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

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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_c). 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_p)
8 mesenchymal-like phenotype was confirmed by CD90⁺/CD73⁺/CD44⁺ expression and multipotent-like
9 differentiation ability. When VIC_p were co-cultured with ECFCs, they formed microvessels by differentiating
10 into perivascular cells both *in vivo* and *in vitro*. VIC_p and VIC_c 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_p-CM and confirmed at the mRNA level in VIC_p compared to VIC_c.
13 CM from VIC_p induced *in vitro* a significant increase in ECFC proliferation, migration and sprouting compared
14 with CM from VIC_c. These effects were inhibited by blocking VEGF-A with blocking antibody or siRNA
15 approach, confirming VIC_p 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_p 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** α SMA: α -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_c: control VIC
- 12** VIC_p: 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 aging¹. 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^{2,3,4}. 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 calcification⁵. 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)⁶ and circulating fibrocytes⁷ are able to behave as perivascular cells and to support
16 vasculogenesis *via* the release of angiogenic factors⁸, two mesenchymal subtypes isolated from CD133 cells
17 have been described as giving rise to endothelial cells. They are respectively hemangioma stem cells^{9,10} isolated
18 from infantile tumors and very small embryonic-like stem cells isolated from human bone marrow^{11,12,13}. 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 phenotype⁵. In 2016, Arevalos *et al.*
21 showed that porcine VICs exhibited a pericyte-like behavior as they stabilized early VEC vasculogenic
22 networks, expressing α -smooth muscle actin (α -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 guidelines¹⁴) 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_p) and control VICs (VIC_c) were obtained after type-I collagenase digestion of the aortic
19 valve, as previously described¹⁵, from patients after surgery and after autopsy for controls, respectively. VICs
20 were seeded on fibronectin-coated plates (0.4 µg/cm², 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% CO₂ 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^{16,17,18}. 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 characterization¹⁹. The medium was replaced every 2 or 3 days until confluence. Angiogenic
10 assays have been described elsewhere^{12,15,20} 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_p in monolayer cultures showed the elongated, spindle-shaped morphology characteristic of
4 mesenchymal cells in culture²¹ (Figure 1A). ECFCs served as positive controls for endothelial and vasculogenic
5 cells. VIC_p 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 α_v , β_1 and β_3 . Furthermore, mesenchymal, endothelial and hematopoietic marker gene expression was quantified
8 by RT-qPCR (Figure 1C). Cultured VIC_p 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_p
10 expressed low levels of endothelial markers such as FLT1 and KDR, contrary to ECFCs. All isolated and
11 cultured MSCs, VIC_p 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_p. In an osteogenic medium, VIC_p underwent osteogenic differentiation firstly revealed by ALP activity
14 and then by calcium deposits as detected by Alizarin Red staining (Figure 1D). Human VIC_p were cultured on
15 fibronectin-coated tissue culture plastic because our objectives were to explore the angiogenic properties and
16 vasculogenic differentiation of VIC_p. 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_p 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_p 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_p 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_p were able to differentiate into endothelial and/or perivascular lineages,
26 VIC_p alone, ECFCs alone, or VIC_p+ECFCs were suspended in Matrigel and injected into nude mice as previously
27 described^{12,22} (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_p+ECFCs, contrary to those containing VIC_p or ECFCs alone, that did not form vessels (Figure 2B).
3 Interestingly, matrigel explants containing VIC_p+ECFCs showed similar microvessel density (0.012 ±0.002
4 vessels/μm²) than control explants MSCs+ECFCs (0.017 ±0.003 vessels/μm², p=0.23, Figure 2B, right panel).
5 In these explants, immunofluorescence analysis for αSMA and human-specific CD31 showed perivascular cells
6 positive for αSMA surrounding human CD31 blood vessels. On the contrary, explanted Matrigel containing
7 ECFCs alone contained CD31-positive cells but no αSMA-positive cells. These results suggest that no αSMA-
8 positive cells migrated from the mouse into the matrigel plugs during the experiment.

9 Matrigel explants with VIC_p alone did not show either any vascularization (Figure 2C) in contrast to what we
10 previously observed with hemangioma stem cells¹⁰ or with very small embryonic-like stem cells¹¹. To confirm
11 this finding, we investigated the ability of VIC_p to differentiate *in vitro* into endothelial lineages under culture
12 in the presence of VEGF-B, as previously described^{11,23}. 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_p to differentiate into perivascular αSMA 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_p 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_p 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_p acquired a perivascular
20 phenotype confirmed by the induction of αSMA, NG2 and calponin protein in confocal analysis (Figure 3C).
21 VIC_p 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 signaling²⁴, we investigated PDGF-BB
24 involvement in VIC_p/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_p 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^{2,25}, we compared the vasculogenic
2 effects of conditioned media (CM) from VIC_c isolated from normal aortic valves unsuitable for grafting and
3 VIC_p. First, CM from VIC_p induced *in vitro* a significant increase in ECFC proliferation compared to CM from
4 VIC_c (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_p
6 induced a significant increase in ECFC migration compared to CM from VIC_c (Figure 4B, Boyden chamber).
7 Then, to evaluate the ability of VIC_p 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 gel²⁶. For the present experiment, MSCs,
10 VIC_p and VIC_c 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_p and MSCs, but not
12 by VIC_c (Figure 4D, E and F), confirming the proangiogenic phenotype of VIC_p 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_c and VIC_p 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_p compared to VIC_c (Table 1),
17 further confirmed at the mRNA level (Figure 5A). In order to assess whether VEGF-A plays a role in VIC_p
18 angiogenic functions and in adult postnatal vasculogenesis, an inhibition strategy targeting VEGF-A expression
19 in VIC_p was set up using monoclonal blocking antibodies (mAb VEGF-A) and RNA interference (siVEGFA).
20 ECFCs were exposed for 72h to CM from VIC_p incubated with and without mAb VEGF-A. A significant
21 decrease in ECFC proliferation and migration was reported when CM from VIC_p were preincubated with
22 blocking mAb VEGF-A (Figure 5B and 5C, p<0.05). We further used CM from VIC_p 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_p transfected with siVEGFA, confirming VEGF-A involvement in VIC_p-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_p 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_p from CAVD patients and promotes vasculogenesis.

3

4 **VEGF-A modulation in VIC_p does not alter their osteogenic or perivascular differentiation**

5 Firstly, we investigated VEGF-A involvement in VIC_p 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_p 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_p osteogenic potential during CAVD²⁷ and VEGFA gene
9 expression²⁸. We explored whether RUNX2 could modulate VEGFA expression in VIC_p 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_p+ECFCs coculture (Figure 7B) and we did not observe any significant difference
13 between α 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_p significantly overexpressed HIF1a compared to VIC_c ($p=0.0159$, 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_p 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_p 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_p 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-A². 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 tobacco⁴. Furthermore, Porras *et*
16 *al.* used biomaterial environment models of CAVD³⁰ 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^{34,35,36}. 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 properties⁵.
25 Recently, Arevalos *et al.* showed a pericyte-like behavior of porcine VIC in stabilizing early VEC vasculogenic
26 networks with α -SMA and NG2 expression³⁷. 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_p isolated from

1 CAVD to differentiate into endothelial cells, in contrast to other plastic mesenchymal subtypes. MSCs have
2 been shown to support angiogenesis⁶ through the release of angiogenic factors, in particular VEGF-A⁸, and
3 enhance angiogenesis by differentiation into perivascular cells but are incapable of endothelial differentiation²².
4 In the present study, we demonstrated that VICs are CD34⁻CD133⁻ and show an mRNA profile comparable to
5 MSCs and/or fibrocytes. VIC_p 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 phenotype³⁸, especially during valve formation in the embryo
11 and injured/diseased valves. Additionally, c-Kit⁺ progenitor cells have been observed in healthy and pathological
12 valves³⁹, and bone marrow-derived stem cells migrate to human and mouse cardiac valves and differentiate into
13 fibroblast-like cells^{40,41}. Our results are in agreement with the hypothesis that VICs are bone marrow-
14 mesenchymal derived cell valve residents^{40,43,44}. Other progenitor cells isolated from the heart are involved in
15 cardiac repair by enhancing angiogenesis in a paracrine manner, such as c-Kit⁺ cardiac stem cells and
16 MSCs^{44,45,46}.

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 material⁵. 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 regeneration⁴⁷ 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 leaflets⁴⁸. 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^{49,50}. 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^{4,51}. 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_c were not performed. VIC_c enabled us to identify changes
10 in the paracrine secretion of VIC_p in CAVD. These control valves are clearly validated as a high-quality control
11 material⁵ and this is a strong argument to validate our results. Moreover, results from these experiments were
12 validated in VIC_p since VEGF-A inhibition was clearly associated to a modified phenotype and abolished
13 functional differences between VIC_p and VIC_c. XXXX rajouter un mot sur l'absence de vrai modèle *in vivo*

14 **Conclusion**

15 Our study demonstrates that VIC_p 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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14

15 Disclosures

16 The authors do not have any conflicts of interest to declare.

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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_p show increased VEGF-A secretion compared to VIC_c.**

3 Conditioned media from VIC_p contained a significant higher level of VEGF-A compared to VIC_c. Results are

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

5 Mann-Whitney test.

6

Protein	VIC_c	VIC_p	VIC_c vs VIC_p (<i>p</i>-value)
Angiopoietin-1 - pg/10⁵ cells	68.42 ±124.7	175.0 ±106.3	<i>p</i> =0.065
Angiopoietin-2 - pg/10⁵ cells	411.1 ±495.8	116.6 ±118.4	<i>p</i> =0.32
VEGF-A - pg/10⁵ cells	37.59 ±22.3	575.0 ±83.0	<i>p</i>=0.008
VEGF-B - pg/10⁵ cells	0	0	<i>NA</i>
PlGF - pg/10³ cells	2.88±2.8	0.90±1.2	<i>p</i> =0.40
IL-6 - ng/10⁵ cells	0.34 ±0.5	6.55 ±14.8	<i>p</i> =0.25
Thrombospondin-1 - ng/10⁵ cells	26.0±24.4	43.9±26.9	<i>p</i> =0.39
soluble VEGFR-1 - pg/10⁵ cells	4.02±4.2	8.89±5.1	<i>p</i> =0.25
soluble VEGFR-2 - pg/10⁵ cells	0.0	0.0	<i>NA</i>
soluble EPCR - ng/10⁵ cells	48.2±28.37	63.0±51.5	<i>p</i> =0.88
Endothelin-1 - pg/10⁵ cells	1.18±0.8	0.51±0.2	<i>p</i> =0.57
soluble endoglin - ng/10⁵ cells	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_p in monolayer culture presenting a spindle morphology at moderate confluent.

5 Scale bar = 400 μm.

6 B) Isolated aortic VIC_p 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_p were negative for CD144, Tie-2 and CD31 (endothelial markers) under culture
9 conditions. MSCs and VIC_p 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_p were positive at
11 different degrees of intensity for selected integrins (α_v , β_1 and β_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_p 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_p, 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_p 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_p with ECFCs (1.5×10^6 of each cell, 1:1) were co-injected and 3×10^6 VIC_p 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_p+ECFCs after 10 days, but not in implants receiving VIC_p or ECFCs alone. On
28 the right panel, implants containing VIC_p+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_p or ECFCs alone.
3 Data for microvessel density are means \pm 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_p 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_p) 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_p alone are not able to form vessel structures.

10
11 **Figure 3. Pathological VICs (VIC_p) differentiate into perivascular cells when cocultured with cord blood**
12 **endothelial progenitor cells (ECFCs).**

13 A) MSCs, VIC_p, or VIC_p+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_p cultured alone. Results represent the mean \pm SEM
17 of 4 independent experiments using VIC_p 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_p cocultured with ECFCs (labeled using von Willebrand factor in red) acquire a perivascular phenotype
20 confirmed by induction of α SMA, NG2 and calponin proteins (green).

21
22 **Figure 4. Angiogenic potential of VIC_p on ECFCs.**

23 A) Proliferation of ECFCs cultured in CM from VIC_c (VIC_c-CM) or from VIC_p (VIC_p-CM) evaluated after three
24 days by measuring cellular phosphatase activity. Results represent the mean \pm 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_c-CM or VIC_p-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 \pm 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_p or VIC_c 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_p and MSCs induced a significant
8 increase in ECFC capillary length compared with VIC_c. Statistical tests used: Kruskal-Wallis test.
9 F) Quantification of ECFC branches per bead for each feeder. VIC_p and MSCs induced a significant increase in
10 ECFC branches compared with VIC_c. 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_p-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_p
17 in contrast to VIC_c. Results represent the mean ± SEM of 5 independent experiments using VIC_p and VIC_c from
18 different donors. Statistical tests used: Mann-Whitney test.
19 B) mAb VEGF-A abolished ECFC proliferation induced by VIC_p conditioned media.
20 C) mAb VEGF-A abolished ECFC migration induced by VIC_p conditioned media.
21 D) siVEGFA in VIC_p abolished proliferation and E) migration of ECFCs induced by VIC_p 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

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27 **Figure 6. Silencing VEGFA abolished VIC_p-induced ECFC sprouting.**

1 A) Confocal laser scanning images of ECFC sprouting with feeder conditions using VIC_p transfected with
2 siVEGFA (VIC_p-siVEGFA) or scrambled (VIC_p-Scr).

3 B) Quantification of ECFC capillary length per bead for each feeder evaluated. VIC_p-siVEGFA induced a
4 significant decrease in ECFC capillary length in contrast to VIC_p-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_p-siVEGFA induced a significant
7 decrease in ECFC branches compared with VIC_p-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_p did not alter their osteogenic or perivascular differentiation.**

13 A) Calcium deposit by VIC_p 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_p 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_p from different donors. Statistical tests used: Mann-Whitney test.

17 B) Fluorescence intensity of α SMA and Calponin (green) when VIC_p 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 α SMA with ImageJ software. No significant differences between
20 α 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_p 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_p 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_p**

- 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_p in
5 contrast to VIC_c. Results represent the mean ± SEM of 5 independent experiments using VIC_p and VIC_c
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_p and VIC_c (p>0.05). Results represent the mean ± SEM of 5 independent experiments using VIC_p and
10 VIC_c 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_p and VIC_c (p>0.05) Results represent the mean ± SEM of 5 independent experiments using VIC_p and
13 VIC_c 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_p and VIC_c (p>0.05). Results represent the mean ± SEM of 5 independent experiments using VIC_p and
16 VIC_c 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, r²=0.9119). Results represent the mean ± SEM of 5 independent
19 experiments using VIC_p and VIC_c from different donors. Statistical tests used: Pearson's correlation
20 coefficient.

Figure 1

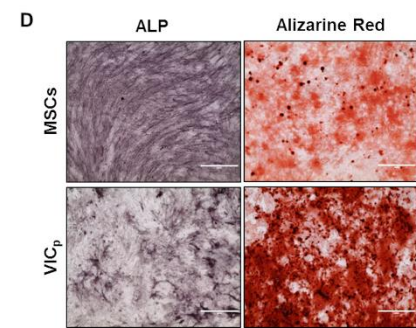
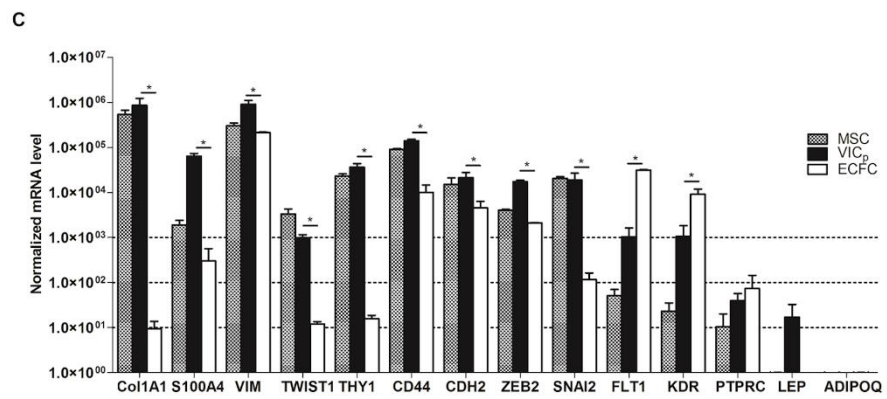
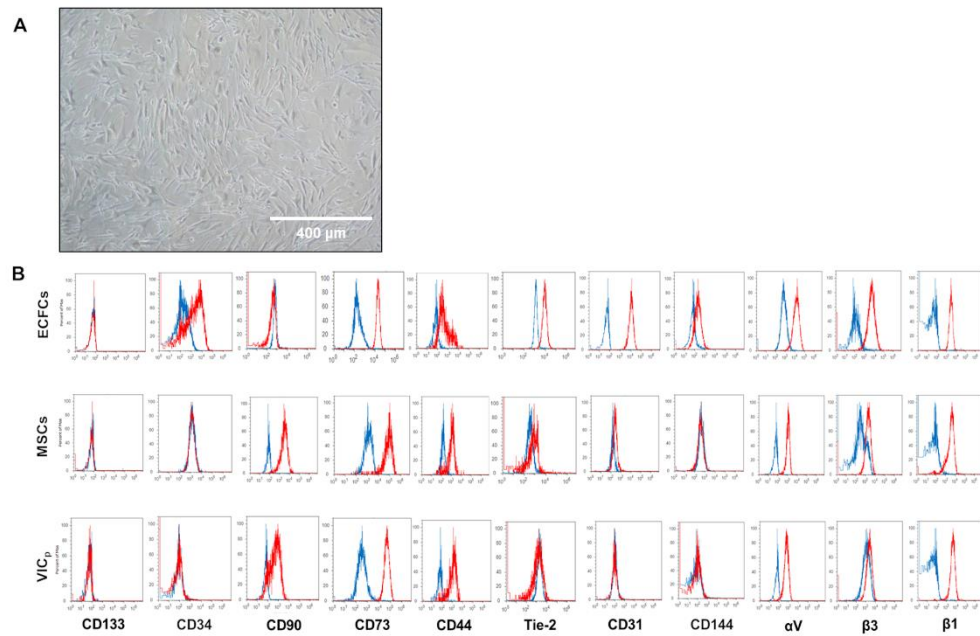
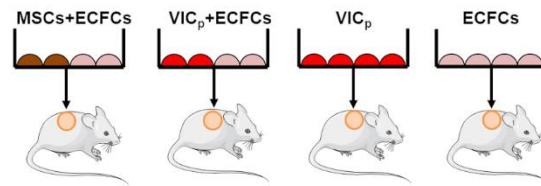
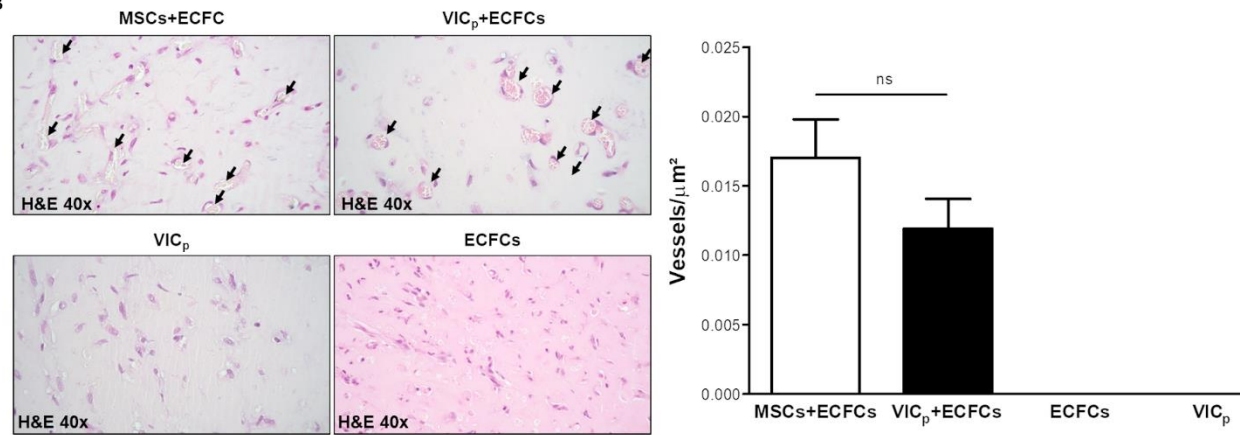


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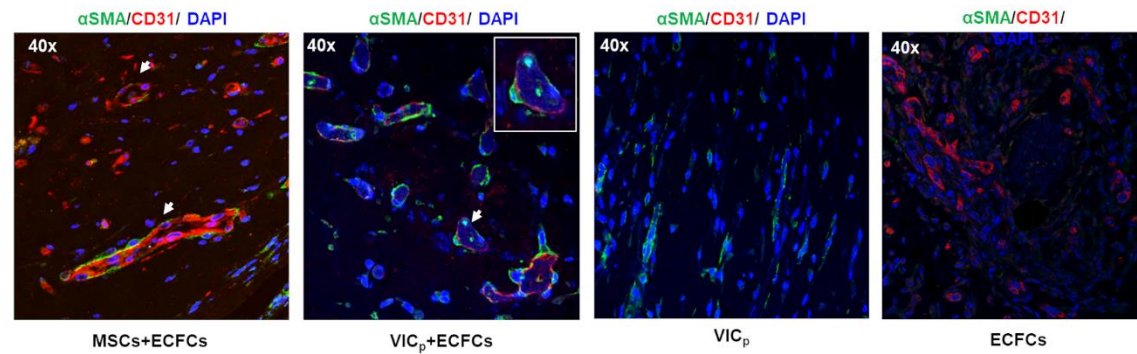
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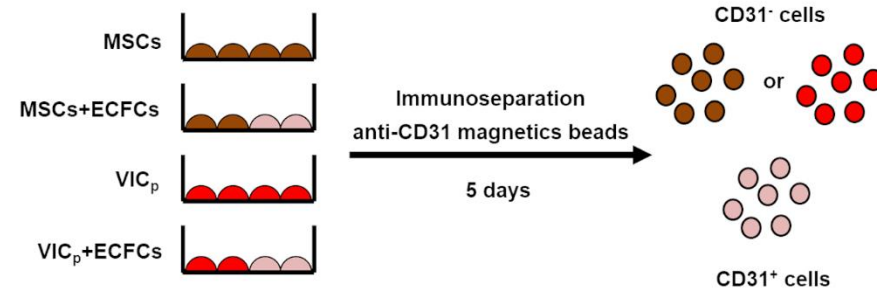


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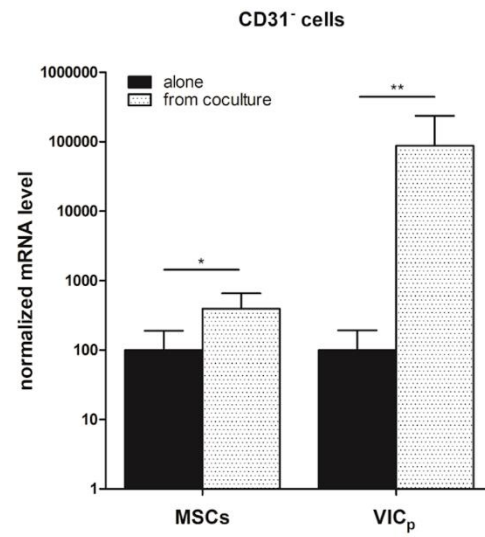


A

Figure 3



B



C

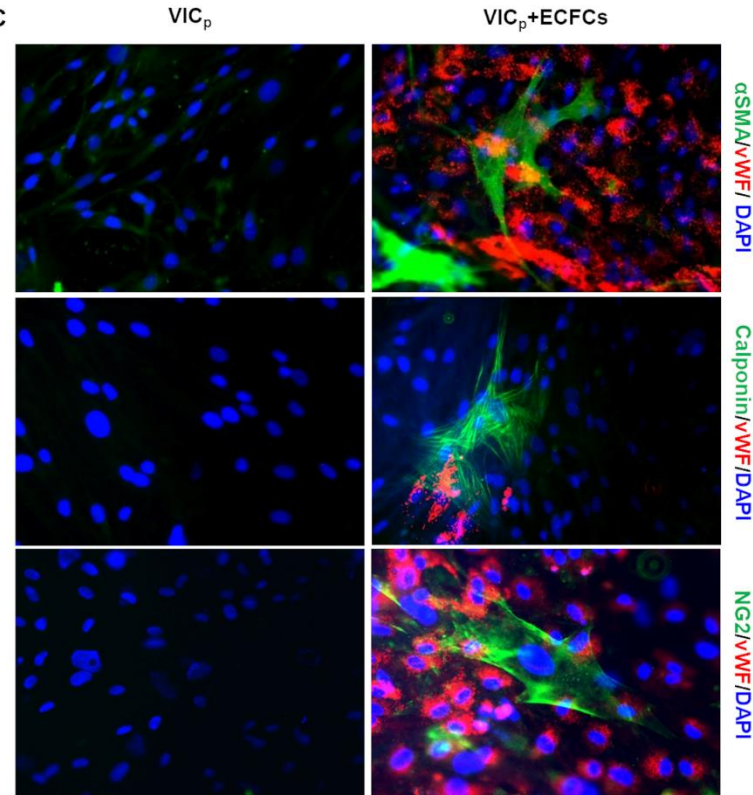


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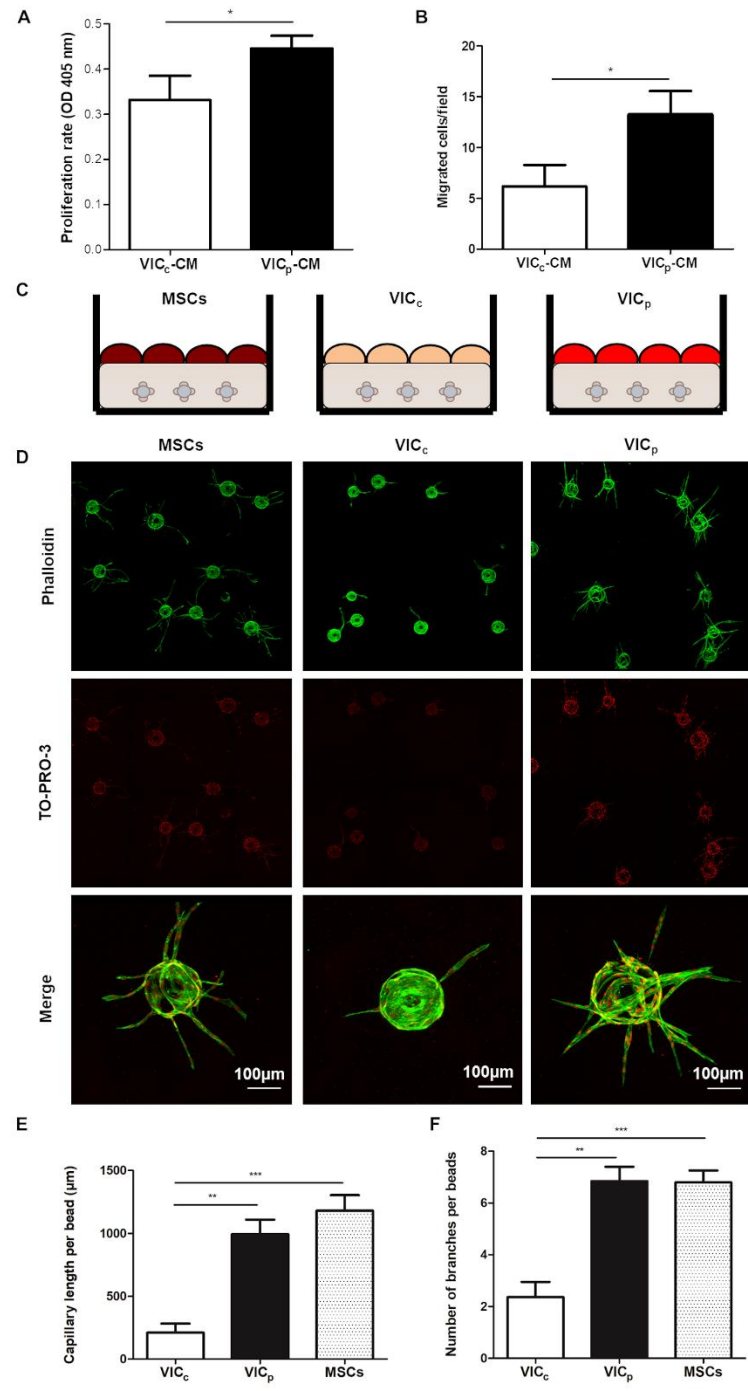


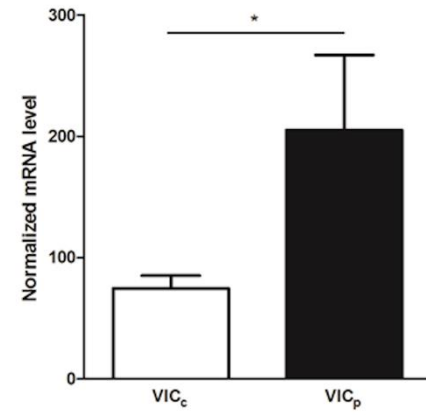
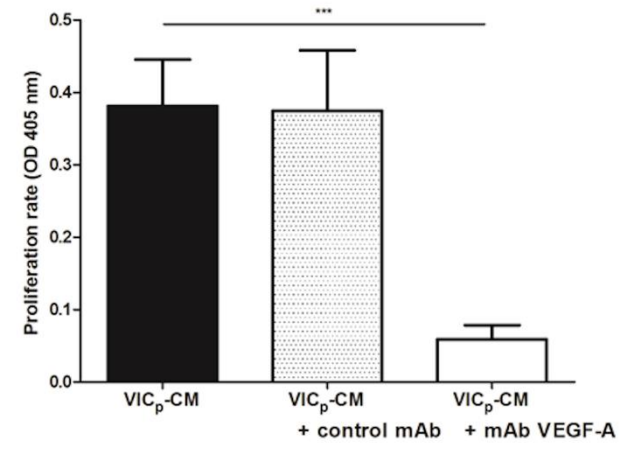
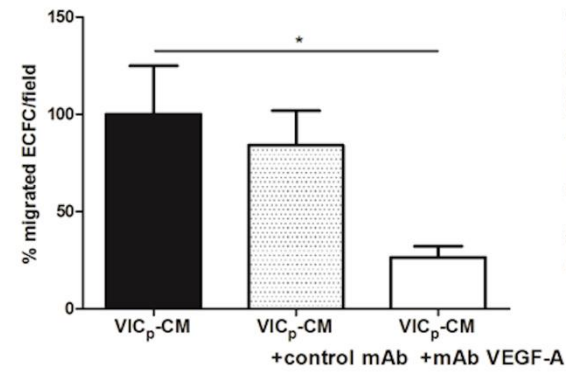
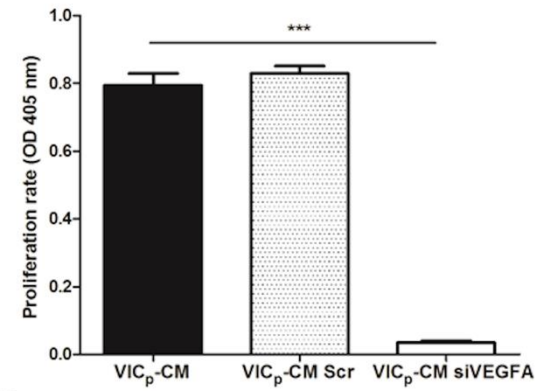
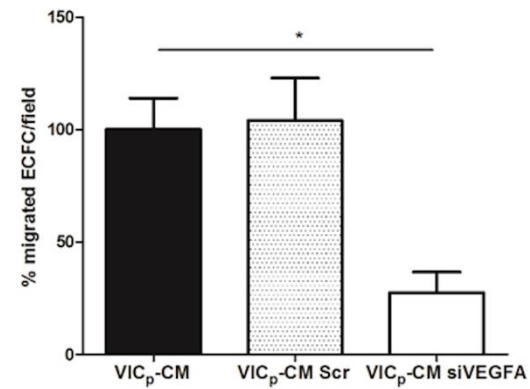
Figure 5**A****B****C****D****E**

Figure 6

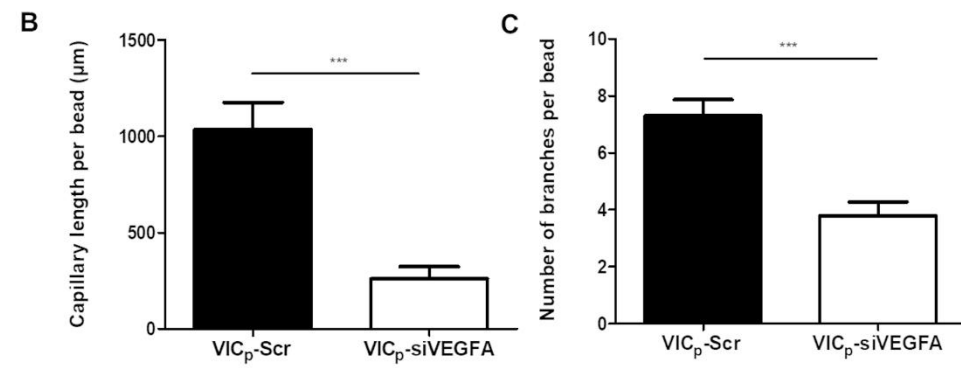
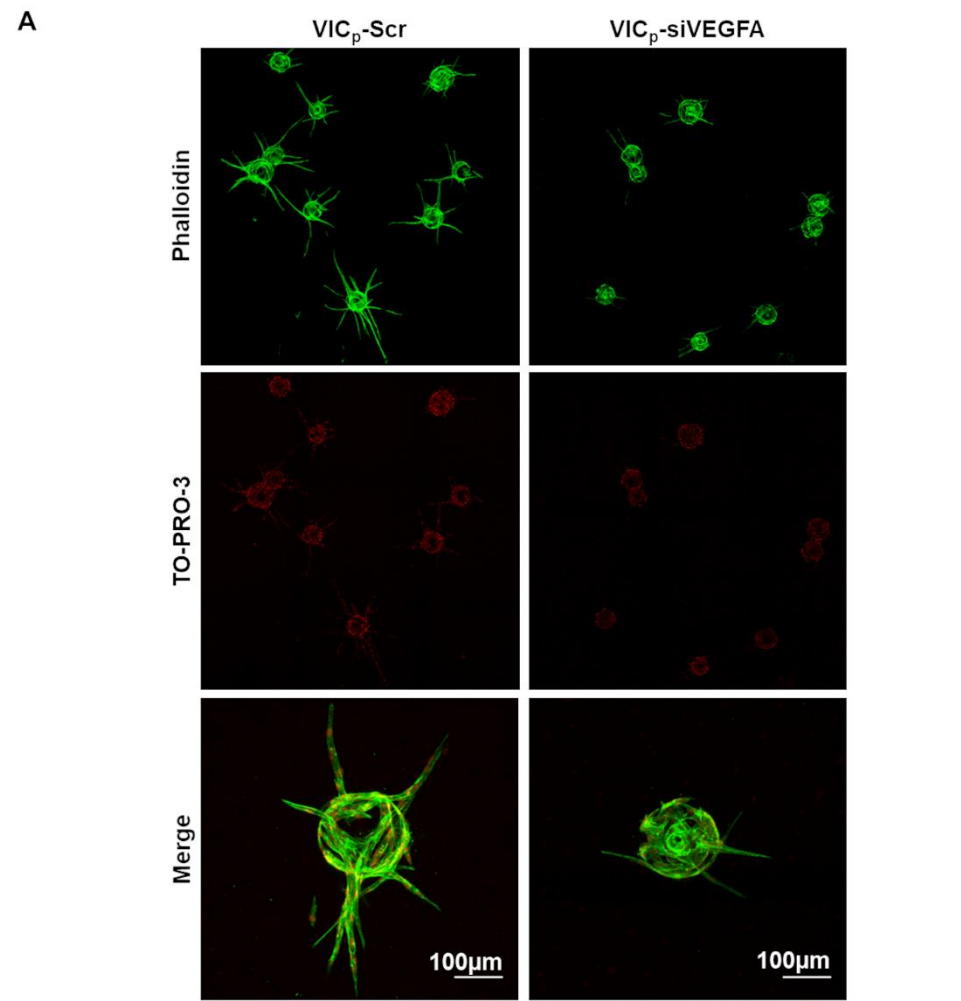


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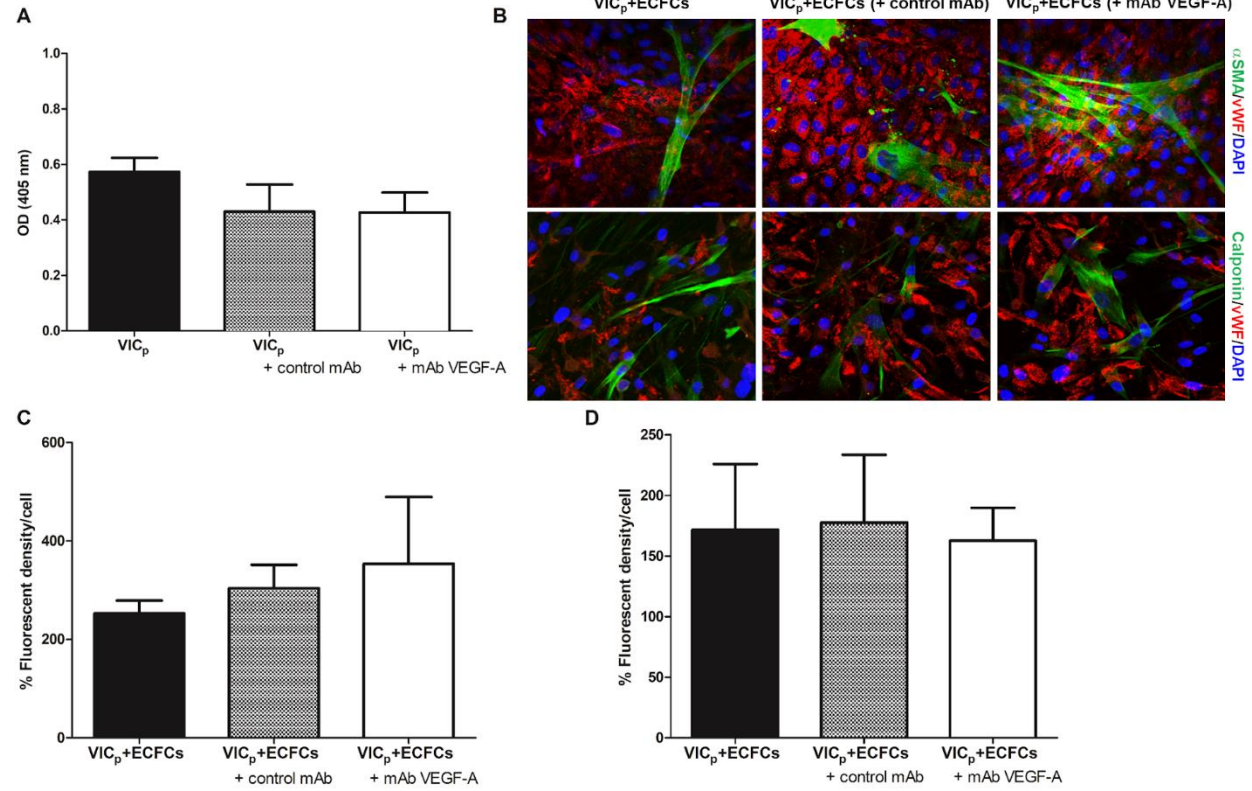
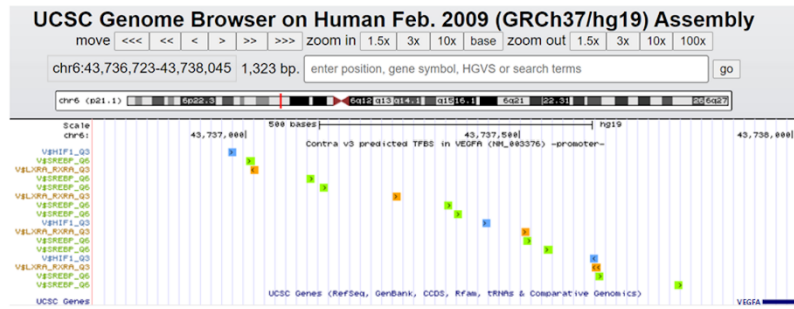
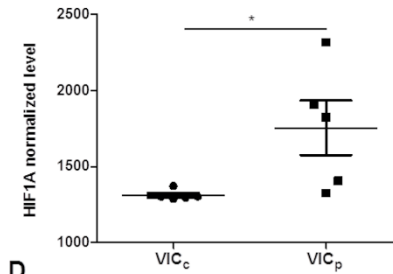


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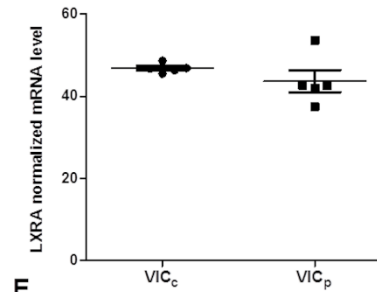
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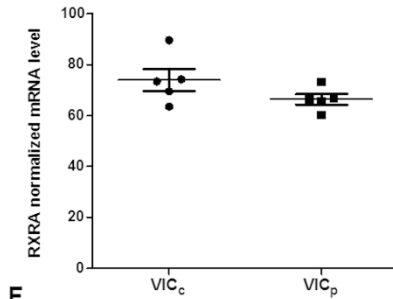
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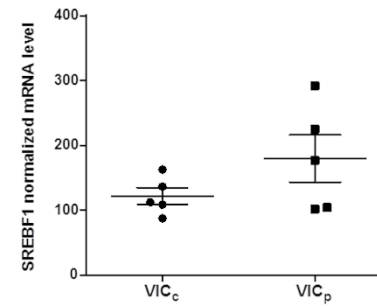
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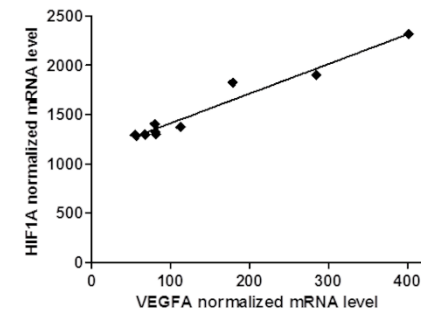
D



E



F



VICs isolated from patients with CAVD (VIC_p)

