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ORIGINAL ARTICLE

# Influence of periostin-positive cell-specific Klf5 deletion on aortic thickening in DOCA-salt hypertensive mice

Hirofumi Zempo[1], Jun-ichi Suzuki[2], Masahito Ogawa[3], Ryo Watanabe[1], Katsuhito Fujiu[4], Ichiro Manabe[4], Simon J Conway[5], Yoshiaki Taniyama[6], Ryuichi Morishita[6], Yasunobu Hirata[2], Mitsuaki Isobe[1] and Ryozo Nagai[7]

**Chronic hypertension causes vascular remodeling that is associated with an increase in periostin- (postn) positive cells, including fibroblasts and smooth muscle cells. Krüppel-like factor (KLF) 5, a transcription factor, is also observed in vascular remodeling; however, it is unknown what role KLF5 plays in postn-positive cells during vascular remodeling induced by deoxycorticosterone-acetate (DOCA) salt. We used postn-positive cell-specific *Klf5*-deficient mice (*Klf5*$^{Postn}$KO: *Klf5*$^{flox/flox}$; *Postn*$^{Cre/-}$) and wild-type mice (WT: *Klf5*$^{flox/flox}$; *Postn*$^{-/-}$). We implanted a DOCA pellet and provided drinking water containing 0.9% NaCl for 8 weeks. The DOCA-salt treatment induced hypertension in both genotypes, as observed by increases in systolic blood pressure. In WT animals, DOCA-salt treatment increased the aortic medial area compared with the non-treated controls. Similarly, *Tgfb1* was overexpressed in the aortas of the DOCA-salt treated WT mice compared with the controls. Immunofluorescence staining revealed that fibroblast-specific protein 1 (FSP1)$^+$-α smooth muscle actin (αSMA)$^+$ myofibroblasts exist in the medial area of the WT aortas after DOCA-salt intervention. Importantly, these changes were not observed in the *Klf5*$^{Postn}$KO animals. In conclusion, the results of this study suggest that the presence of KLF5 in postn-positive cells contributes to the pathogenesis of aortic thickening induced by DOCA-salt hypertension.**
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## INTRODUCTION

Hypertension is the most common risk factor associated with cardiovascular diseases. Approximately 25% of the world's adult population have hypertension, and this rate is likely to increase to 29% by 2025.[1] Chronic hypertension is known to lead to cardiovascular dysfunction through myocardial hypertrophy and vascular remodeling;[2–9] however, the pathophysiology of this disease has yet to be elucidated.

Krüppel-like factor 5 (KLF5; also known as BTEB2 and IKLF) is a key mediator of cardiovascular remodeling.[10–15] KLF5 binds to GC boxes and SP1 sites at a number of gene promoters to regulate their transcription.[14,16,17] Previously, our group showed that KLF5 overexpression resulted in the proliferation of vascular smooth muscle cells (SMCs). Gene silencing of KLF5 using RNA interference showed marked suppression of cyclin D1 expression and decreased vascular SMC growth *in vitro*.[14] We also demonstrated that overexpression of KLF5 in rats subjected to carotid balloon injury increased neointimal formation and proliferating cell nuclear antigen-positive rates.[14] Furthermore, we found that angiotensin II infusion suppressed the degrees of arterial-wall thickening, angiogenesis, cardiac hypertrophy and interstitial fibrosis in *Klf5*-knockout mice.[10]

Periostin (postn) is a well known as a useful marker of non-cardiomyocyte lineages, and it is observed on fibroblasts[18] and SMCs.[19] Postn has been investigated not only in the cardiovascular system but also in the ischemic brain.[20] It is not usually expressed in normal physiological conditions, but is induced by tissue injury, contributing to cardiac remodeling.[21,22] Therefore, it has been suggested that KLF5 in fibroblasts and SMCs has a pivotal role in vascular remodeling. Recently, we generated postn-positive cell-specific *Klf5* null mice.[15] However, the role of KLF5 in fibroblasts

[1]Department of Cardiovascular Medicine, Tokyo Medical and Dental University, Tokyo, Japan; [2]Department of Advanced Clinical Science and Therapeutics, The University of Tokyo, Tokyo, Japan; [3]Westmead Millennium Institute for Medical Research, Westmead, New South Wales, Australia; [4]Department of Cardiovascular Medicine, University of Tokyo, Tokyo, Japan; [5]Developmental Biology and Neonatal Medicine Program, Herman B Wells Center for Pediatric Research, Indiana University of Medicine, Indianapolis, IN, USA; [6]Department of Clinical Gene Therapy, Osaka University, Osaka, Japan and [7]Jichi Medical University, Shimotsuke City, Tochigi, Japan
Correspondence: Dr J Suzuki, Department of Advanced Clinical Science and Therapeutics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan.
E-mail: junichisuzuki-circ@umin.ac.jp
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and SMCs on vascular remodeling is still unknown. The aim of this study is to investigate the role of KLF5 in postn-positive cells on vascular remodeling by using DOCA-salt hypertension in mice.

## MATERIALS AND METHODS

### Generation of postn-positive cell-specific Klf5-deficient mice

Mice containing the *Klf5*$^{flox}$ allele were crossed with mice expressing Cre recombinase under the control of the Postn promoter to generate mice.[15] The presence of the *Klf5*$^{flox/flox}$ and *Postn*$^{Cre/-}$ double-transgene was determined using PCR analysis of the genomic DNA from ear tips. The *Klf5* deletion of the fibroblasts was validated by western blot analysis of *Klf5*$^{Postn}$KO mice using a KLF5 monoclonal antibody hybridoma supernatant (KM1784).[15]

### DOCA pellet implantation

We used 8-week-old male postn-positive cell-specific *Klf5*-deficient mice (*Klf5*$^{Postn}$KO: *Klf5*$^{flox/flox}$; *Postn*$^{Cre/-}$) and wild-type littermate mice (WT: *Klf5*$^{flox/flox}$; *Postn*$^{-/-}$). The mice were provided access to a standard diet and water. This study was approved by the Animal Care and Use Committee of the Tokyo Medical and Dental University. A DOCA pellet (25 mg, Innovative Research of America, Sarasota, USA) was implanted subcutaneously in the backs of mice under anesthesia as previously described.[23] Mice receiving a DOCA pellet were given 0.9% NaCl to drink. Treatment with DOCA-salt continued for 8 weeks. The control groups, consisting of WT and *Klf5*$^{Postn}$KO mice, did not receive DOCA or saline drinking water. Each group consisted of 10–12 mice. Mice were killed after 8 weeks of treatment, and tissue samples were collected for analysis.

### Systolic blood pressure

Systolic blood pressure (SBP) was measured weekly between 1000 and 1200 h using a tail-cuff system (BP-98A, Softron, Tokyo, Japan).[24,25] Non-anesthetized mice were prewarmed for 10 min at 37 °C in a thermostatically controlled heating cabinet. An average of five individual recordings was taken.

### Histopathology

The aortas were harvested immediately after the mice were killed. Five transverse sections per organ were obtained for each histological examination. The aortic samples were stained with Elastica van Gieson (EvG) staining.[26] The areas were measured using a computerized analyzer (Scion Image beta 4.0.2: NIST, Gaithersburg, MD, USA).

### RNA extraction and real-time PCR

Total RNA was extracted according to the manufacturer's protocol using TRIsure (Bioline, Tokyo, Japan). Complementary DNA was prepared using a reverse transcriptase-PCR (RT-PCR) kit (QIAGEN, Tokyo, Japan).[27] PCR was performed using a PCR kit with oligo-primers for transforming growth factor beta 1 (*Tgfb1*, Mm01178820_m1) and collagen1a1 (*Col1a1*, Mm01302043_g1). The sequences of the PCR primers were predesigned and inventoried by TaqMan Gene Expression Assays (Life Technologies Japan, Tokyo, Japan). The results were obtained from three independent experiments (five samples in each group).

### Immunofluorescence staining

Immunohistochemistry was performed to examine CD11b (1561-01, SouthernBiotech), α-smooth muscle actin (αSMA-FITC: F3777, Sigma-Aldrich, St Louis, MO, USA) and fibroblast-specific protein 1 (FSP1; also known as S100A4, ab27957, Abcam, Cambridge, UK) expression in the aorta. The sections were incubated overnight at 4 °C with primary antibodies and washed using PBST. Secondary antibodies were then applied for 60 min at room temperature. After washing in PBST, the sections were counterstained with DAPI (CS-201-06, InnoGenex, San Ramon, CA, USA).

### Statistics

Statistical analysis was performed using SPSS Base System 14.0J for Windows (IBM Japan, Tokyo, Japan). All the data were expressed as the mean ± s.e.; statistical comparisons were performed using a one-way ANOVA with Tukey HSD *post hoc* test or a one-way ANOVA repeated measures with Sidak's multiple comparison test. $P<0.05$ was considered statistically significant.

## RESULTS

### Blood pressure

Figure 1 shows the SBP for the four groups of mice over an 8-week period. Despite genotype differences, the control WT and *Klf5*$^{Postn}$KO mice had comparable SBPs. Similarly, DOCA-salt treatment increased the SBP of the WT and *Klf5*$^{Postn}$KO mice. There was no difference in the SBP of the WT-DOCA and *Klf5*$^{Postn}$KO-DOCA mice.

### Histopathology of the aortas

It is known that DOCA-salt hypertension increases aortic wall thickness.[6] Thus, we examined whether DOCA-salt treatment increased the aortic medial area in the *Klf5*$^{Postn}$KO mice (Figures 2a and b). In the control groups, there was no significant difference in the aortic wall medial area between the WT and *Klf5*$^{Postn}$KO mice. DOCA-salt treatment significantly increased the aortic wall medial area in the WT animals. However, the treatment did not alter the aortic wall medial area in the *Klf5*$^{Postn}$KO animals. This result indicates that the *Klf5*$^{Postn}$KO mice do not have histopathological changes after an 8-week DOCA-salt intervention.

### mRNA expression in the aorta

We examined whether DOCA-salt intervention alters gene expression in the aorta (Figure 2c). In the aortas of the control group, there was no significant difference in *Tgfb1* mRNA levels between the WT and *Klf5*$^{Postn}$KO mice. DOCA-salt treatment significantly increased *Tgfb1* mRNA levels in the WT animals ($P<0.001$). However, the treatment did not alter *Tgfb1* mRNA levels in the *Klf5*$^{Postn}$KO animals. There was no significant difference in the mRNA levels of *Col1a1* mRNA of the WT-DOCA and *Klf5*$^{Postn}$KO-DOCA mice ($P=0.193$). These observations indicate that DOCA-salt treatment increases *Tgfb1* in the aortas of the WT mice, but not in the *Klf5*$^{Postn}$KO mice.

### Immunofluorescence staining

Figure 3 shows representative immunofluorescence staining. CD11b$^{+}$ lymphocyte infiltration was observed only in the tunica adventitia. Next, immunofluorescence staining showed that αSMA was equally expressed in the aortic media of all groups (Figure 3b). In contrast,

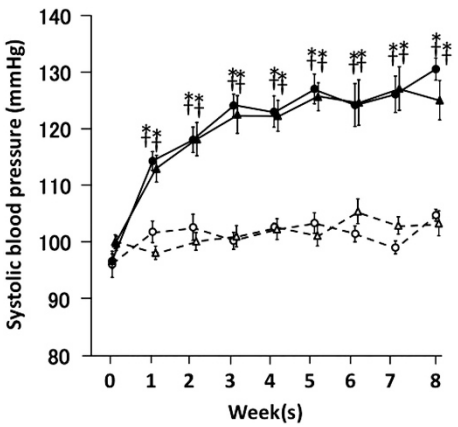


**Figure 1** Systolic blood pressure during the 8-week DOCA-salt treatment period. Open circle, WT-control mice ($n=10$); open triangle, *Klf5*$^{Postn}$KO-control mice ($n=12$); solid circle, WT-DOCA-salt mice ($n=11$); solid triangle, *Klf5*$^{Postn}$KO-DOCA-salt mice ($n=10$). Values are the mean ± s.e. *$P<0.05$ *vs.* WT-Cont. †$P<0.05$ *vs.* *Klf5*$^{Cre/-}$-Cont.

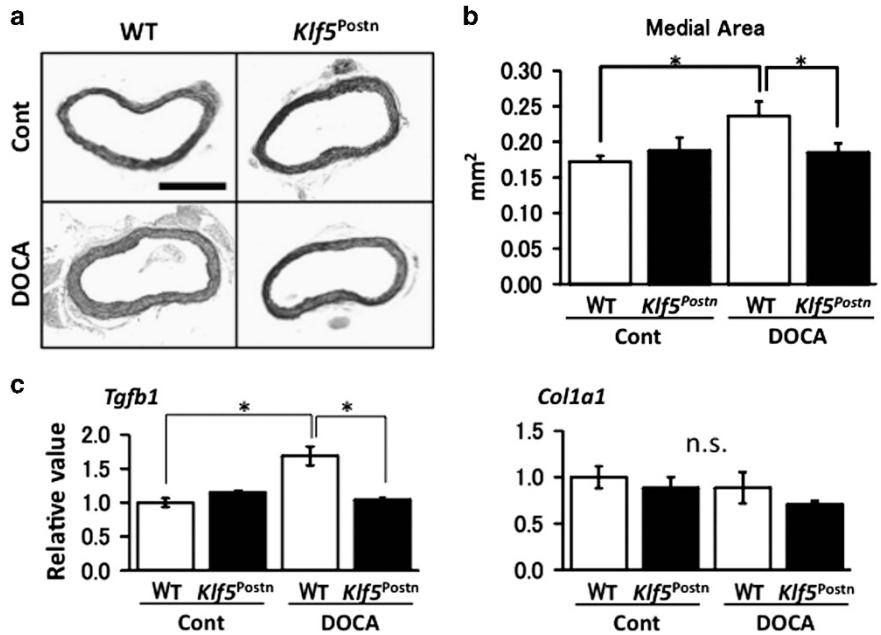


**Figure 2** Myofibroblasts localize in the thickened aortic media after 8 weeks of DOCA-salt treatment. (**a**) Representative EvG staining of the aorta (×100). Bar, 500 μm. (**b**) Aortic medial wall area. $n=10$ per group. $^*P<0.05$. (**c**) mRNA expression of *Tgfb1* and *Col1a1* in the aorta. $n=5$ per group. Values are the mean ± s.e. $^*P<0.05$.

FSP1 was observed in the aortic media of the WT-DOCA mice compared with the WT-Cont, *Klf5*$^{Postn}$KO-Cont and *Klf5*$^{Postn}$KO-DOCA mice. These results show that FSP1 cells are co-expressed with αSMA in the aortic media.

## DISCUSSION

The present study revealed that only the aortic medial area of WT mice increased, while DOCA-salt treatment similarly increased the SBP of the WT and *Klf5*$^{Postn}$KO mice. Furthermore, our data revealed that many FSP1$^+$ αSMA$^+$ myofibroblasts are found in the thickened aortic media during DOCA-salt treatment. Additionally, in contrast to WT murine aortas, *Klf5*$^{Postn}$KO mice exhibited lower FSP1 protein expression induction by DOCA-salt treatment. These results suggest that KLF5 participates in myofibroblast conversion and/or migration to the aortic media during DOCA-salt induced hypertension.

TGFβ1 expression is known to be induced by DOCA treatment and by endothelin-1.[28,29] Consistent with these previous observations, DOCA-salt hypertension is also associated with endothelin-1 dependence.[30–33] An earlier study demonstrated that endothelin-1 also directly upregulates KLF5 expression.[13] Additionally, we have reported that *Tgfb1* expression is significantly lower in the hearts of systemic *Klf5*-knockout mice than in WT mice following angiotensin II infusion, suggesting that TGFβ1 lies downstream of KLF5.[10] Therefore, DOCA-salt treatment enhances the expression of KLF5 via an endothelin-1 mechanism within myofibroblasts; KLF5 in myofibroblasts might be involved in aortic thickening via a *Tgfb1* expression pathway.

Our group previously showed that pressure overload using transverse aortic constriction suppressed the degree of cardiac hypertrophy and interstitial fibrosis in Klf5-knockout mice.[15] In this study, we used a DOCA-salt induced hypertension model in mice; however, there was no difference in the heart weights of WT and Klf5PostnKO mice (data not shown). A reason for the differences of these studies may depend on the pressure intensities. Indeed, systolic blood pressure was approximately 130 mm Hg in the present study. Although these studies used different models (DOCA-salt or transverse aortic constriction), the results suggest that KLF5 is necessary for pressure load-induced cardiovascular remodeling.

Myofibroblasts are involved in wound healing and tissue repair.[34] Previous studies demonstrate that most myofibroblasts express αSMA and that the expression of αSMA and collagen type I in these cells is regulated coordinately by TGFβ1.[35] However, the results of the present study show that DOCA-induced aortas and myofibroblasts did not increase Col1a1 gene expression. This result suggests that DOCA-treated myofibroblasts tend to be the contraction type rather than the extracellular-matrix producing type.

A study showed that transient receptor potential melastatin 7 promotes vascular adventitial remodeling in transverse aortic constriction rats,[36] while the present study suggests that KLF5 involves the aortic medial wall. Zhang *et al.* demonstrated that KLF5 expression increased with the phenotypic switching of vascular SMCs from a contractile to a proliferative state in clinical aortic wall samples of atherosclerotic aortas. They also showed significantly increased KLF5 gene and protein expression in cultured vascular SMCs from atherosclerotic donors. These results suggest that a more proliferative state of vascular SMCs from patients with atherosclerosis may be associated with a higher expression of KLF5.[37] Endothelin-1 is known to have an especially prominent role in DOCA-salt hypertension.[38] One important effect of this situation is to increase total peripheral resistance by contracting the arteries and arterioles.[39] Prepro-endothelin-1 mRNA expression and immunoreactive endothelin-1 content of the aorta are increased in DOCA-salt rats,[40–42] suggesting that one mechanism of endothelin-1-induced arterial constriction in hypertension is to increase the levels of peptide around the arterial smooth muscles. However, there is currently no direct evidence regarding endothelin-1 regulation of aortic SMC proliferation via KLF5. Recently, Courboulin *et al.*[43] demonstrated that endothelin-1 triggered KLF5 activation in pulmonary artery SMCs. They showed that the pulmonary artery

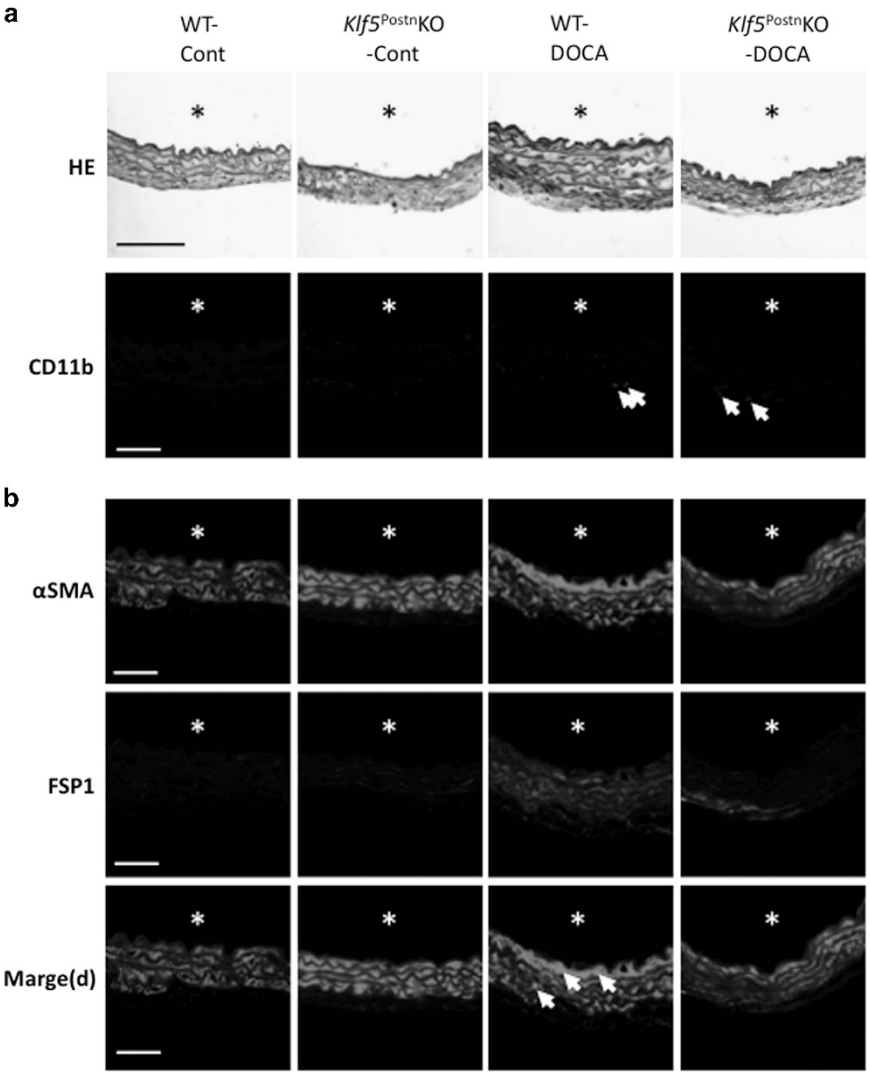


**Figure 3** DOCA-salt treatment induced myofibroblast transition in the aortic media of WT, but not *Klf5*$^{Postn}$KO mice. (**a**) Representative EvG (magnification, ×400; scale bar, 100 μm) and immunofluorescence staining (magnification, ×600; scale bar, 50 μm) of the aorta. Arrows point to CD11b$^{+}$ cells. (**b**) Immunofluorescence staining of the aorta revealed an increased number of αSMA$^{+}$ FSP1$^{+}$ cells in WT-DOCA-salt mice. Scale, ×600; bar, 50 μm. The asterisks indicate the lumen. A full color version of this figure is available at the *Hypertension Research* journal online.

SMCs with increased KLF5 were implicated in the pro-proliferative phenotype. Thus, the DOCA-endothelin-1-KLF5 pathway may be critical in this pathophysiology. Because arterial remodeling includes different pathological phenomena, further investigation is needed to clarify the detailed mechanism.

In conclusion, the results of this study suggest that the presence of KLF5 in postn-positive cells contributes to the pathogenesis of the aortic thickening induced by DOCA-salt treatment hypertension.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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