Hirosaki Med. J. 73 : 20—29, 2023

ORIGINAL ARTICLE

Wnt5a pathway was not involved in the progression of valve calcification in calcific aortic valve stenosis

Zaiqiang Yu[1)], Kazuyuki Daitoku[1)], Ikuo Fukuda[1)], Tadaatsu Imaizumi[2)], Masahito Minakawa[1)], Ken-Ichi Furukawa[3)], and Kazuhiko Seya[2)]

Abstract

Objective: Wingless/integrase 5a (Wnt5a) pathway is known to regulate the osteogenesis. In this study, we aimed to clarify the role of Wnt5a pathway in aortic valve ectopic calcification.

Methods: Human aortic valve interstitial cells (HAVICs) were obtained from calcified aortic valves of patients with calcific aortic valve stenosis (CAVS). HAVICs were separated to CD34-negative and -positive cells by flow cytometry. We measured real-time PCR, alkaline phosphatase (ALP) activity, Alizarin Red S staining as an index of calcification. Immunohistochemical staining was performed to confirm the distribution of Wnt5a on calcified and normal aortic valves.

Results: HAVICs, especially, CD34-negative cells are highly sensitive to tumor necrosis factor-α (TNF-α, 30 ng/mL), and which accelerated the Wnt5a gene expression. Wnt5a antagonist, box5, did not down-regulate HAVIC calcification induced by TNF-α. Further, Wnt5a agonist, foxy5, did not accelerated HAVIC calcification induced by TNF-α. There was no significant difference in the TNF-α-induced acceleration of ALP activity between the cells treated with foxy5 and box5. Furthermore, the proportion of Wnt5a positive cells was no difference between calcified and normal valves.

Conclusions: We confirmed that Wnt5a pathway does not regulate the TNF-α-induced aortic valve calcification in HAVICs obtained from CAVS patients.

Hirosaki Med. J. 73 : 20—29, 2023

Key words: Calcific aortic valve stenosis; Aortic valve calcification; Wingless/integrase 5A; Bone morphogenetic protein 2.

1. Introduction

In the aging section of the society, the incidence of calcific aortic valve stenosis (CAVS) is gradually increasing, and it is accelerated by ectopic aortic valve calcification (AVC). The most readily available treatment for CAVS is valve replacement via open surgery; however, it is an extremely invasive procedure[1)]. Incidentally, in Japan, a less invasive, transcatheter aortic valve implantation (TAVI) has been performed for high-risk patients with heart disease and patients aged ≥80 years[2)]. Nevertheless, in the future, if the durability issues of prosthetic valves owing to paravalvular leaks or damage caused by calcification can be overcome, then TAVI may be performed for young patients with CAVS or a low-risk. Therefore, it is essential to rapidly develop an effective but non-invasive medical treatment for inhibiting the acceleration of AVC, and to implement this, the detailed mechanism of AVC needs to be elucidated.

Previously, we elucidated the role of tumor necrosis factor (TNF)-α, an inflammatory

[1)] Department of Thoracic and Cardiovascular Surgery, Hirosaki University Graduate School of Medicine, Hirosaki, Japan
[2)] Department of Vascular Biology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan
[3)] Department of Pharmacology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan

Correspondence: Z. Yu, MD, and K. Seya
Received for publication, August 30, 2022
Accepted for publication, October 19, 2022

✉ : Zaiqiang Yu Department of Thoracic and Cardiovascular Surgery, Hirosaki University Graduate School of Medicine
E-mail: yuzaiqiang@hirosaki-u.ac.jp
✉ : Kazuhiko Seya Department of Vascular Biology, Hirosaki University Graduate School of Medicine
E-mail: seya@hirosaki-u.ac.jp

cytokine, in accelerating alkaline phosphatase (ALP) activity and the progression of AVC via the bone morphogenic protein (BMP)2 pathway[3]. Incidentally, high phosphate levels (high-Pi) also accelerate AVC. Moreover, AVC is an actively regulated process associated with an osteoblast phenotype involving BMP2 and the matrix Gla protein (MGP), both of which may be keys for controlling AVC via development of novel medical therapies in the future[4]. Reportedly, a wingless/integrase 1 (*Wnt*) gene (*Wnt5a*) encodes a 38–43-kDa cysteine-rich putative glycoprotein. Wnt5a is a prototypical WNT of the β-catenin-independent branch and is intensely related to osteogenesis, but the role of the Wnt5a pathway in AVC remains unclear. Hence, we aimed to clarify the relationship of the Wnt5a pathway with AVC and attempted to establish a new target for the development of novel medical therapies that will inhibit the acceleration of AVC.

2. Methods

2.1 Declaration

The study was approved by the Institutional Review Board of Hirosaki University Hospital and performed according to the Declaration of Helsinki. Written informed consent was obtained from all patients with CAVS enrolled in the study.

2.2 Materials

Commercial sources of reagents: α-modified Eagle's medium (α-MEM), Nacalai Tesque (Kyoto, Japan); fetal bovine serum (FBS), Biofluids™ (Rockville, MD, USA); penicillin and streptomycin, Gibco™ (Gaithersburg, MD, USA); collagenase type V, Sigma-Aldrich (St. Louis, MO, USA); foxy5 and box5, Wako (Tokyo, Japan); and Power SYBR® Green PCR Master Mix, TOYOBO (Osaka, Japan). All primers used in quantitative real-time polymerase chain reaction (qPCR) were obtained from Fasmac (Kanagawa, Japan).

2.3 Isolation and culture of human aortic valve interstitial cells (HAVICs)

In this study, we used human aortic valves obtained from four patients with CAVS who had undergone surgical aortic valve replacement at Hirosaki University Hospital (Aomori, Japan). The mean age of the patients (3 males aged 78, 68, and 51 years and 1 female aged 79 years) was 68.3 ± 5.8 years.

First, we gently cut the specimens into small pieces (2 ± 1 mm) and washed them twice with 1% phosphate buffered saline (PBS). Subsequently, human aortic valve interstitial cells (HAVICs) were isolated from these specimens by incubating them with collagenase type V (1 mg/mL) for 2 h at 37° C with 95% O_2 and 5% CO_2. The HAVICs were cultured in α-MEM consisting of 10% FBS, 100 U/mL sodium penicillin G, and 100 μg/mL streptomycin in 12-well plates for 3 days, that is, until they reached 80%–90% confluence. Thereafter, they were further cultured in the presence or absence of TNF-α (30 ng/mL) for 7–14 days[3, 5]. Previously, we found that HAVICs isolated form patients with CAVS were similar to mesenchymal stem cells, including ossification differentiation potential and colony formation ability[6]. CD34-positive and -negative cells were isolated from HAVICs using flow cytometry according to cellular maker CD34, as previously reported by Nomura et al[7].

2.4 Identification of aortic valve calcification

The degree of calcification of the HAVICs was measured by Alizarin Red S staining. We used a microscope equipped with a digital camera (Canon, Tokyo, Japan) to calculate the number of stained cells. The amount of stain released from the extracellular matrix after incubation in 100 mM aqueous cetylpyridinium chloride solution was quantified using a spectrophotometer by

measuring the absorbance at 550 nm[8].

2.5 ALP activity assay

Cultured HAVICs were lysed on days 0 and 14 using 100 μL of cell-lysis buffer containing 0.05% Triton X-100. Thereafter, ALP activity was measured using theLabAssay ALP Kit from Wako Pure Chemicals (Osaka, Japan), according to the manufacturer's protocol.

2.6 Measurement of gene expression

The total RNA was isolated from HAVICs using QuickGene RNA Cultured Cell kit S (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions. An aliquot of the total RNA was subjected to reverse transcription using random primers. For real-time PCR analysis, the cDNA was amplified on a CFS^{TM} real-time system (Bio-Rad, Hercules, CA, USA) using the following protocol: initial denaturation at 95°C for 1 min, followed by 40 cycles of amplification at 95°C for 15 s and at 60 °C for 1 min. The 20-μL reaction mixture comprised 3 μL of the first-strand reaction product (1:4 dilution), 0.3 μL each of 10 μM forward and reverse primers, 2.9 μL of pure water, and 5 μL of SYBR qPCR reagent. The primers used for the amplification of *Wnt5a*, *BMP2*, and glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) genes (Table 1) were designed using the National Center for Biotechnology Information (NCBI) Primer BLAST (Bethesda, MD, USA). A housekeeping gene *G3PDH* was used as the normalization standard. qPCR data are presented as cycle threshold (Ct) levels; they were normalized using individual *G3PDH* control Ct values. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method[9].

2.7 Effects of foxy5 and box5 on TNF-α accelerated the calcification of HAVICs

After culturing the HAVICs in 24-well plates for 3 days until they reached 80%–90% confluence, the cells were treated with a Wnt5a pathway agonist foxy5 (50 μM) or a Wnt5a pathway antagonist box5 (100 μM) for 3 h. Subsequently, the cells were treated with TNF-α (30 ng/mL) for 14 days. On day 7, we used real-time PCR to determine the change in gene expression. Additionally, on day 14, alterations in the calcification of HAVICs and their ALP activity were examined.

2.8 Immunohistochemical staining of aortic valve specimens

Each valve tissue was fixed with paraformaldehyde, embedded in paraffin, and cut into thin sections of 4-μm thickness. The tissue sections were treated with xylene followed by ethanol for deparaffinization, the endogenous peroxidases were blocked using 0.3% hydrogen peroxide (H_2O_2) in methanol at room temperature for 10 min, and finally, the sections were washed with PBS. Subsequently, the sections were blocked with normal 1% bovine serum albumin at room temperature for 30 min and were incubated with primary antibodies (Table 2). Thereafter, the sections were incubated with a secondary antibody and tertiary agents of a streptavidin biotin-peroxidase detection kit (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan) following the manufacturer's protocol, and lightly counterstained with hematoxylin. Hoechst 33342 was used as a fluorescent nuclear stain. For each tissue section, we obtained images of three separate visual fields using a 40× objective. To obtain the abundance ratio of each type of marker-positive cells, the number of positive cells was divided by the number of nuclei and expressed as a percentage.

2.9 Statistical analysis

All statistical analyses were performed using KyPlot 5.0 software (Kyenslab, Tokyo, Japan). Group comparisons were performed using one-way analysis of variance (ANOVA) along with

Student–Newman–Keuls post hoc correction procedure. Comparisons between independent datasets were made using Student's *t*-test. The results of all replicates are presented as mean ± standard error of mean (SEM), and differences with $P < 0.05$ were considered to be statistically significant.

3. Results

3.1 TNF-α induces the calcification of human aortic valve interstitial cells

After culturing the human aortic valve interstitial cells (HAVICs) isolated from patients with CAVS until 80%–90% confluence, they were cultured in a medium containing TNF-α (30 ng/mL) for 14 days. Thereafter, Alizarin Red S staining of the cells revealed that TNF-α significantly accelerates the calcification of HAVICs at 14 days (Figure 1A).

We also measured the change in *Wnt5a* gene expression with the calcification of HAVICs accelerated by TNF-α. When the HAVICs were 80%–90% confluent, they were further cultured for 8 hours, with and without TNF-α (30 ng/mL) in the culture medium. The *Wnt5a* gene expression was upregulated upon TNF-α treatment (Figure 1B). These findings indicate that *Wnt5a* has a strong relationship with TNF-α-induced calcification of HAVICs from calcific aortic valves.

3.2 CD34-negative cells had higher sensitive to TNF-α

The HAVICs obtained from patients with CAVS are CD45-negative and CD73/90/105-positive with ossification differentiation ability that is similar to that of mesenchymal stem cells. Upon further sorting of these cells using flow cytometry, according to the cellular marker CD34, we discovered that the CD34-negative cells were more sensitive to TNF-α than the CD34-positive cells at 14 days, but weak results

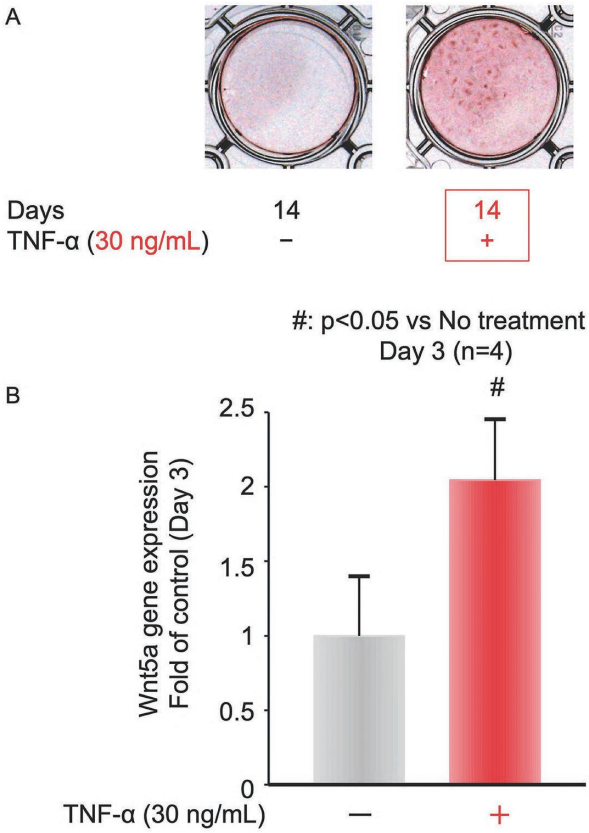

Fig. 1 Tumor necrosis factor-α (TNF-α)-induced calcification of human aortic valve interstitial cells (HAVICs) isolated from patients with aortic stenosis confirmed by alizarin Red S staining. Wingless/integrase 5A (*Wnt5a*) gene expression was accelerated by TNF-α.

A: We cultured HAVICs in α-modified Eagle's medium (α-MEM) consisting of 10% fetal bovine serum (FBS) until they reached 80%–90% confluence (day 0), after which they were further cultured in medium containing TNF-α (30 ng/mL) for 14 days (day 14). The representative Alizarin Red S staining image of HAVICs from patients with calcific aortic valve stenosis (CAVS) is displayed here.

B: Wingless/integrase 5A (*Wnt5a*) expression is accelerated by TNF-α on day 3.
Wnt5a expression in TNF-α-induced calcification of mesenchymal stem cell-like cells (MSCLCs) obtained from patients with calcific aortic valve stenosis (CAVS) was investigated on day 3. On day 3, the levels were calculated versus the level in cells that were not treated with TNF-α. Relative gene expression levels were determined by normalizing the measured values to those obtained for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*). Gray bar: non-treated cells and red bar: cells treated with TNF-α (30 ng/mL) (+). Bars present mean ± standard error of the mean (SEM; n = 4). The significant difference ($P < 0.05$) in comparison to the non-treated cells at day 3 is denoted by "#".

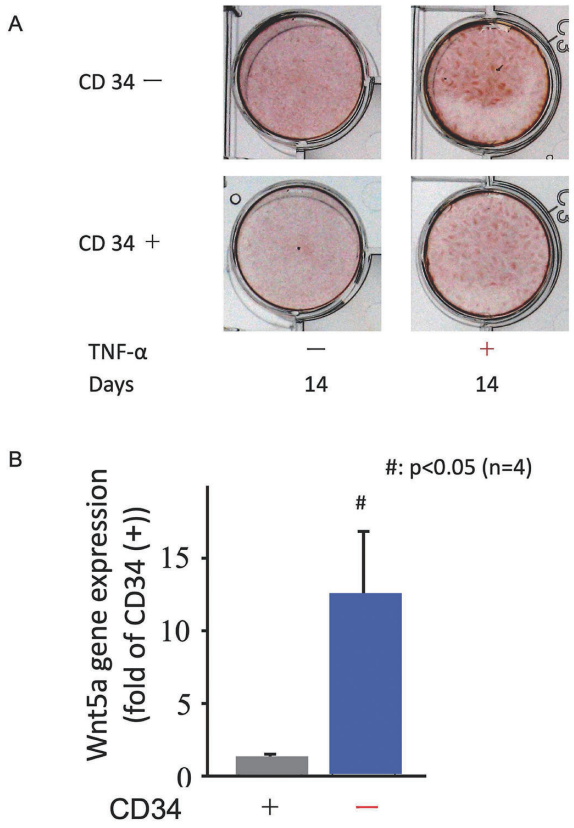

Fig. 2 Alizarin Red S staining confirmed that CD34-negative cells (MSCLCs) are more sensitive to TNF-α than the CD34-positive cells. Wingless/integrase 5A (*Wnt5a*) gene expression in CD34-negative cells is higher than that in CD34-positive cells.

A: The HAVICs were obtained from patients with calcific aortic valve stenosis (CAVS) and cultured in α-modified Eagle's medium (MEM) consisting of 10% fetal bovine serum (FBS) until they reached 80%–90% confluence (day 0). Subsequently, the HAVICs were cultured with and without TNF-α (30 ng/mL) for 14 days (day 14). Alizarin Red S staining confirmed that in patients with CAVS, CD34-negative HAVICs are more reactive to TNF-α than CD34-positive cells, but with weak reactivity.

B: Comparison of *Wnt5a* expression between CD34-negative and CD34-positive cells of HAVICs from the same patient. The CD34-negative cells presented higher levels of *Wnt5a* expression than the CD34-positive cells. Gray bar: CD34-positive cells from patients with CAVS. Blue bar: CD34-negative cells from patients with CAVS. Bars are mean ± standard error of the mean (SEM; n = 4). A significant difference ($P < 0.05$) was observed, compared with *Wnt5a* expression in CD34-positive HAVICs of patients with calcific aortic valve stenosis (CAVS), which is depicted by "#".

were obtained (Figure 2a).

The gene expression patterns of CD34-negative and CD34-positive cells isolated from the same aortic valves of a patient with CAVS revealed that the former have a higher *Wnt5a* expression than the latter (Figure 2b). These findings indicated that *Wnt5a* was intensely associated with CD34-negative cells with respecting to the calcification activity.

3.3 Foxy5 and box5 did not influence TNF-α-induced calcification of HAVICs

To clarify the role of *Wnt5a* in aortic valve calcification (AVC), we used foxy5 and box5, to observe the changes of *Wnt5a* in the TNF-α-induced calcification of HAVICs. The HAVICs were cultured in a TNF-α medium (30 ng/mL) combined with foxy5 (50 μM) or box5 (100 μM), and Alizarin Red S staining was performed after 14 days. The results indicated that the calcification of the HAVICs induced by TNF-α was not affected by either foxy5 or box5 (Figure 3A). The amount of stain released from the extracellular matrix was quantified, the calcification of the HAVICs induced by TNF-α was not affected significantly (Figure 3B).

3.4 Foxy5 and box5 did not influence ALP activity during TNF-α-induced calcification of HAVICs

As the ALP activity is elevated during TNF-α-induced calcification of HAVICs, we examined the effect of foxy5 or box5 on ALP activity at 14 days. Although TNF-α increased the ALP activity of HAVICs, there was no significant difference among the cells treated with or without foxy5 or box5 combined culture with TNF-α (Figure 4). These data showed that Wnt5a did not accelerate TNF-α-induced calcification of HAVICs.

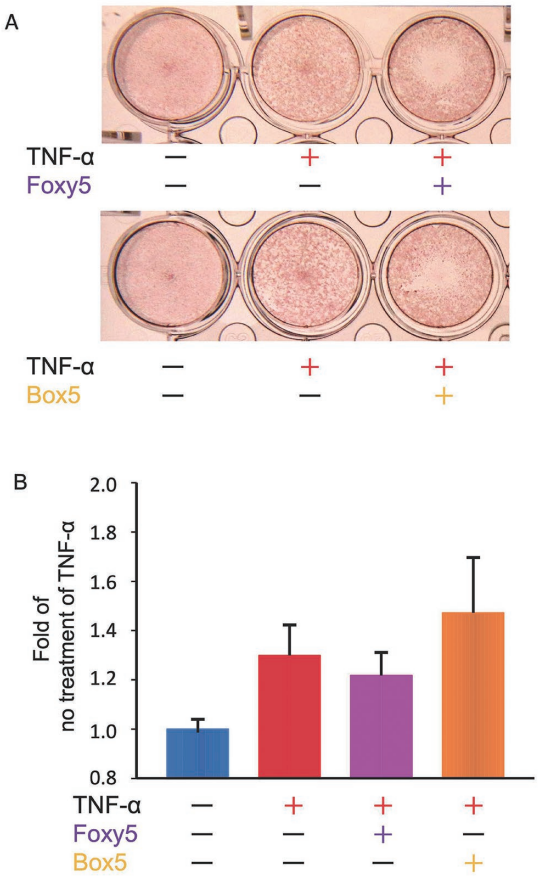

Fig. 3 TNF-α-induced calcification of HAVICs isolated from patients with aortic stenosis does not change upon co-culture with foxy5 or box5

A: We cultured MSCLCs obtained from patients with calcific aortic valve stenosis (CAVS) in α-modified Eagle's medium (MEM) + 10% fetal bovine serum (FBS) until they reached 80%–90% confluence (day 0). We further cultured these HAVICs for 14 days (day 14).
Representative Alizarin Red S staining images of HAVICs obtained from 3 patients with CAVS treated with TNF-α on day 14. The TNF-α-induced calcification of HAVICs was not influenced by foxy5 or box5, agonist and antagonist of *Wnt5a*, respectively.

B: Quantification of Alizarin Red S staining at day 14 via extraction with cetylpyridinium chloride. The amount of released dye was quantified by spectrophotometry at 550 nm. Each staining ratios was calculated relative to the day 14 non treatment group. Each bar indicates the mean ± SEM; n = 4. *: $p < 0.01$ compared with compared with TNF-α administration group.

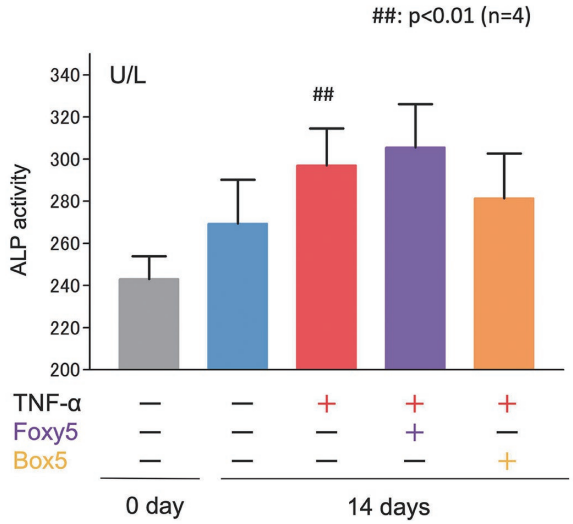

Fig. 4 ALP activity does not change significantly in TNF-α-treated HAVICs isolated from patients with calcific aortic valve stenosis (CAVS) upon culture with foxy5 or box5
Analysis of ALP activity in HAVICs isolated from patients with CAVS by culturing them in the presence or absence of foxy5 or box5, agonist and antagonist of wingless/integrase 5A, respectively, with TNF-α (30 ng/mL) for 14 days (day 14). We calculated all levels versus the level in cells that were not treated with TNF-α on day 14. Gray bar: non-treated cells on day 0; blue bar: non-treated cells on day 14; red bar: cells treated with TNF-α (30 ng/mL) only; purple bar: cells treated with TNF-α (30 ng/mL) and foxy5 (50 μM); and yellow bar: cells treated with TNF-α (30 ng/mL) and box5 (100 μM). Bars present mean ± standard error of the mean (SEM; n = 4). Compared with the non-treated cells, a significant difference ($P < 0.01$) is indicated with "##"

3.5 No difference in the proportion of Wnt5a-positive cells between normal and calcified aortic valves

If Wnt5a is strongly associated with the accelerated TNF-α-induced calcification of HAVICs isolated from patients with CAVS, the proportion of Wnt5a-positive HAVICs should be higher in the calcified valves than in the non-calcified or normal aortic valves isolated from patients with aortic regurgitation. However, an immunohistochemical comparison of the non-calcified and calcified aortic valve tissues revealed that the calcified aortic valves obtained from patients with CAVS do not exhibit an increase in the number of Wnt5a-positive HAVICs (Figure 5). These findings indicate that the proportion of Wnt5a-positive cells may not be related to TNF-α induced calcification of HAVICs.

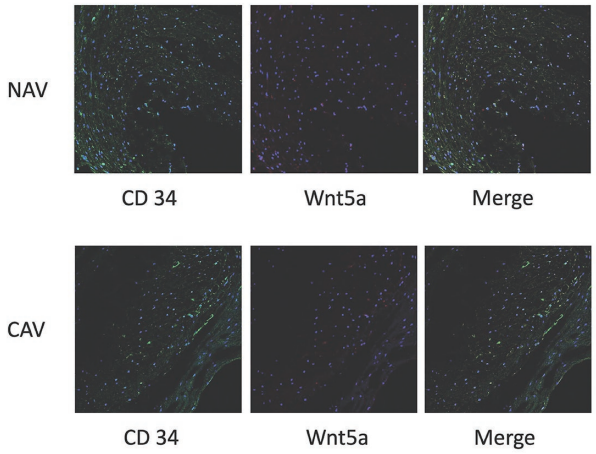

Fig. 5 Proportion of wingless/integrase 5A (Wnt5a) -positive cells does not change between normal and calcific aortic valves. The Wnt5a-positive HAVICs are abundantly present in human aortic valve specimens. There is no difference in their number between normal aortic valve (NAV) and calcified aortic valve (CAV). Sections of NAV and CAV were incubated with various primary antibodies for stem cell markers, followed by detection using secondary antibodies with fluorescent dyes. Typical double immunohistochemical staining of CAV from patients with calcific aortic valve stenosis (CAVS) and NAV from patients with aortic regurgitation was performed based on Wnt5a (Red) and CD34 (Green). Left panels indicate CD34-positive HAVICs with nuclei staining, middle panels indicate Wnt5a-positive cells with nuclei staining, and right panels indicate merged, overlaying figures of the two panels. Hoechst 33342 (blue) was used to stain the cell nuclei.

4. Discussion

We found, in the present study, that Wnt5a gene expression was increased in TNF-α-induced calcification. However, a Wnt5a agonist foxy5 did not accelerate TNF-α-induced calcification and ALP activity. In addition, Wnt5a positive cells in calcific aortic valves was not increased when compared with non-calcified aortic valves. These observations in this study suggest that Wnt5a signaling does not have deep relationship with TNF-α-induced calcification.

A previous study has reported that TNF-α accelerates aortic valve calcification by upregulating ALP activity, but is not associated with the Wnt pathway [3]. Additionally, the CD34-negative cells of HAVICs isolated from the calcified aortic valves of patients with CAVS are more sensitive to high-Pi than the CD34-positive cells [7]. However, in this study, *Wnt5a* gene expression was increased in TNF-α-induced calcification of HAVICs, CD34-negative cells of HAVICs also showed with higher gene expression of Wnt5a than positive cells. So we further investigated the role of Wnt5a in aortic valve

Table 1. Primers used for guantitative real-time PCR.

Gene symbol	GenBank Accession no.	Sequences (5`-3`)
Wnt5a	NM_001377	Forward: cctcgccatgaagaagtcca Reverse: attacaacctgggcgaagga
G3PDH	NM_002046	Forward: tgcaccaccaactgcttagc Reverse: ggcatggactgtggtcatgag

Table 2. List of antibodies for immunofluorescence studies.

Primary antibodies	Supplier	species	type	dilution	references
CD34	Invitrogen	mouse	Monoclonal	1/1000	MA1-19229
WNt5a	Invitrogen	rabbit	Monoclonal	1/1000	MA5-14946
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

calcification. Although the calcification of HAVICs was accelerated by TNF-α, it was not accelerated by foxy5, an agonist of Wnt5a activity. Furthermore, the proportion of Wnt5a-positive cells was not significantly higher in the calcified aortic valves obtained from patients with CAVS than that in the non-calcified aortic valves. Wnt signaling is vital during heart valve formation [10-12], but we could not confirm that Wnt5a signaling activity was increased in TNF-α-induced calcification of HAVICs derived from patients with CAVS.

Aortic valve calcification is an active process associated with several bone formation genes and proteins, including BMP2 and ALP [13, 14]. Interestingly, previous studies have reported that Wnt signaling is closely related to cardiac disorders, including osteogenic signaling and aortic valve disease [15, 16]. In fact, Wnt5a plays an important role during embryogenesis and various inflammatory disorders, such as rheumatoid arthritis [17], which involve the non-canonical Wnt pathway that includes Wnt-Ca^{+2} signaling without β-catenin [18]. Moreover, Wnt5a regulates vascular calcification by initiating arterial calcification [19, 20]. We presumed that Wnt5a accelerates TNF-α induced calcification. However, according to our experimental results, the TNF-α-induced aortic valve calcification and ALP activity were not significantly accelerated by foxy5, an agonist of the Wnt5a pathway. So we though that *Wnt5a* expression was high combined with the valves calcified via TNF-α-induced pathways, but not accelerated the aortic valves calcification induced by TNF-α.

As our study confirmed the presence of a large number of Wnt5a-positive cells in normal aortic valves, Wnt5a may be associated with aortic valve formation or the endothelial-to-mesenchymal transition of CD34-positive to CD34-negative cell [21]. Interestingly, the proportion of Wnt5a-positive cells did not increase in the calcified aortic valves in spite of upregulated *Wnt5a* expression in the CD34-negative HAVICs compared with that in the CD34-positive cells in the calcified aortic valves. As the CD34-negative cells are highly sensitive to various calcification stimuli [12], we believe that Wnt5a is possibly associated with the transformation of CD34-positive cells to CD34-negative cells at the beginning of calcification in the normal aortic valves, but not with the acceleration of the calcification process in the aortic valves. The limitation of this study was that we did not confirm how and when Wnt5a signaling induced the transformation of CD34 positive cells to negative cells. More clinical data of CAVS patients need to be collected for confirming our experimental outcome rightfulness.

5. Conclusions

In summary, we demonstrated that *Wnt5a* expression is enhanced in the CD34-negative cells of HAVICs isolated from calcified aortic valves, However, we did not observe significant association between acceleration of *Wnt5a gene* expression and TNF-α-induced calcification. In the future, we intend to perform further investigations regarding the role of Wnt5a in AVC for establishing the new therapeutic drugs target.

Informed consent

Written informed consent was obtained from all patients with CAVS enrolled before operation in the study.

Conflict of interest

All authors have no financial conflicts of interest to declare.

Acknowledgments

This work was supported by grants from JATS award for transitional clinical research (2018), JAPES KAKENHI (Grant numbers 17K10916, 18K16381, 19K09089, 20K09117, and 21K08813), and grants-in-aid of the Hirosaki University Educational Improvement and Promotional Aid, Hirosaki, Japan. The authors are grateful to the entire staff of the Department of Thoracic and Cardiovascular Surgery, Hirosaki University School of Medicine, for extending their support for this study.

Author Contributions

Study conception: all authors
Writing: YZ, SK
Critical review and revision: all authors
Final approval of the article: all authors
Accountability for all aspects of the work: all authors

References

1) Lindman BR, Clavel MA, Mathieu P, Iung B, Lancellotti P, Otto CM, Pibarot P. Calcific aortic stenosis. Nat Rev Dis Primers. 2016;2:16006.

2) Ando T, Takagi H, Grines CL. Transfemoral, transapical and transcatheter aortic valve implantation and surgical aortic valve replacement: a meta-analysis of direct and adjusted indirect comparisons of early and mid-term deaths. Interact Cardiovasc Thorac Surg. 2017;25:484-92.

3) Yu Z, Seya K, Daitoku K, Motomura S, Fukuda I, Furukawa K. Tumor necrosis factor-α accelerates the calcification of human aortic valve interstitial cells obtained from patients with calcific aortic valve stenosis via the BMP2-Dlx5 pathway. J Pharmacol Exp Ther. 2011;337:16-23.

4) Chiyoya M, Seya K, Yu Z, Daitoku K, Motomura S, Imaizumi T, Fukuda I, et al. Matrix Gla protein negatively regulates calcification of human aortic valve interstitial cells isolated from calcified aortic valves. J Pharmacol Sci. 2018;136:257-65.

5) Seya K, Furukawa K-I, Chiyoya M, Yu Z, Kikuchi H, Daitoku K, Motomura S, et al. 1-Methyl-2-undecyl-4(1H)-quinidone, a derivative of quinolone alkaloid evocarpine, attenuates high phosphate-induced calcification of human aortic valve interstitial cells by inhibiting phosphate cotransporter PiT-1. J Pharmacol Sci. 2016;131:51-7.

6) Liu X, Yu Z, Daitoku K, Fukuda I, Motomura S, Matsumiya T, Imaizumi T, et al. 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. J Pharmacol Sci. 2021;145:213-21.

7) Nomura A, Seya K, Yu Z, Daitoku K, Motomura S, Murakami M, Fukuda I, et al. CD34-negative mesenchymal stem-like cells may act as the cellular origin of human aortic valve calcification. Biochem Biophys Res Commun. 2013;440:780-5.

8) Stanford CM, Jacobson PA, Eanes ED, Lembke LA, Midura RJ. Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106-01 BSP). J Biol Chem. 1995;270:9420-8.

9) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25:402-8.

10) Woldt E, Terrand J, Mlih M, Matz RL, Bruban V, Coudane F, Foppolo S, et al. The nuclear hormone receptor PPARγ counteracts vascular calcification by inhibiting Wnt5a signalling in vascular smooth muscle cells. Nat Commun. 2012;3:1077.

11) Bhatt PM, Malgor R. Wnt5a: a player in the pathogenesis of atherosclerosis and other inflammatory disorders. Atherosclerosis. 2014;237:155-62.

12) Albanese I, Yu B, Al-Kindi H, Barratt B, Ott L, Al-Refai M, de Varennes B, et al. Role of noncanonical Wnt signaling pathway in human aortic valve calcification. Arterioscler Thromb Vasc Biol. 2017;37:543-52.

13) Merryman WD, Schoen FJ. Mechanisms of calcification in aortic valve disease: role of mechanokinetics and mechanodynamics. Curr

Cardiol Rep. 2013;15:355.

14) Li C, Xu S, Gotlieb AI. The progression of calcific aortic valve disease through injury, cell dysfunction, and disruptive biologic and physical force feedback loops. Cardiovasc Pathol. 2013;22:1-8.

15) Chen JH, Chen WL, Sider KL, Yip CY, Simmons CA. β-Catenin mediates mechanically regulated, transforming growth factor-β1-induced myofibroblast differentiation of aortic valve interstitial cells. Arterioscler Thromb Vasc Biol. 2011;31:590-7.

16) Rajamannan NM. Oxidative-mechanical stress signals stem cell niche mediated Lrp5 osteogenesis in eNOS(-/-) null mice. J Cell Biochem. 2012;113:1623-34.

17) Bhatt PM, Lewis CJ, House DL, Keller CM, Kohn LD, Silver MJ, McCall KD, et al. Increased Wnt5a mRNA expression in advanced atherosclerotic lesions, and oxidized LDL treated human monocyte-derived macrophages. Open Circ Vasc J. 2012;5:1-7.

18) Kilian B, Mansukoski H, Barbosa FC, Ulrich F, Tada M, Heisenberg CP. The role of Ppt/Wnt5 in regulating cell shape and movement during zebrafish gastrulation. Mech Dev. 2003;120:467-76.

19) Guan S, Wang Z, Xin F, Xin H. Wnt5a is associated with the differentiation of bone marrow mesenchymal stem cells in vascular calcification by connecting with different receptors. Mol Med Rep. 2014;10:1985-91.

20) Khan K, Yu B, Kiwan C, Shalal Y, Filimon S, Cipro M, Shum-Tim D, et al. The role of Wnt/β-Catenin pathway mediators in aortic valve stenosis. Front Cell Dev Biol. 2020;8:862.

21) Weinstein N, Mendoza L, R.Alvarez-Buylla E. A computational model of the endothelial to mesenchymal transition. Front Genet. 2020;11:40.