

**Human aortic valve interstitial cells obtained from
patients with aortic valve stenosis are vascular
endothelial growth factor receptor 2 positive and
contribute to ectopic calcification**

(大動脈弁狭窄症患者より得た大動脈弁間質細胞は
VEGFR2陽性であり、異所性石灰化に寄与する)

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Abstract

Since aortic valve stenosis (AVS) is the most frequent and serious valvular heart disease in the elderly, and is accompanied by irreversible valve calcification, medicinal prevention of AVS is important. Although we recently demonstrated that human aortic valve interstitial cells (HAVICs) obtained from patients with AVS were highly sensitive to ectopic calcification stimulation, the cell types contributing to calcification are unknown. We aimed to immunocytochemically characterize HAVICs and identify their contribution to valve calcification. HAVICs were isolated from patients with AVS and cultured on non-coated dishes. Immunocytochemical features and HAVIC differentiation were analyzed in passage 1 (P1). The immunohistochemical features of the calcified aortic valve were analyzed. Most cultured P1 HAVICs were CD73-, CD90-, and CD105-positive, and CD45- and CD34-negative. HAVICs were vascular endothelial growth factor receptor 2 (VEGFR2)-positive; however, approximately half were α -smooth muscle actin (SMA)-positive, colonized, and easily differentiated into osteoblastic cells. Calcified aortic valve immunohistochemistry showed that all cells were positive for VEGFR2 and partly α -SMA. Further, VEGFR2-positive cells were more sensitive to tumor necrosis factor- α -induced ectopic calcification with or without α -SMA positivity. We conclude that HAVICs obtained from patients with AVS are VEGFR2-positive undifferentiated mesenchymal cells and may contribute to aortic valve ectopic calcification.

Keywords: aortic valve stenosis, aortic valve ectopic calcification, vascular endothelial growth factor receptor 2, α -smooth muscle actin, tumor necrosis factor- α

1. Introduction

Calcified aortic valve disease is one of the main causes of cardiovascular death.¹ Aortic valve calcification is one of the major causes of aortic valve stenosis (AVS). With the gradual aging of the population, calcified AVS has become an increasingly common disease in recent years and is the most common type of valvular heart disease in developed countries. A Swedish study estimated that the prevalence of calcified AVS in the 65–79-year age group was between 2% and 4%, compared with almost 10% in the 80-year age group.² At present, there is no effective medical means to slow or stop AVS. Currently, aortic valve replacement is the only effective treatment, but it is costly and risky, and some patients cannot tolerate open surgery. Although transcatheter aortic valve replacement is used for high-risk patients, approximately 20% of patients with AVS are unsuitable for valve replacement because of its invasiveness.³ Thus, development of effective drug therapies is crucial. This requires elucidation of the characteristics of human aortic valve interstitial cells (HAVICs), which induce calcification.

Aortic valve calcification is a complex and multifactorial process of ectopic calcification.⁴ Many ectopic calcification-related factors and signaling pathways are involved in the pathophysiological process, of which the inflammatory response is considered the first important factor in the development of aortic valve calcification.⁵ Inflammatory stimuli significantly upregulate important genes involved in promoting HAVIC calcification, such as bone morphogenetic protein 2 (BMP2) and runt-related transcription factor 2.⁶ Our previous study revealed that the acute inflammatory response caused by tumor necrosis factor- α (TNF- α) accelerated calcification of HAVICs isolated from patients with calcified AVS via the BMP2-alkaline phosphatase pathway.⁷ Some studies have shown that in the presence of high inorganic phosphate, inflammatory factors accelerate ectopic calcification.^{8,9} We also demonstrated that Matrix Gla protein negatively regulates calcification of HAVICs isolated from calcified aortic valves.¹⁰ However, the cell group which aortic valve ectopic calcifications originate is unknown.

Osteogenic endothelial progenitor cells, such as vascular endothelial growth factor receptor-2 (VEGFR2)/CD34-positive cells, contribute to aortic valve calcification in patients with atherosclerosis.¹¹⁻¹³ Furthermore, two recent studies reported that aortic valve calcification is associated with ectopic ossification induced by various bone marrow-derived circulating osteogenic progenitor cells, such as type I collagen/CD45-positive and/or osteocalcin/CD45-positive cells.^{14, 15} However, it has not yet been known whether these progenitor cells present in the aortic valve cause valve calcification. We used fluorescence-activated cell sorting to isolate CD73-, CD90-, and CD105-positive and CD45-negative undifferentiated cells from valve samples. These cells were further separated into CD34-negative and CD34-positive cells. Compared with CD34-positive cells, CD34-negative cells were more sensitive to high inorganic phosphate (3.2 mM) and were prone to calcification. In addition, immunohistochemical staining showed that the number of CD34-negative cells was significantly greater compared with the number of CD34-positive cells.¹⁶ However, the recovery rate of these cells by cell sorting was low (approximately 10%). Thus, the effects of HAVICs on valve ectopic calcification is yet to be clarified.

In this study, we aimed to clarify the phenotype and differentiation potential of HAVICs using immunofluorescent staining, and to identify the role of HAVICs on ectopic calcification.

2. Materials and Methods

2-1 Materials

The following reagents were sourced from commercial suppliers: α -modified Eagle's medium (α -MEM; Nacalai Tesque, Kyoto); fetal bovine serum (FBS) and SN-50 (Sigma-Aldrich, St. Louis, MO, USA), penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA, USA); TNF- α , Hoechst 33342, dorsomorphin, and other analytical grade reagents (Fujifilm,

Tokyo); collagenase type V (Sigma-Aldrich); and Power SYBR Green PCR Master Mix (TOYOBO, Osaka). All primers used for quantitative real-time PCR were obtained from Eurofins Genomics (Tokyo). All chemicals were of the highest purity commercially available. All solutions were freshly prepared and were of sufficiently high concentrations to allow addition of only very small volumes to the culture medium.

2-2 Isolation and culture of human aortic valve interstitial cells

All patients gave written informed consent, and the study was approved by the Institutional Review Board of the Hirosaki University Hospital. Human aortic valves were obtained from 15 patients (7 males with a mean age of 68.9 ± 3.5 years and eight females with a mean age of 71.9 ± 2.5 years) with calcified AVS who underwent surgical aortic valve replacement at Hirosaki University Hospital (Aomori) after providing informed consent. There was no significant difference in age between females and males. Human aortic valve specimens were gently cut into 2 ± 1 -mm-long pieces. HAVICs were isolated by collagenase digestion of aortic valve pieces as previously described.⁷ Briefly, valve pieces were digested with collagenase Type V (approximately 650 U/mL) and incubated for 1.5 hours at 37°C. Cells were isolated through 70 μ m filter, and isolated cells were cultured on non-coated dishes in α -MEM containing 10% FBS. Medium was changed every 3–4 days.

2-3 Immunocytochemical staining of human aortic valve interstitial cells

After coating a 12-mm-diameter glass-base dish (AGC Inc., Tokyo) with 0.1% gelatin aqueous solution, cells (3×10^3) were seeded on a dish in α -MEM supplemented with 10% FBS, and cultured overnight. After washing with 100 μ L phosphate-buffered saline (PBS) for three times at room temperature, cells were fixed with 10% neutral buffered formalin (NBF) for 15 minutes. Then, cells were permeabilized with methanol at -20°C for 10 minutes. After washing

with PBS, 200 μ L PBS solution containing 1% bovine serum albumin (BSA) was added for blocking. After 30 minutes, 200 μ L of primary antibody solution (diluted with 1% BSA in PBS) was used for replacement (Table 1). After 1 hour at room temperature, the sample was refrigerated at 4°C overnight. The next day, after washing with PBS, 200 μ L of secondary antibody solution (diluted with 1% BSA in PBS) was replaced and stored in the dark at room temperature for 1 hour. After PBS washing, nuclear staining reagent (Hoechst 33242) was applied for 10 minutes. After washing with distilled water, a fluorescence microscope was used to image stained cells. To obtain the abundance ratio of each marker in positive cells, the number of positive cells was divided by the number of nuclei and calculated as a percentage.

2-4 Assessment of the differentiation potential of human aortic valve interstitial cells

For colony formation, isolated HAVICs (1×10^5) were seeded on a 10-cm petri dish and cultured in α -MEM containing 10% FBS. Medium was changed every 3 days, and cells were stained with 0.5% crystal violet after 14 days. Stained cells were imaged using a digital camera (Canon, Tokyo).

For osteogenic differentiation, isolated HAVICs (1×10^5) were cultured in a 10-cm petri dish. To induce osteogenic differentiation of HAVICs, 10 mmol/L glycerophosphate, 50 μ g/mL ascorbic acid and 1 nmol/L dexamethasone were added to complete medium, and osteogenic medium was changed every 3 days for 21 days. At the same time, a non-treatment group was set and cultured with complete medium for 21 days. The degree of calcification was measured using Alizarin Red S stain (Sigma-Aldrich) at 3 weeks.¹⁷ Stained cells were imaged using a digital camera. Alizarin Red S dye released from the extracellular matrix following incubation of cells in 100 mM aqueous cetylpyridinium chloride was quantified by spectrophotometry at 550 nm.¹⁷

For adipogenic induction, isolated HAVICs (1×10^5) were cultured in a 10-cm petri

dish in adipogenic medium containing 0.1 mmol/L indomethacin, 100 nmol/L dexamethasone, and 0.5 mmol/L isobutyl methylxanthine. Medium was changed every 3 days. Three weeks later, cells were stained with Oil Red O solution (Sigma-Aldrich) to observe induction of adipocyte differentiation. At the same time, the non-treatment cells were cultured in non-adipogenic medium containing 10% FBS in α -MEM medium for 21 days. Stained cells were imaged using a digital camera.

For chondrogenic differentiation, 2×10^5 HAVICs were placed in 15-mL test tubes and centrifuged at 450 *g* for 10 minutes to form a pellet. Medium was removed and replaced with 0.4 mL cartilage-forming medium containing complete medium and 10 ng/mL transforming growth factor- β_2 , 1 μ g/mL BMP-2, and 100 nmol/L dexamethasone. Cartilage-forming medium was changed every 3 days. At the same time, the non-treated group was set up and cultured in complete medium for 21 days. After 21 days of culture, cell pellets were imaged using a digital camera. Cell pellets were fixed in 10% NBF, embedded in paraffin and sectioned, and stained with Toluidine blue to detect proteoglycans.

2-5 Immunohistochemical staining of aortic valve specimens

Aortic valve specimens were fixed with paraformaldehyde, embedded in paraffin, and cut into 4- μ m sections. After deparaffinization with xylene followed by ethanol, endogenous peroxidases were blocked using 0.3% hydrogen peroxide in methanol at room temperature for 10 minutes and then washed with PBS. Next, sections were blocked with normal serum at room temperature for 30 minutes and incubated with primary antibodies (Table 1). Sections were incubated with secondary and tertiary agents from a streptavidin biotin-peroxidase detection kit (Histofine SAB-PO Kit; Nichirei, Tokyo) and lightly counterstained with hematoxylin. Hoechst 33342 was used as a fluorescent nuclear stain. Images of three separate visual fields were obtained from each insert with a 40 \times objective. To obtain the abundance ratio of each marker,

the number of positive cells was divided by the number of nuclei and calculated as a percentage.

2-6 Identification of calcification

We measured Smad1/5/8 phosphorylation and TNF- α -induced ectopic calcification as an index of HAVIC calcification. To measure Smad1/5/8 phosphorylation, cultured passage 1 (P1) HAVICs were seeded on a gelatin-coated 12-mm-diameter glass-base dish. The next day, TNF- α (30 ng/mL) was added in the absence or presence of dorsomorphin (3 μ M), which is an inhibitor of Smad1/5/8 phosphorylation, for 6 hours. This experiment was performed in the presence of 0.1% dimethyl sulfoxide, which did not induce cell viability up to day 1 (data not shown). Dorsomorphin was added 1 hour before TNF- α administration. After reaction, cells were stained as above with combinations of anti-VEGFR2 and anti-phosphorylated Smad1/5/8 or anti- α -SMA and anti-phosphorylated Smad1/5/8 (Table 1). To assess calcification, the number of cells with nuclear-localized phosphorylated Smad1/5/8 was divided by the total number of cells and shown as a percentage. To measure ectopic calcification, cells were seeded in a 24-well plate and cultured for 3 days until confluent. Cells were further cultured with or without 30 ng/mL TNF- α for 14 days. The degree of calcification was quantified using Alizarin Red S stain, which was visualized using a digital camera (Nikon, Tokyo). Alizarin Red S dye released from the extracellular matrix following incubation of cells in 100 mM aqueous cetylpyridinium chloride was quantified by spectrophotometry at 550 nm.¹⁷

2-7 ALP activity assay

HAVICs were seeded into 12-well plates and grown for 3 days (until 80%–90% confluency) and then were further cultured in the presence or absence of TNF- α (30 ng/mL). After 2 weeks of culture, proteins were collected from the cells by using a cell-lysis buffer containing 80 μ L of 0.05% Triton X-100, and ALP activity was measured using a LabAssay

ALP Kit from Wako Pure Chemicals (Osaka, Japan).

2-8 Measurement of gene expression

Total RNA was isolated from HAVICs by using a QuickGene RNA Cultured Cell kit S (Promega, Fitchburg, WI, USA). An aliquot of the total RNA was reverse-transcribed into cDNA by using random primers. For real-time PCR analysis, cDNA was amplified (on a CFSTM Real-Time System; Bio-Rad, Hercules, CA, USA) using this protocol: initial denaturation at 95°C for 1 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. The 10-μL reaction volume included 1.5 μL of a 1:4 dilution of the first-strand reaction product, 0.3 μL of 10 μM forward and reverse primers, 2.9 μL of pure water, and 5 μL of SYBR qPCR reagent. The primers used for NF-κB, BMP2, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) genes (Table 2) were designed using NCBI Primer BLAST (The National Center for Biotechnology Information, Bethesda, MD, USA). Amplification of the housekeeping gene G3PDH served as a normalization standard. Real-time PCR data were represented as cycle threshold (Ct) levels and normalized by the individual G3PDH control Ct values. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

2-9 Western blot analysis

Cytoplasmic extracts were obtained by lysing cells in 20 mM Tris-HCl, pH 7.4, containing 0.05% Triton X-100. After the BCA protein assay (TAKARA, Tokyo, Japan), aliquots of the solutions were mixed with loading buffer and heated at 70°C for 15 min. The samples (0.5 μg for NF-κB p65 and 3 μg for phosphor-NF-κB p65 protein) were subjected to electrophoresis. To measure the released BMP2, cell culture supernatants (20 μL) were mixed with loading buffer and heated at 70°C for 15 min. Electrophoresis of each sample was subjected on a 5–20% SDS-polyacrylamide gel (ATTO Co., Tokyo, Japan), and proteins were

transferred onto PVDF membranes by using an iBlot™ Dry Blotting System (Thermo Fisher Scientific, Waltham, MA, USA). The membranes were incubated with primary antibodies [anti-phospho-p65, 1:1000 and anti-p65, 1:1000 (MEDICAL & BIOLOGICAL LABORATORIES CO., LTD, Nagoya, Japan); anti-BMP2, 1:1000 (Santa Cruz Biotechnology, Dallas, TX, USA)] and then with anti-rabbit or anti-mouse secondary antibody (MEDICAL & BIOLOGICAL LABORATORIES CO., LTD, Nagoya, Japan) by using an iBind™ Western System (Thermo Fisher Scientific, Waltham, MA, USA). The immunoreactive bands were detected by an enhanced chemiluminescence (ECL) detection system. The integrated optical density of the immunoreactive band was quantified using ImageJ 1.53e software (National Institute of Health, Bethesda, MD, USA).

2-10 Statistical analysis

All statistical analyses were performed using KyPlot 6.01 software (Kyenslab, Tokyo). Comparisons between groups were assessed by analysis of variance (ANOVA) with Student–Newman–Keuls post-hoc correction. Two-way ANOVA followed by the Bonferroni post-hoc test was performed to determine statistical differences between groups. Comparisons between two independent datasets were assessed using the Student's *t*-test. Data are presented as mean \pm standard error of the mean. A *P* value of <0.05 was considered statistically significant.

3. Results

3-1 Immunocytochemical properties of human aortic valve interstitial cells from patients with aortic valve stenosis

Figure 1 shows that almost all cultured HAVICs were CD105-positive in all cell areas. Thus, anti-CD105 antibody was used as a positive control.¹⁶ The mesenchymal stem cell markers, CD90 ($95.1\% \pm 0.2\%$) and CD73 ($90.0\% \pm 0.7\%$), were positive in almost all HAVICs.

The localization area of CD73 in each cell was narrow. Conversely, the hematopoietic stem cell markers, CD45 (0%) and CD34 ($0.9\% \pm 0.9\%$), were negative in almost all cells. Although VEGFR2, an endothelial cell marker, was positive (100.0%), the smooth muscle and fibroblast cell marker, α -SMA, was positive in $43.7\% \pm 0.8\%$ of HAVICs.

3-2 Localization of human aortic valve interstitial cells in calcified aortic valve tissues

VEGFR2-positive HAVICs were abundant in calcified aortic valve specimens from patients with AVS (Figure 2) and widely localized in the endothelium, which was similar to CD105 ($95.9\% \pm 0.5\%$). On the other hand, aortic valve specimens included α -SMA-negative area (middle images) and α -SMA-positive area (bottom images). All areas of these specimens were positive on Alizarin Red staining. In totally, there were a few α -SMA-positive cells in aortic valve specimens from patients with calcified AVS ($14.5\% \pm 4.6\%$). Although the localization properties of mesenchymal stem cell markers (CD73, CD90, and CD105) and hematopoietic stem cell marker (CD45) in calcified aortic valve specimens were similar to those reported in a previous study,¹⁶ there were few CD34 positive cells as with isolated P1 HAVICs (data not shown).

3-3 Differentiative properties of human aortic valve interstitial cells

To establish the differentiative properties of HAVICs as a mesenchymal stem cell, we investigated various differentiation abilities of HAVICs.¹⁸ After 14 days of culture, colony formation of HAVICs isolated from patients with AVS was confirmed (Figure 3A). Next, we used osteogenic medium to investigate whether HAVICs isolated from patients with AVS had the ability to undergo osteogenic differentiation. Alizarin Red S stain was applied after 21 days of culture. Alizarin Red S binding in HAVICs cultured in osteogenic medium was more marked compared with HAVICs cultured in non-treatment medium (Figure 3B and 3C). However, with

both chondrocyte and adipocyte induction, HAVICs were less able to differentiate compared with the non-treatment group (Figures 3D and 3E). The data shows that HAVICs isolated from AVS tissues are undifferentiated, and have colony-forming and differentiation abilities.

3-4 Dual staining of vascular endothelial growth factor receptor 2 and α -smooth muscle actin in human aortic valve interstitial cells

To confirm the localization of α -SMA in HAVICs, P1 HAVICs isolated from patients with AVS were cultured overnight on gelatin-coated glass-base dishes. All α -SMA-positive cells were positive for VEGFR2. The rates of α -SMA and VEGFR2 positivity were $45.4\% \pm 2.3\%$ and 100.0% , respectively (Figure 4).

3-5 Tumor necrosis factor- α -induced calcification of vascular endothelial growth factor receptor 2-positive cells

HAVICs were cultured in the presence or absence of TNF- α (30 ng/mL) for 6 hours. Marked nuclear translocation of phosphorylated Smad1/5/8 was observed in VEGFR2-positive HAVICs (Figures 5A and 5B). Nuclear translocation of phosphorylated Smad1/5/8 by TNF- α was significantly suppressed by dorsomorphin (3 μ M). Furthermore, TNF- α induced ectopic calcification of HAVICs by 14 days of culture, which was inhibited by dorsomorphin (3 μ M) (Figures 6A and 6B). TNF- α also activated alkaline phosphatase (ALP), a typical index of calcification (Figure 6C). We confirmed that TNF- α accelerated BMP2 gene expression and BMP2 release from cells, one of markers of HAVIC calcification (Figures 6D and 6E). We further demonstrated that NF- κ B expression, a transcription factor of BMP2, and the phosphorylation of p65, one of the NF- κ B family, also enhanced in the presence of TNF- α (Figures 6D and 6F).

3-6 Tumor necrosis factor- α -induced Smad1/5/8 phosphorylation in α -smooth muscle actin-positive and -negative human aortic valve interstitial cells

HAVICs were cultured in the presence or absence of TNF- α (30 ng/mL) for 6 hours. Nuclear translocation of phosphorylated Smad1/5/8 was significantly observed in both α -SMA-positive and α -SMA-negative HAVICs; specifically, VEGFR2-positive HAVICs (Figures 7A and 7B). Phosphorylated Smad1/5/8 expression induced by TNF- α was significantly suppressed by dorsomorphin (3 μ M). There was no significant difference in Smad1/5/8 phosphorylation between α -SMA-positive and α -SMA-negative cells.

4. Discussion

In a previous study, we have demonstrated that CD73-, CD90-, and CD105-positive undifferentiated cells are localized to the aortic valves obtained from patients with AVS using a highspeed sorter (FACSARIA[™]; BD Biosciences). Cells were negative for CD45, while only some cells expressed CD34.¹⁶ However, these results did not affect the features of cultured HAVICs because of the low recovery rate of this sorting method and the low cell viability, which prohibited continuous culture. Further, the differentiation ability of these cells is unknown. Thus, we performed immunocytochemical staining of cultured HAVICs obtained from patients with AVS, in the present study.

We acknowledge that almost all P1 HAVICs isolated from aortic valves of patients with AVS expressed CD73, CD90, and CD105, and these cells stained negative for CD45; these features indicate mesenchymal stem cells.¹⁸ HAVICs also did not express CD34, which is a marker of hematopoietic stem cells. Further, we confirmed that these undifferentiated cells had colony-forming and osteoblastic-like cell differentiation abilities.

To identify the cellular markers that contribute to ectopic calcification, we performed immunocytochemistry to stain for α -SMA, which is a marker of smooth muscle cells and

fibroblasts, and VEGFR2, which is a marker of vascular endothelial cells. Interestingly, all HAVICs were VEGFR2-positive, while only half of cells were α -SMA-positive. Using immunohistochemistry, we also confirmed that aortic valve specimens obtained from patients with AVS contained α -SMA-positive cells. Further, VEGFR2-positive cells were abundant; this population consisted of both endothelial cells and valvular interstitial cells. CD105, namely, endoglin, is a TGF- β -binding protein in endothelial cells. These results indicate that HAVICs isolated from patients with AVS are undifferentiated; thus, it is possible that HAVICs originate from aortic valve endothelial cells.

In this study, the ratio of α -SMA-positive cells in culture was greater compared with calcified aortic valve tissue. Latif et al. reported that the ratio of α -SMA-positive cells in adult aortic valve specimens was very low, but after these cells were isolated and cultured, the rate of α -SMA-positive cells increased because of differentiation.¹⁹ Although our results support their observations, the molecular mechanism of differentiation from α -SMA-negative cells to α -SMA-positive cells is still unknown.

Although the cellular origin of aortic valve ectopic calcification was not clear until now, several hypotheses have been proposed. One hypothesis is that aortic valve interstitial cells, including fibroblasts and smooth muscle cells, differentiate into osteoblasts in response to certain stimuli, such as inflammatory cytokines, resulting in aortic valve ectopic calcification. Although various molecular mechanisms involving BMP2 and other osteogenic proteins have been proposed, an effective medical therapy to inhibit aortic valve calcification has not yet been established.²⁰ Recently, Valgimigli et al. reported that aortic valve calcification is associated with ectopic ossification induced by CD34-positive and VEGFR2-positive endothelial progenitor (EP) cells.¹² Egan et al. proposed that aortic valve ectopic calcification is induced by CD45 and circulating osteocalcin-positive (COP) cells.¹⁵ However, the precise roles of EP and COP cells in aortic valve calcification have not yet been determined. Lin et al. demonstrated

that CD34-negative cells in adipose tissue-derived mesenchymal stroma had a greater ability to differentiate into osteogenic lineages compared with CD34-positive cells.²¹ In our previous study, we confirmed that calcification of CD34-negative undifferentiated cells was rapidly induced by medium containing a high concentration of inorganic phosphate (3.2 mM).¹⁶ In this study, almost all HAVICs isolated from patients with AVS were CD34-negative. In addition, all HAVICs were VEGFR2-positive. These results suppose that aortic valve endothelial cells are closely related to the cellular origin of aortic valve ectopic calcification. In future studies, more detailed information on the origin of HAVICs in terms of the endothelial–mesenchymal transition is important to identify novel therapeutic strategies for AVS.

This study also indicated that, in VEGFR2-positive HAVICs, TNF- α accelerated the transcription factor, NF- κ B activation and induced ectopic calcification via BMP2-ALP pathway. Furthermore, TNF- α induced expression of a calcification marker, phosphorylated Smad1/5/8, in VEGFR2-positive HAVICs with or without α -SMA positivity, which was inhibited in the presence of the Smad1/5/8 phosphorylation inhibitor, dorsomorphin. In the previous study, we have already demonstrated that TNF- α induced the passage 4 HAVIC calcification and suggested that fibroblastic cells in HAVICs were differentiated into osteoblastic cells by TNF- α , resulting in ectopic aortic valve calcification. However, the cell group which aortic valve ectopic calcifications originate was unknown.⁷ In this study, we further investigated the characteristics of calcified cells derived from HAVICs, and we found that all of the calcified cells derived from passage 1 HAVICs are VEGFR2-positive and CD34-negative, and α -SMA-positive cells do not contribute to osteoblastic differentiation. However, the differentiation mechanism from VEGFR2-positive and CD34-negative cells to osteoblastic-like cells is yet unknown. Further studies may be required to clarify the details of the role of HAVICs in ectopic calcification of aortic valve.

There are two limitations of our study. First, in a previous study, we confirmed that

CD34-negative mesenchymal undifferentiated cells contribute to aortic valve ectopic calcification.^{22, 23} However, in non-calcified aortic valve, CD34-positive cells are abundant.¹⁶ We think this differentiation of valve interstitial cells from CD34-positive to CD34-negative and from VEGFR2-negative to VEGFR2-positive may be closely related to the endothelial–mesenchymal transition. To solve this problem, we prepared aortic endothelial cells because it is known that the embryonic origin of aortic valve endothelial cells is aortic endothelial cells. However, these cells are CD34-, CD73-, and CD90-negative (data not shown). To solve this problem, it is necessary to plan a novel strategy using normal aortic valve endothelial cells obtained from rodents. Second, this study did not explain why α -SMA-positive cells do not contribute to osteoblastic differentiation. The most well-known hypothesis is that differentiation of fibroblasts and smooth muscle cells into osteoblasts in the aortic valve induces ectopic calcification. In cultured HAVICs, the ratio of α -SMA-positive cells may not be constant. Thus, it is very difficult to separate α -SMA-positive cells using the cell-sorting method. In future, we should investigate using VEGFR2 and/or α -SMA knockdown or overexpression systems to identify the contribution of α -SMA-positive cells to ectopic calcification.

In conclusion, HAVICs isolated from patients with AVS express CD73, CD90, CD105, and VEGFR2, but do not express CD45 and CD34. Thus, HAVICs are undifferentiated cells that can easily differentiate into osteoblastic-like cells. Although these cells are highly sensitive to calcification stimuli, α -SMA-positive cells may not contribute to aortic valve ectopic calcification. Furthermore, large numbers of VEGFR2-positive cells localized not only to the endothelium, but also to the interstitium in calcified aortic valve obtained from patients with AVS. These results suggest that HAVICs isolated from patients with AVS are derived from aortic valve endothelial cells and that differentiation of these cells may contribute to aortic valve ectopic calcification. Based on these results, it is necessary to further elucidate the mechanism of aortic valve ectopic calcification and develop new therapies to suppress AVS progression.

Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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Figure legends

Figure 1. HAVICs isolated from aortic valves of patients with AVS were VEGFR2 positive. HAVICs were seeded on gelatin-coated glass-base dishes and cultured overnight. Primary antibodies for CD markers, VEGFR2 and α -SMA were administrated and detected using

secondary antibodies with fluorescent dyes. A: Representative double immunocytochemical staining of HAVICs from patients with AVS with each marker (CD90, CD73, CD45, CD34, VEGFR2, and α -SMA). Left panels indicate various positive markers with nuclear staining, and middle panels indicate CD105-positive cells with nuclear staining. Right panels indicate merged figures, which are overlays of the two panels. Cell nuclei were stained blue with Hoechst 33342. Almost all HAVICs were CD73, CD90, and CD105 positive, and CD34 and CD45 negative. HAVICs were VEGFR2 positive. Some cells were α -SMA positive, while others were α -SMA negative. B: The expression ratio of each positive cell to its nucleus was calculated. Each bar indicates the mean \pm SEM ($n = 4$). *: $p < 0.001$ compared with VEGFR2-positive cells.

Figure 2. VEGFR2-positive cells were abundantly present in aortic valve specimens. Aortic valve sections were incubated with various primary antibodies for markers and detected using secondary antibodies with fluorescent dyes. A: Typical double immunohistochemical staining of VEGFR2 (top images) and α -SMA (middle and bottom images) in calcified aortic valve specimens from patients with AVS. The left panels indicate various positive markers with nuclear staining, and the middle panels indicate CD105-positive cells with nuclear staining. The right panels indicate merged figures, which are overlays of the two panels. Cell nuclei were stained blue with Hoechst 33342. Aortic valve sections include α -SMA-negative area (middle images) and α -SMA-positive area (bottom images). B: The expression ratio of each positive cell to its nucleus was calculated. Each bar indicates the mean \pm SEM ($n = 8$). *: $p < 0.001$ compared with VEGFR2-positive cells.

Figure 3. HAVICs differentiate into osteoblastic cells. To confirm the differentiative properties of HAVICs as mesenchymal stem cells, HAVICs (1×10^5 cells) were cultured for 14 or 21 days. All panels indicate typical data. A: Colony formation of HAVICs isolated from patients with

AVS was confirmed after 14 days of culture. B: Differentiation into osteoblastic-like cells was confirmed after 21 days of culture in the presence of glycerophosphate (10 mM) and dexamethasone (1 nM). The left panel shows the non-treatment group, and the right panel shows the ossification induction group. C: Quantification of Alizarin Red S staining at day 21 via extraction with cetylpyridinium chloride. Released dye was quantified by spectrophotometry at 550 nm. Each staining ratio was calculated relative to non-treatment on day 21. Each bar indicates the mean \pm SEM (n = 3). *: p < 0.05 compared with non-treatment. D, E: Differentiation into chondrocytes (D) and adipocytes (E) was not identified. The left panel shows the non-treatment group, and the right panel shows the chondrocyte and adipocyte induction groups.

Figure 4. All α -SMA-positive cells were positively stained for VEGFR2. HAVICs were seeded on gelatin-coated glass-base dishes and cultured overnight. Primary antibodies for VEGFR2 and α -SMA were administrated and detected using secondary antibodies with fluorescent dyes. Double immunocytochemical staining of two distinct HAVICs from patients with AVS was performed using VEGFR2 and α -SMA. Representative data was shown. The left panels indicate α -SMA-positive cells with nuclear staining, and the middle panels indicate VEGFR2-positive cells with nuclear staining. The right panels indicate merged figures, which are overlays of the two panels. Cell nuclei were stained blue with Hoechst 33342. VEGFR2-positive HAVICs were sometimes α -SMA positive and sometimes α -SMA negative. Similar results were confirmed using two samples.

Figure 5. VEGFR2-positive cells were easily calcified. After reaching confluent, VEGFR2-positive cells were further cultured in the presence of TNF- α (30 ng/mL) for up to 6 hours (gelatin-coated glass-base dishes) or 14 days (24-well plates). A: After 6 hours of culture,

primary antibodies for VEGFR2 and phosphorylated Smad1/5/8 (P-Smad) were administrated and detected using secondary antibodies with fluorescent dyes. Representative double immunocytochemical staining of VEGFR2 and P-Smad in HAVICs from patients with AVS was shown. The left panels indicate VEGFR2-positive cells, and the middle panels indicate P-Smad-positive cells. The right panels indicate merged figures, which are overlays of the two panels with nuclear staining. Cell nuclei were stained blue with Hoechst 33342. Each panel indicated as an upper panel, non-treatment; second panels from the top, TNF- α ; third panels from the top, TNF- α + dorsomorphin (Dorso); lower panel, dorsomorphin. In VEGFR2-positive HAVICs, TNF- α accelerated nuclear translocation of P-Smad, which was inhibited in the presence of dorsomorphin. B: The ratio of each P-Smad nuclear translocated cell per nucleus was calculated. Each bar indicates the mean \pm SEM ($n = 4$). *: $p < 0.001$ compared with non-treatment. **: $p < 0.001$ and ***: $p < 0.001$ compared with TNF- α administration.

Figure 6. TNF- α induced the ectopic calcification of VEGFR2-positive HAVICs via BMP2-ALP pathway. A: Representative Alizarin Red S staining of cultured cells at day 14. VEGFR2-positive cells were calcified in the presence of TNF- α , and calcification was completely inhibited in the presence of dorsomorphin. Similar results were confirmed using two samples. B: Quantification of Alizarin Red S staining at day 14 via extraction with cetylpyridinium chloride. Released dye was quantified by spectrophotometry at 550 nm. Each staining ratio was calculated relative to non-treatment on day 14. Each bar indicates the mean \pm SEM ($n = 3$). *: $p < 0.05$ compared with non-treatment. **: $p < 0.01$ compared with TNF- α administration. C: Measurement of ALP activity in AVS-patient HAVICs following 14 days of culture in the presence or absence of TNF- α . All ratios were calculated versus the non-treatment. White bar: non-treatment (NT); red bar: TNF- α . Each bar indicates the mean \pm SEM ($n = 4$). *: $p < 0.05$ compared with non-treatment. D: Time course of each BMP2 and NF- κ B gene expression in

the presence of TNF- α . Gene expression was measured at 3 and 8 hours after TNF- α administration. All ratios were calculated versus the control group on 0 hour. Relative gene-expression levels were determined by normalizing measured values to those obtained for the housekeeping gene G3PDH. Each left and right axis indicated the fold degree of BMP2 and NF- κ B gene expression, respectively. Red symbols: BMP2; white symbol: NF- κ B. Each symbol indicates the mean \pm SEM ($n = 4$). *: $p < 0.05$ compared with 0 hour. #: $p < 0.01$ compared with 0 hour. E: Western blot analysis of released BMP2 on 2 hours in HAVICs isolated from patients with AVS. Lane 1: non-treatment (NT); lane 2: TNF- α (30 ng/mL); lane 3: TNF- α + SN-50 (10 μ M); lane 4: SN-50. Each bar indicates the mean \pm SEM ($n = 5$). *: $p < 0.05$ compared with non-treatment. #: $p < 0.01$ compared with TNF- α . F: Western blot analysis of phospho-NF- κ B P65 (Phospho-p65, upper images) and NF- κ B P65 (p65, lower images) on 2 hours in HAVICs isolated from patients with AVS. Band intensity of Phospho-p65 was normalized relative to p65 levels. Lane 1: non-treatment (NT); lane 2: TNF- α (30 ng/mL). Each bar indicates the mean \pm SEM ($n = 4$). *: $p < 0.05$ compared with non-treatment.

Figure 7. Expression of α -SMA does not accelerate TNF- α -induced calcification of VEGFR2-positive cells. After reaching confluence, VEGFR2-positive cells were further cultured in the presence of TNF- α (30 ng/mL) for up to 6 hours (gelatin-coated glass-base dishes). A: After 6 hours of culture, primary antibodies for α -SMA and phosphorylated Smad1/5/8 (P-Smad) were administrated and detected using secondary antibodies with fluorescent dyes. Double immunocytochemical staining of HAVICs from patients with AVS was performed with α -SMA and P-Smad. Representative data was shown. The left panels indicate α -SMA-positive cells, and the middle panels indicate P-Smad-positive cells. The right panels indicate merged figures, which are overlays of the two panels with nuclear staining. Cell nuclei were stained blue with Hoechst 33342. Each panel indicated as an upper panel, non-treatment; middle panels, TNF- α ;

lower panels, TNF- α + dorsomorphin (Dorso). In VEGFR2-positive HAVICs, TNF- α accelerated nuclear translocation of P-Smad, which was inhibited in the presence of dorsomorphin. B: The ratio of P-Smad nuclear translocation per nucleus in each α -SMA-positive and negative cell was calculated. Each bar indicates the mean \pm SEM (n = 4). *: $p < 0.001$ compared with non-treatment in α -SMA-negative cells. **: $p < 0.001$ compared with TNF- α administration in α -SMA-negative cells. #: $p < 0.001$ compared with non-treatment in α -SMA-positive cells. ##: $p < 0.001$ compared with TNF- α administration in α -SMA-positive cells. There was no significant difference in Smad1/5/8 phosphorylation between α -SMA-positive and negative cells.

Table 1.
List of antibodies used for immunofluorescence studies

Primary antibodies	Supplier	species	type	dilution	references
CD34	Invitrogen	mouse	monoclonal	1/1000	MA1-19229
CD45	Invitrogen	mouse	monoclonal	1/1000	14-9457-82
CD73	Invitrogen	mouse	monoclonal	1/1000	41-0200
CD90	Biolegend	mouse	monoclonal	1/1000	328102
CD105	Invitrogen	rabbit	polyclonal	1/1000	PA5-80582
α -SMA	Nkmax Bio	mouse	monoclonal	1/500	ATGA0358
VEGFR2	Novus	mouse	monoclonal	1/1000	NBP2-36429
VEGFR2	Novus	rabbit	polyclonal	1/1000	NB100-530
Phospho-Smad1/5/8 (9)	Abcam	rabbit	polyclonal	1/1000	ab66737

Secondary antibodies	Supplier	species	type	dilution	references
Alexa Fluor 488 goat anti-rabbit IgG (H+L)	Invitrogen	rabbit	polyclonal	1/1000	A11008
Alexa Fluor 555 goat anti-mouse IgG (H+L)	Invitrogen	mouse	polyclonal	1/1000	A32727

Table 2 Primers used for quantitative real-time PCR

Gene	GenBank	Sequences (5'–3')
	Accession No.	
<i>BMP2</i>	NM_001200	Forward: cggactgcggtctcctaa
		Reverse: ggaagcagcaacgctagaag
<i>NF-κB</i>	NM_003998	Forward: ggatcacagctgctttctgttg
		Reverse: tggcgaccgtgataaccttaa
<i>G3PDH</i>	NM_002046	Forward: tgcaccaccaactgcttagc
		Reverse: ggcatggactgtggcatgag

Fig 1

A

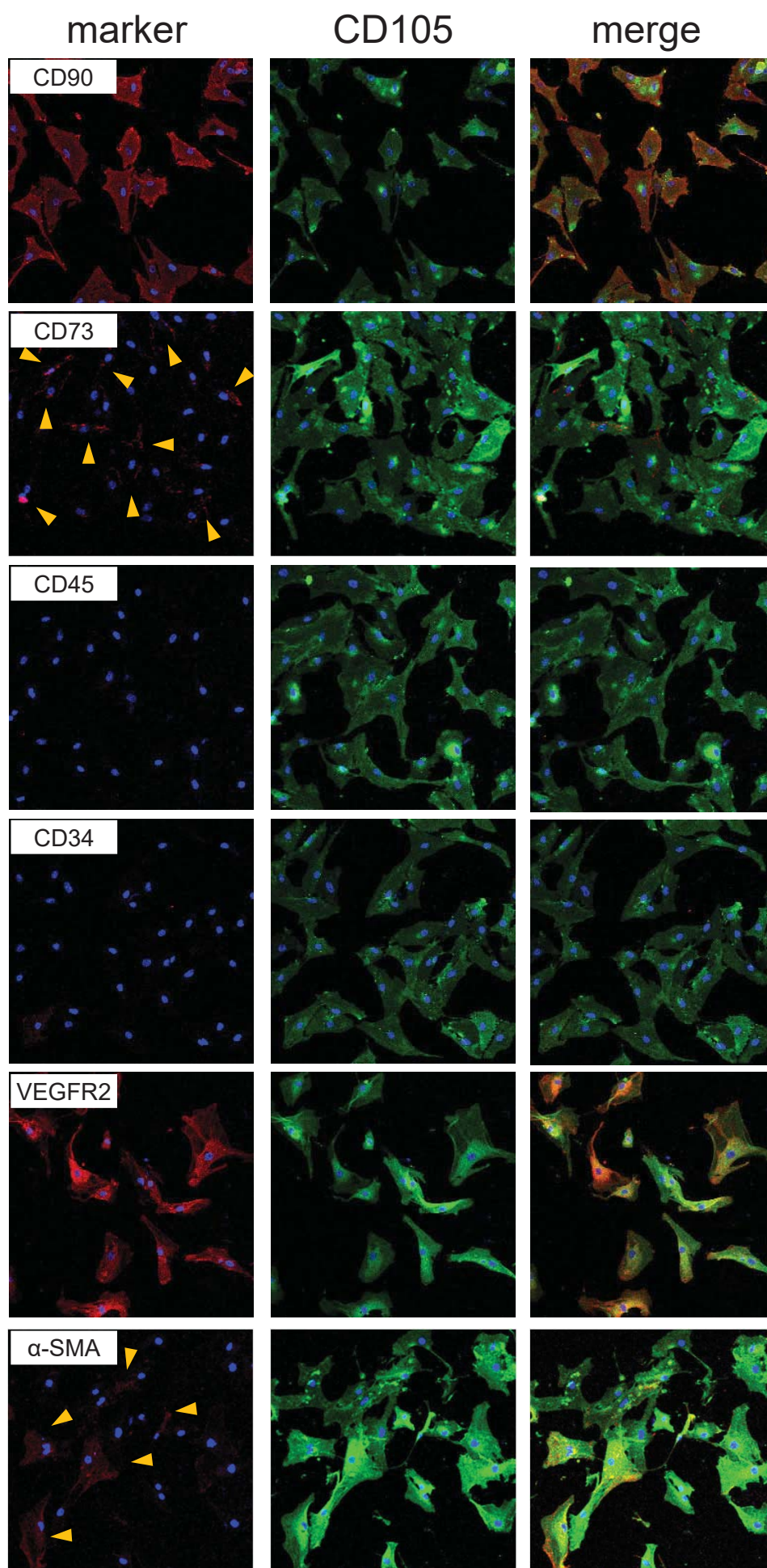


Fig 1

B

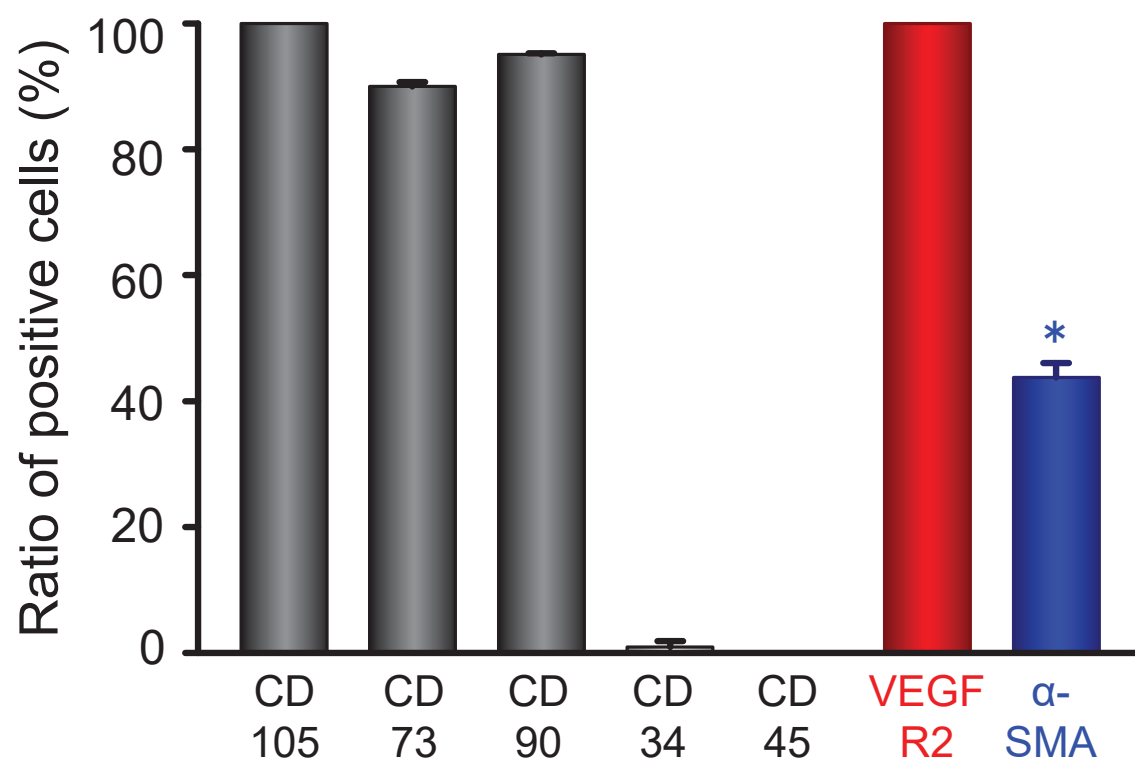
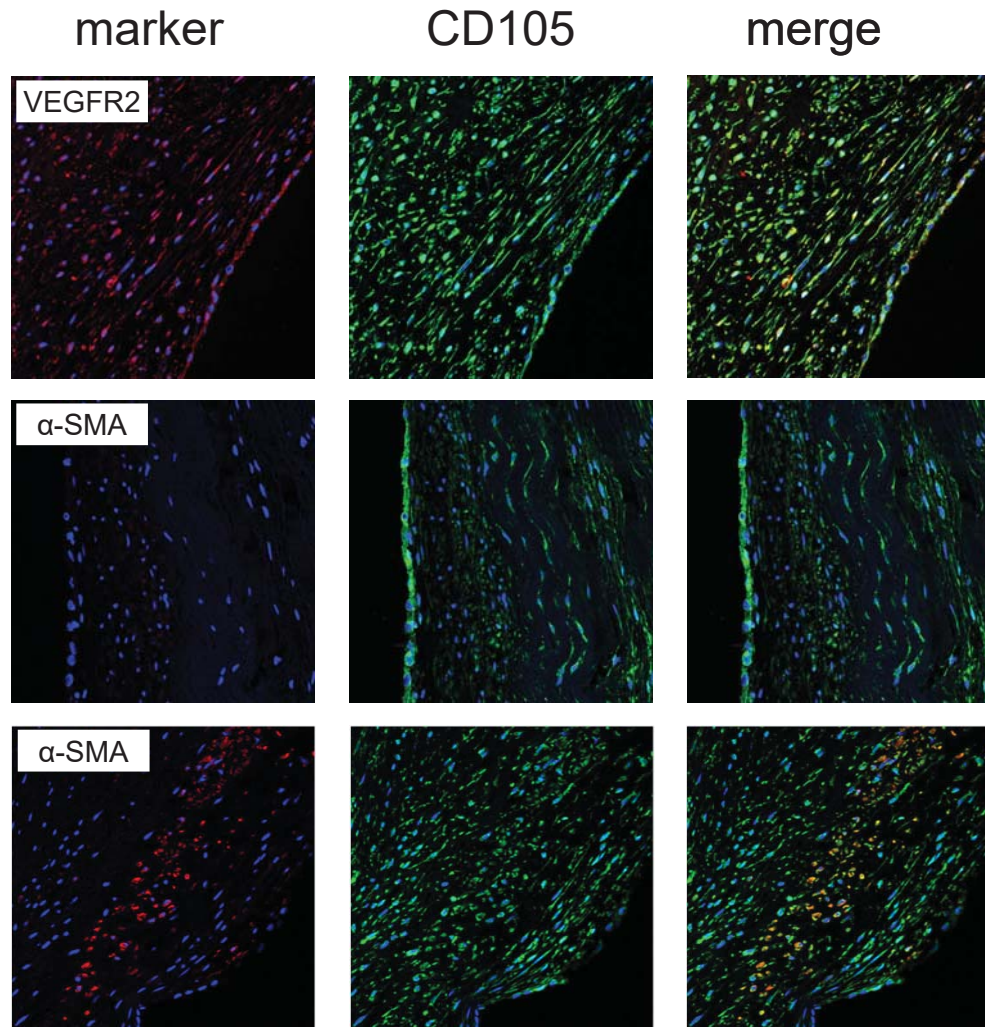


Fig 2

A



B

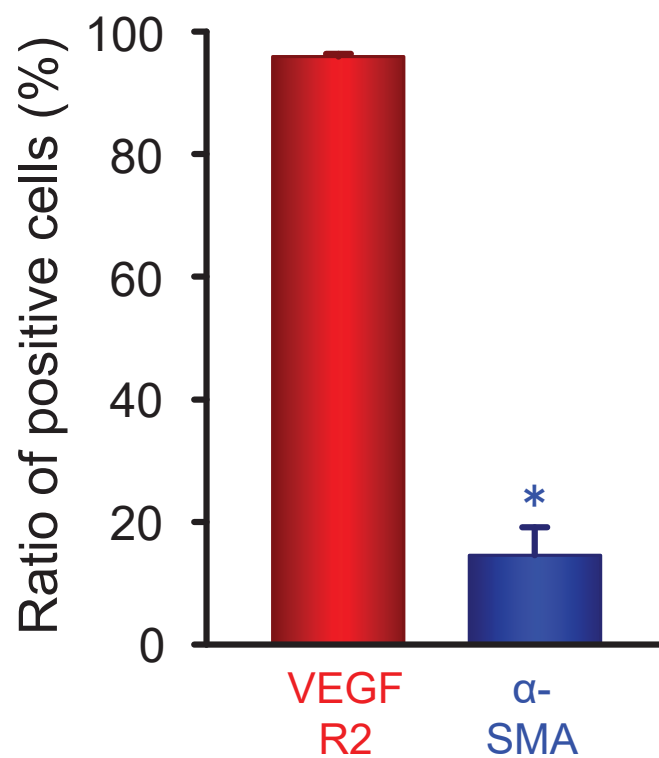


Fig 3

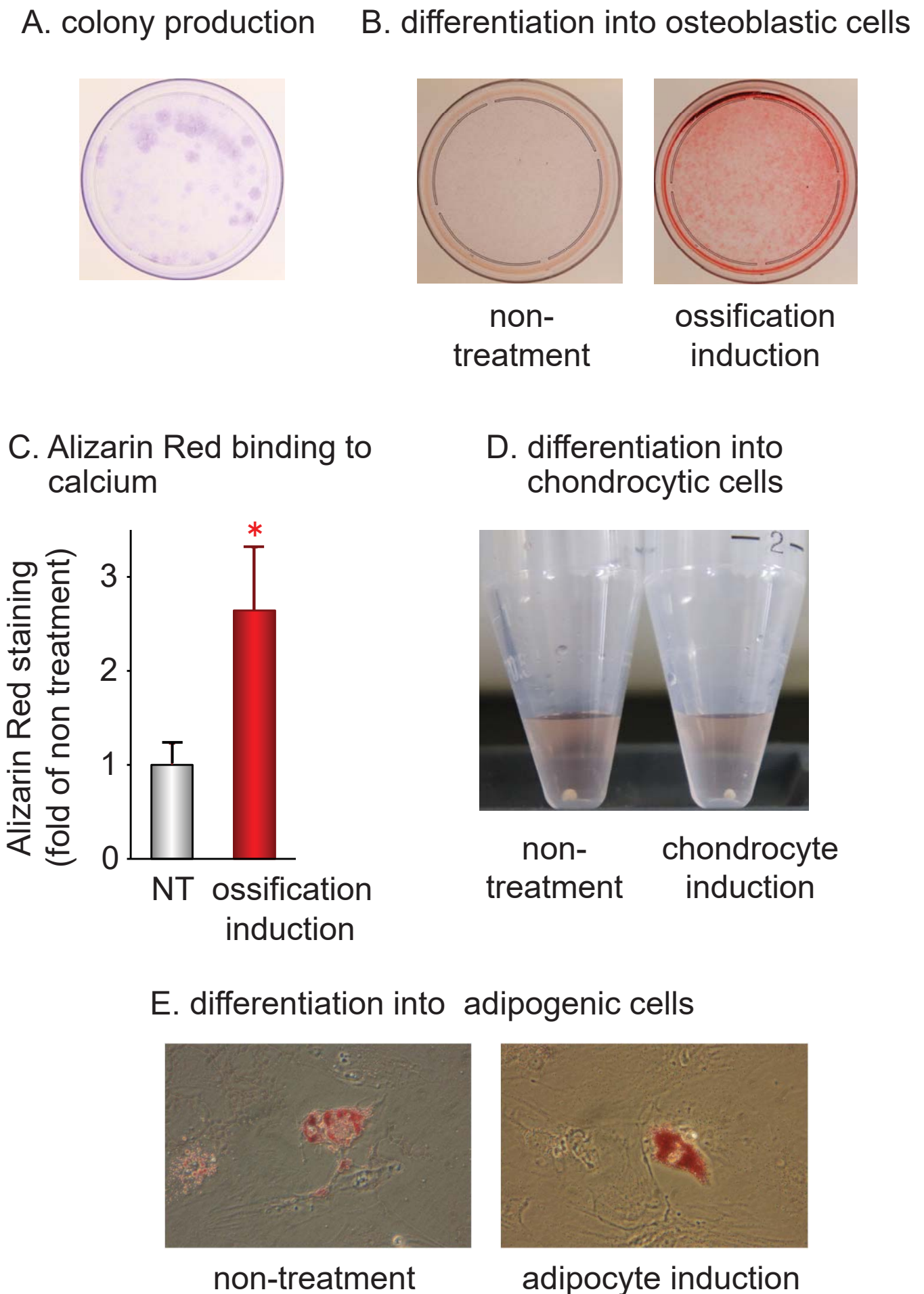


Fig 4

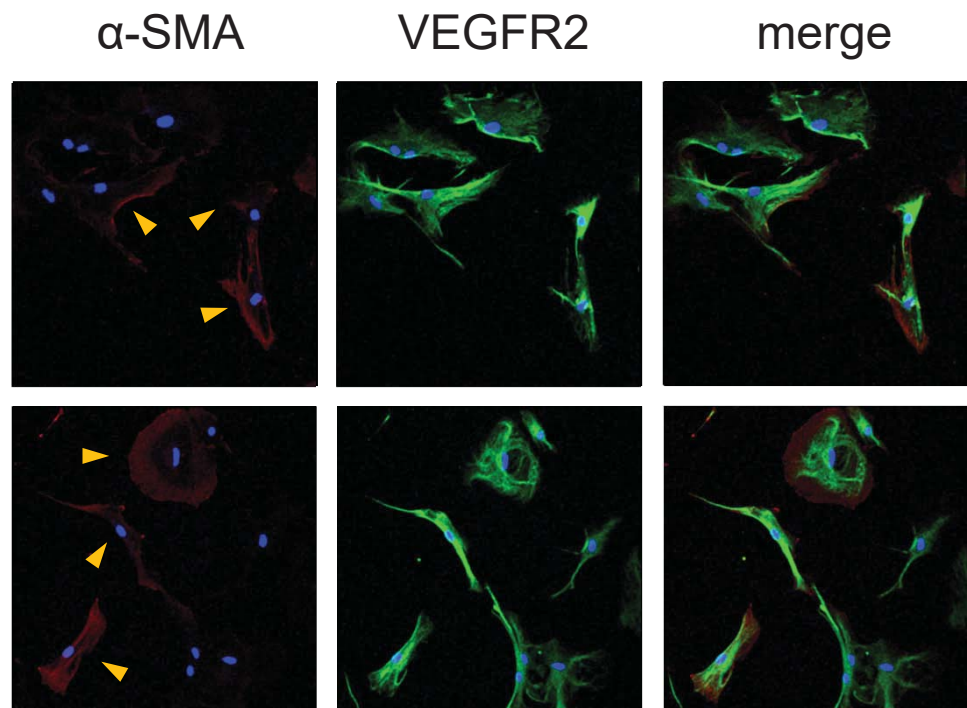


Fig 5

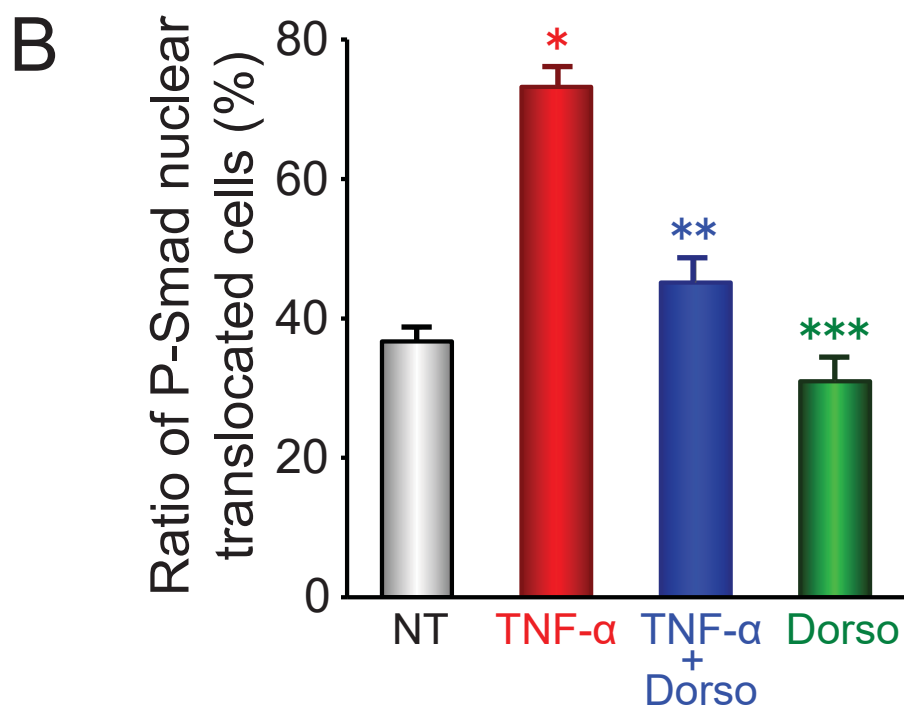
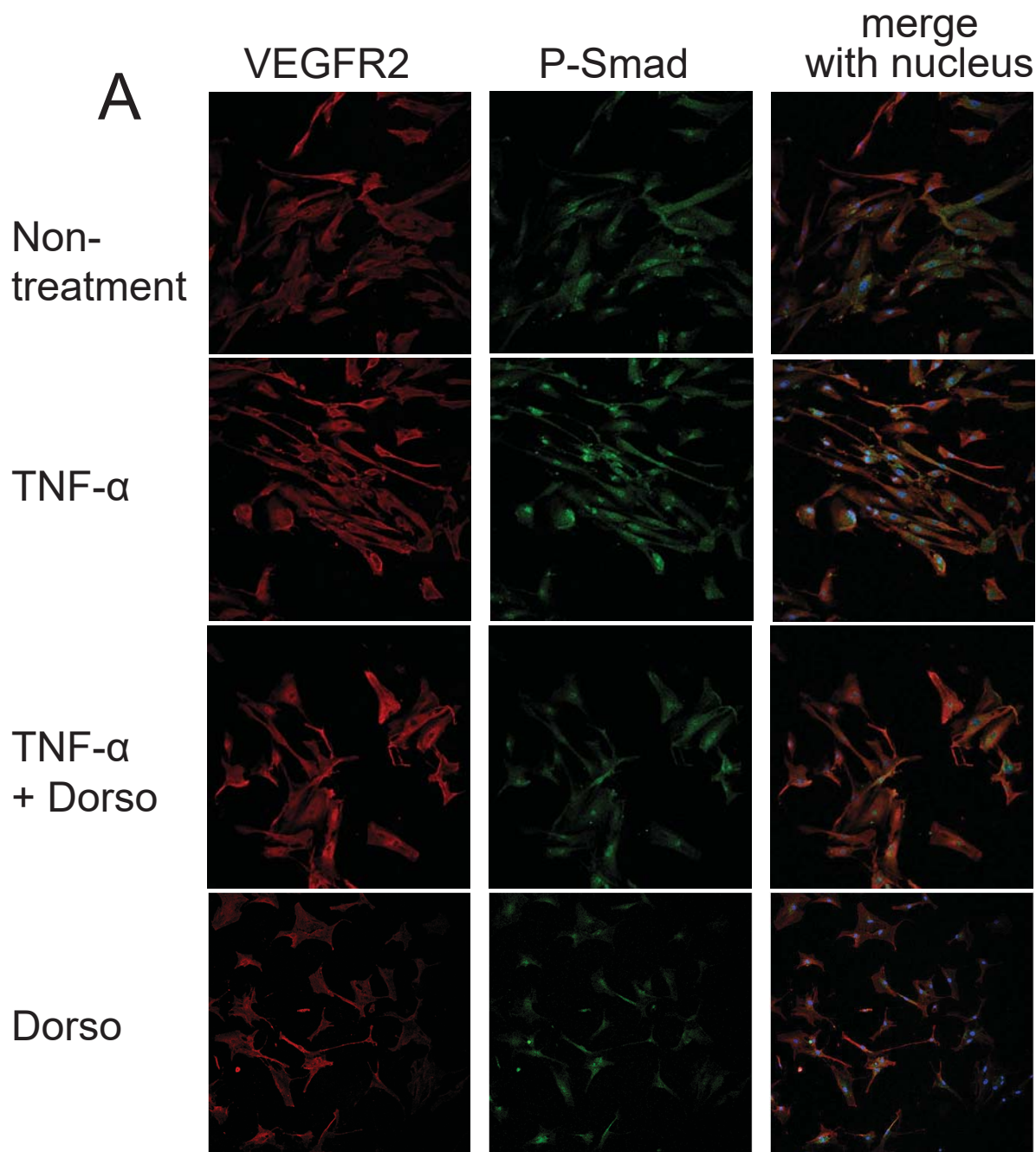
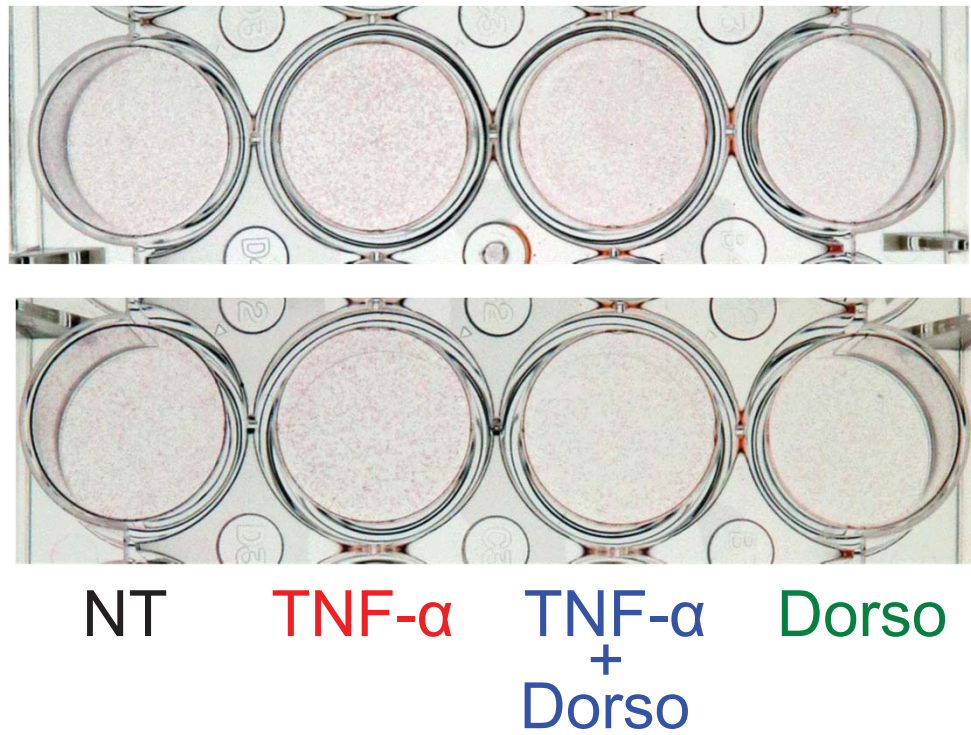


Fig 6

A



B

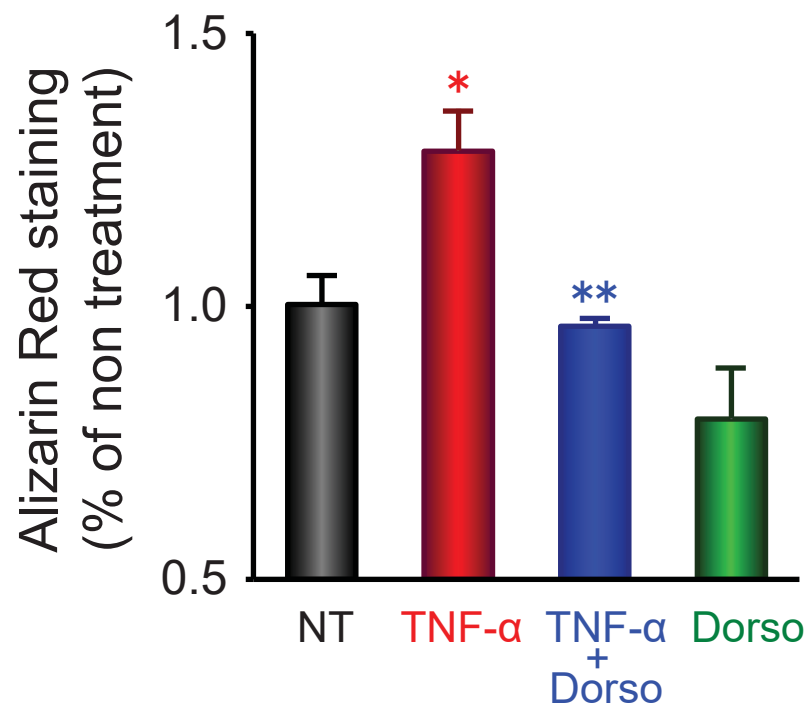


Fig 6

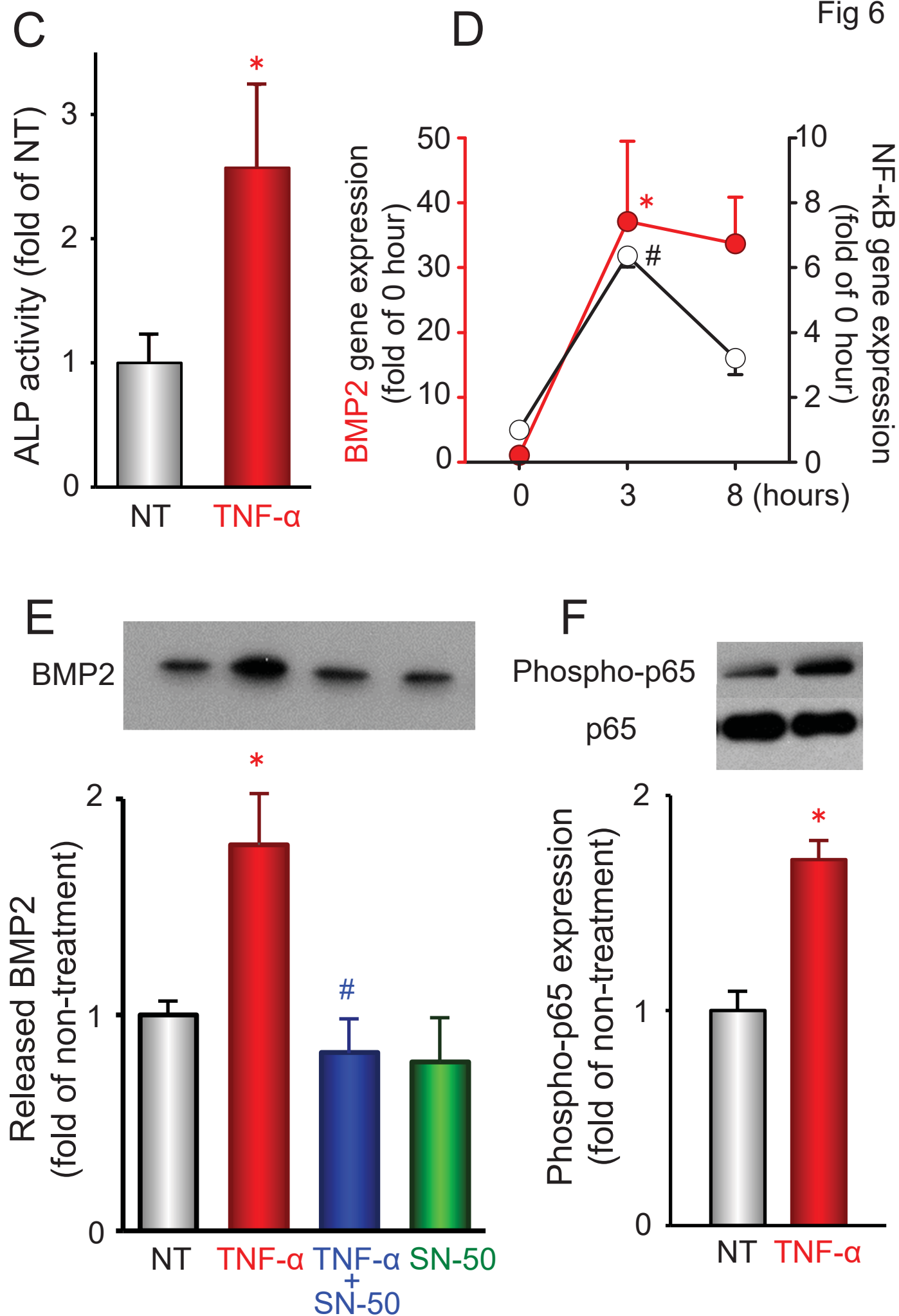


Fig 7

