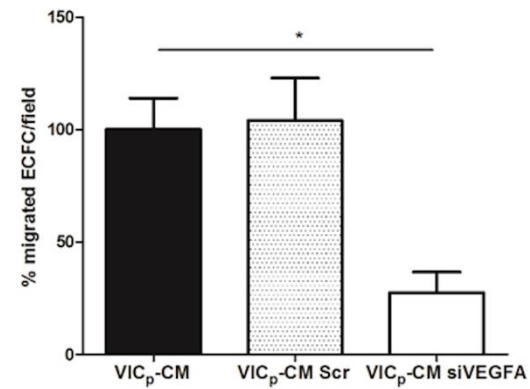
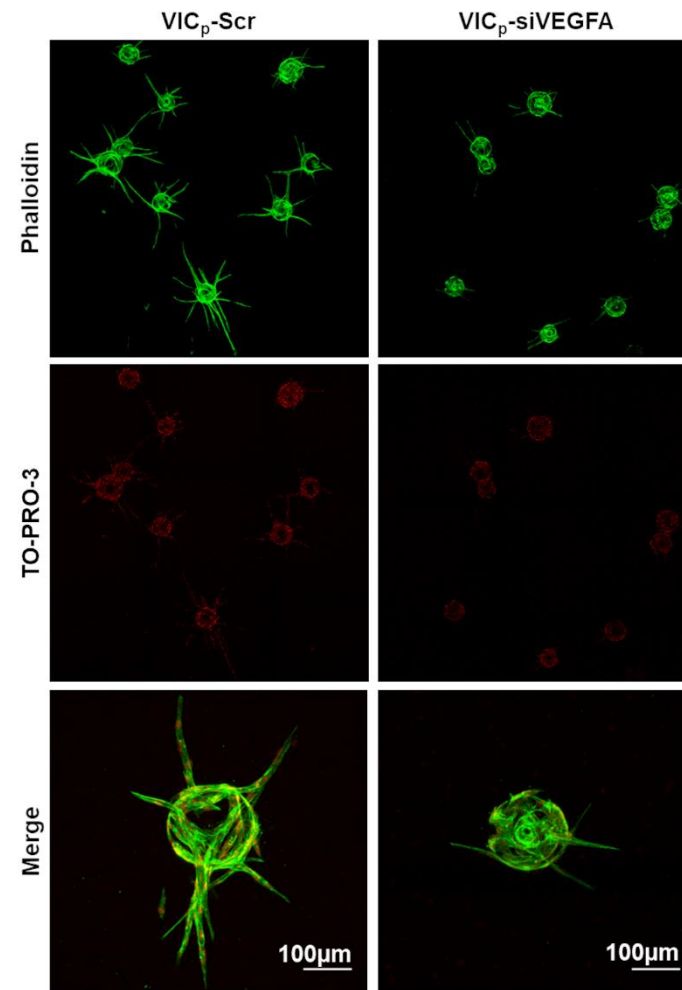
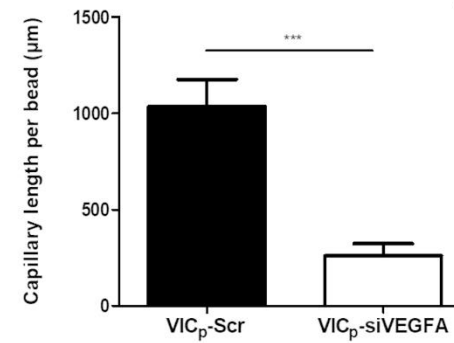
**Figure 5****A****B****C****D****E**

Figure 6

A



B



C

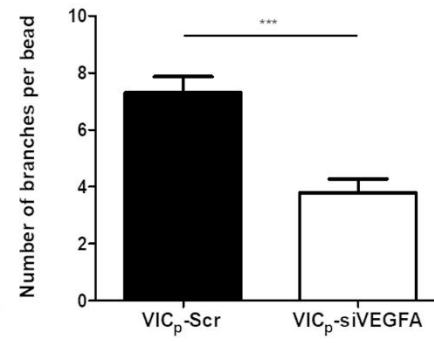
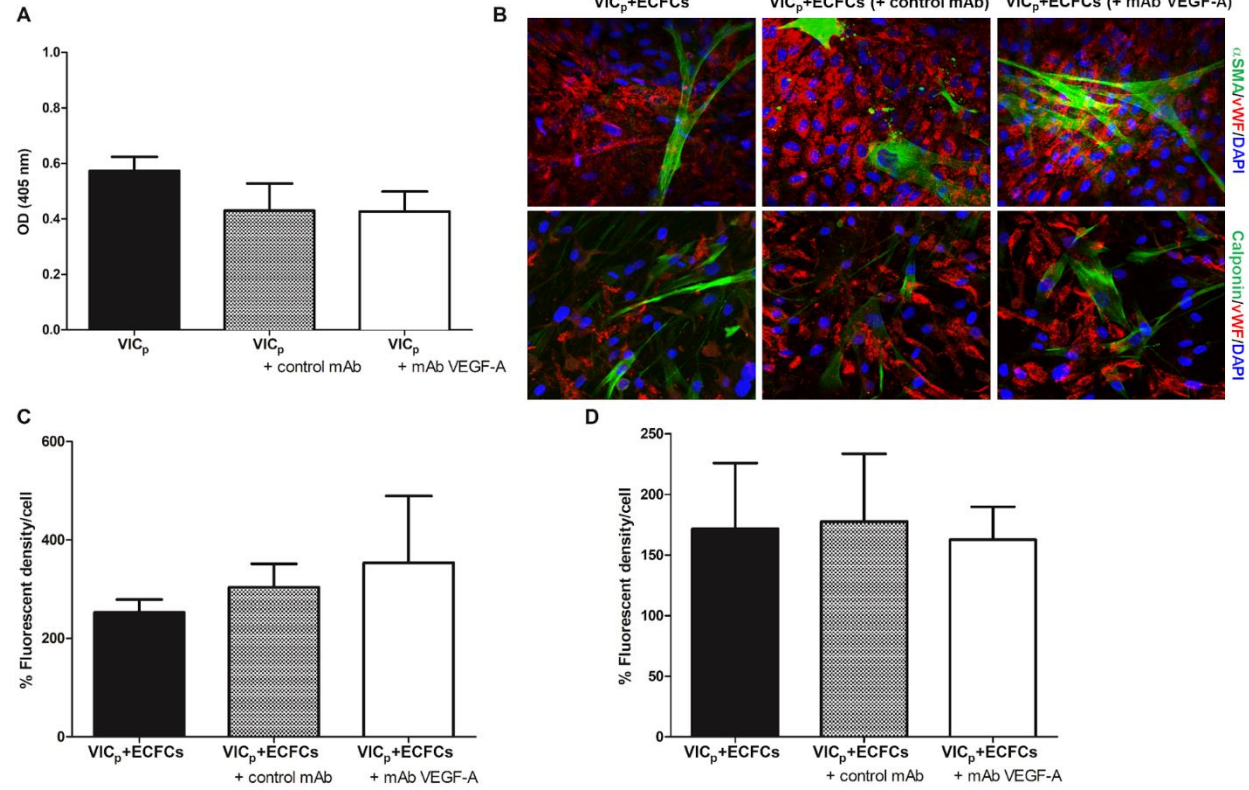
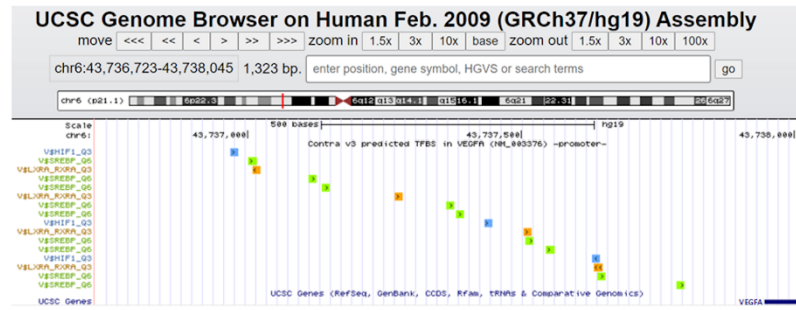


Figure 7

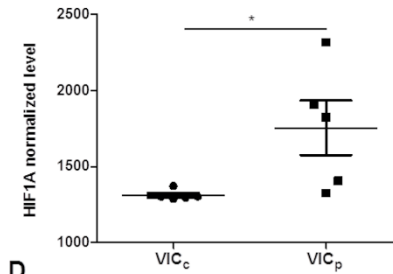


# Figure 8

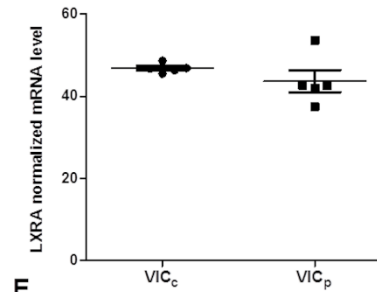
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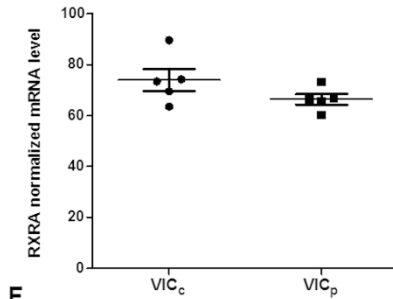
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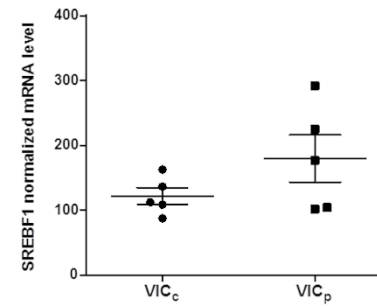
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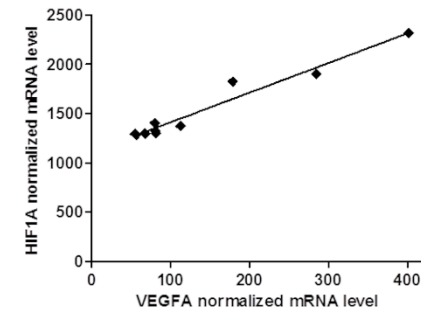
## D



## E



## F



VICs isolated from patients with CAVD (VIC<sub>p</sub>)

