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

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Abstract

Chronic hypertension causes vascular remodeling that is associated with an increase of 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 (*Klf5*$^{\text{Postn}}$KO: *Klf5*$^{\text{flox/flox}}$;*Postn*$^{\text{Cre/−}}$) mice and wild type (WT: *Klf5*$^{\text{flox/flox}}$;*Postn*$^{-/-}$) mice. We implanted a DOCA pellet and provided drinking water that containing 0.9% NaCl for eight-weeks.

The DOCA-salt treatment induced hypertension in both genotype groups, as observed by the increase in systolic blood pressure. In WT, DOCA-salt treatment increased the aortic medial area compared to non-treated controls. Similarly, *Tgf1b* was overexpressed in the aortas of DOCA-salt treated WT mice compared to controls. Immunofluorescence staining revealed that fibroblast specific protein 1 (FSP1)$^{+}$-α smooth muscle actin (αSMA)$^{+}$ myofibroblast exist in the medial area of WT aorta after DOCA-salt intervention. Importantly, these changes were not observed in the *Klf5*$^{\text{Postn}}$KO animals.

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Conflict of Interest: None declared.

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

Keywords

Kruppel-like factors 5; Deoxycorticosterone-acetate salt; Remodeling; Periostin

Introduction

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

Krüppel-like factor 5 (KLF5; also known as BTEB2 and IKLF) is one of the mediators of cardiovascular remodeling [10–15]. KLF5 binds to GC boxes and SP1 site at a number of gene promoters and regulates their transcription [14, 16, 17]. Previously, our group showed that KLF5 overexpression resulted in the proliferation of vascular smooth muscle cell (SMC). Gene silencing of KLF5 with 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 rate [14]. Furthermore, we found that angiotensin II infusion suppressed degrees of arterial-wall thickening, angiogenesis, cardiac hypertrophy and interstitial fibrosis in *Klf5*-knockout mice [10].

Periostin (postn) is known as a useful marker of the noncardiomyocyte lineages, and it observed on fibroblasts [18] and SMCs [19]. It has been well investigated not only in the cardiovascular system but also in the ischemic brain [20]. It is not normally expressed in normal conditions, but it is induced by tissue injury, contributing to cardiac remodeling [21,22]. Therefore, it is suggested that KLF5 in fibroblasts and SMCs plays a pivotal role in the vascular remodeling. Recently, we generated postn positive cell-specific *Klf5* null mice [15]. However, its role in fibroblasts and SMCs on vascular remodeling is still unknown. The aim of this study is to investigate the role of KLF5 in the postn positive cells on the vascular remodeling by using DOCA-salt hypertension in mice.

Methods

Generation of periostin positive cell-specific Klf5 deficient Mice

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

DOCA Pellet Implantation

We used 8-week-old male postn positive cell-specific *Klf5* deficient (*Klf5*$^{\text{Postn}}$KO: *Klf5*$^{\text{flox/flox}}$;*Postn*$^{\text{Cre/−}}$) mice and wild type (WT: *Klf5*$^{\text{flox/flox}}$;*Postn*$^{-/-}$) littermate mice. They were given 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, US) was implanted subcutaneously in the back under anesthesia as described previously [23]. Mice receiving DOCA were given 0.9% NaCl to drink. Treatment with DOCA-salt continued for eight-weeks. The control groups, consisting of WT and *Klf5*$^{\text{Postn}}$KO mice, did not receive DOCA and saline. Each group consisted of 10–12 mice. Mice were sacrificed after eight-weeks of treatment and tissue samples were collected for analysis.

Systolic Blood Pressure

Systolic blood pressure (SBP) was measured by using a tail-cuff system (BP-98A, Softron Co., Tokyo, Japan) [24,25] weekly between 10:00 am to 12:00 pm. Unanesthetized awake mice were prewarmed for 10 min at 37°C in a thermostatically controlled heating cabinet. An average of five recordings were taken at individual value.

Histopathology

Aorta was harvested immediately after the mice were sacrificed. Five transverse sections per organ were obtained for histological examination. 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).

RNA Extraction and Real-time PCR

Total RNA was extracted according to the manufacture's protocol by the TRIsure (Bioline, Tokyo, Japan). Complementary DNA was prepared with a reverse transcriptase-polymerase chain reaction (RT-PCR) kit (QIAGEN, Tokyo, Japan) [27]. The PCR was performed with a PCR-kit in the presence of oligo-primers for transforming growth factor beta 1 (*Tgfb1*, Mm01178820_m1) and collagen1a1 (*Col1a1*, Mm01302043_g1). The sequences of the PCR primers were predesigned inventoried TaqMan Gene Expression Assays (Life Technologies Japan, Tokyo, Japan). Results were obtained from 3 independent experiments (5 samples in each group).

Immunofluorescence Staining

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

Statistics

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

Results

Blood Pressure

Figure 1 shows SBP in four mice groups over an eight-week period. Despite genotype difference, control WT and *Klf5*^{Postn}KO mice showed comparable SBP. Similarly, DOCA-salt treatment increased SBP between the WT and *Klf5*^{Postn}KO mice. There was no difference in SBP between the WT-DOCA and *Klf5*^{Postn}KO-DOCA mice.

Histopathology of Aorta

It has been 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 (Figure 2A and B). In 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 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 histopathologically change because of eight-week DOCA-salt intervention.

mRNA Expressions of Aorta

We examined whether DOCA-salt intervention alters gene expression of aorta (Figure 2C). In the control group aorta, 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 between the WT-DOCA and *Klf5*^{Postn}KO-DOCA mice ($P = 0.193$). These observations indicate that DOCA-salt treatment increases TGFβ1 on aorta in 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 on tunica adventitia. Following this, immunofluorescence staining showed that αSMA was equally expressed in the aortic media in all groups (Figure 3B). In contrast, FSP1 was observed in the aortic media of WT-DOCA mice compared with WT-Cont, *Klf5*^{Postn}KO-Cont, and *Klf5*^{Postn}KO-DOCA mice. These results show that FSP1 cells co-express with αSMA in the aortic media.

Discussion

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

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

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

Myofibroblasts are involved with wound healing and tissue repair [34]. Results 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, results of the present study show that DOCA-induced aorta and myofibroblast did not increase Col1a1 gene expression. This result suggests that DOCA-treated myofibroblasts tend toward being contraction type rather than extracellular-matrix producing type.

A study showed that transient receptor potential melastatin 7 promotes vascular adventitial remodeling in TAC rats [36], while the present study suggests that KLF5 involves the aortic medial wall. Zhang et al. demonstrated that KLF5 expression increased with phenotypic switch of vascular smooth muscle cells from a contractile to a proliferative state in atherosclerotic aortas in clinical aortic wall samples. They also showed that significantly increased KLF5 gene and protein expression in cultured vascular smooth muscle cells from atherosclerotic donors. These results suggest that a more proliferative state of vascular smooth muscle cells from patients with atherosclerosis may be associated with higher expression of KLF-5 [37]. Endothelin-1 is known to have an especially prominent role in DOCA-salt hypertension [38]. One important effect of its situation is to increase total

peripheral resistance by contracting arteries and arterioles [39]. Prepro-endothelin-1 mRNA expression and immunoreactive endothelin-1 content of aorta are increased in DOCA-salt rats [40–42], suggesting that one mechanism of endothelin-1-induced arterial constriction in hypertension is an increased level of peptide around arterial smooth muscles. However, there is no direct evidence from endothelin-1 to the aortic SMC proliferation via KLF-5 at this moment. Recently, Courboulin *et al.* demonstrated that endothelin-1 triggered KLF-5 activation in pulmonary artery smooth muscle cells. They showed that the pulmonary artery smooth muscle cells with enhanced KLF-5 were implicated in the pro-proliferative phenotype [43]. Thus, 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 presents of KLF5 on periostin positive cells contribute to the pathogenesis of the aortic thickening induced by DOCA-salt treated hypertension.

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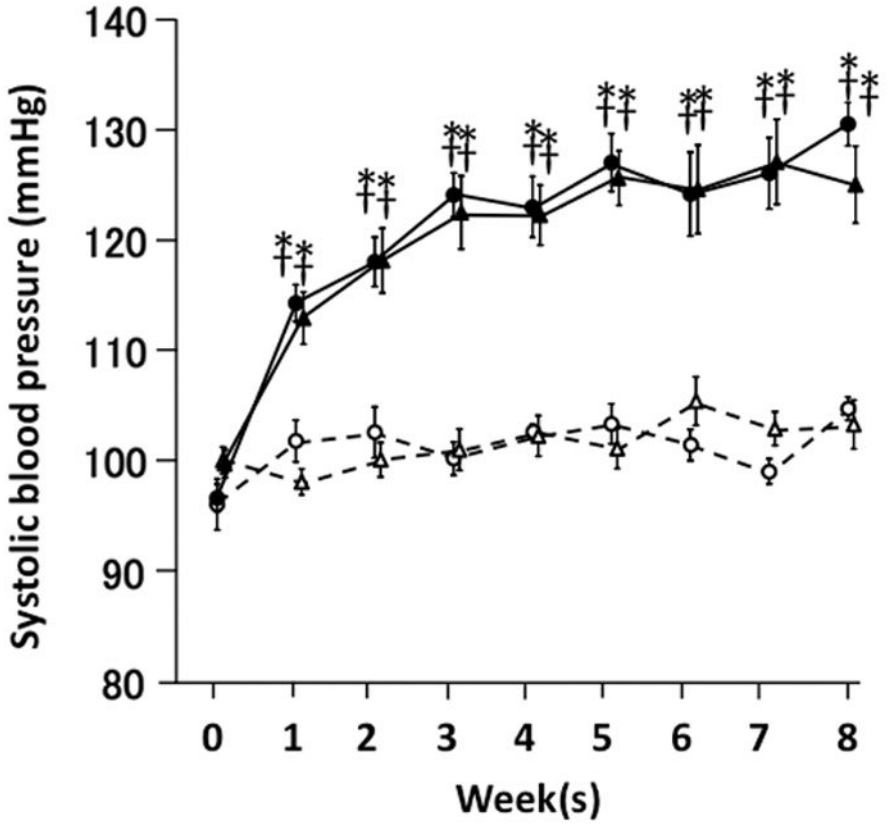

Figure 1.

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

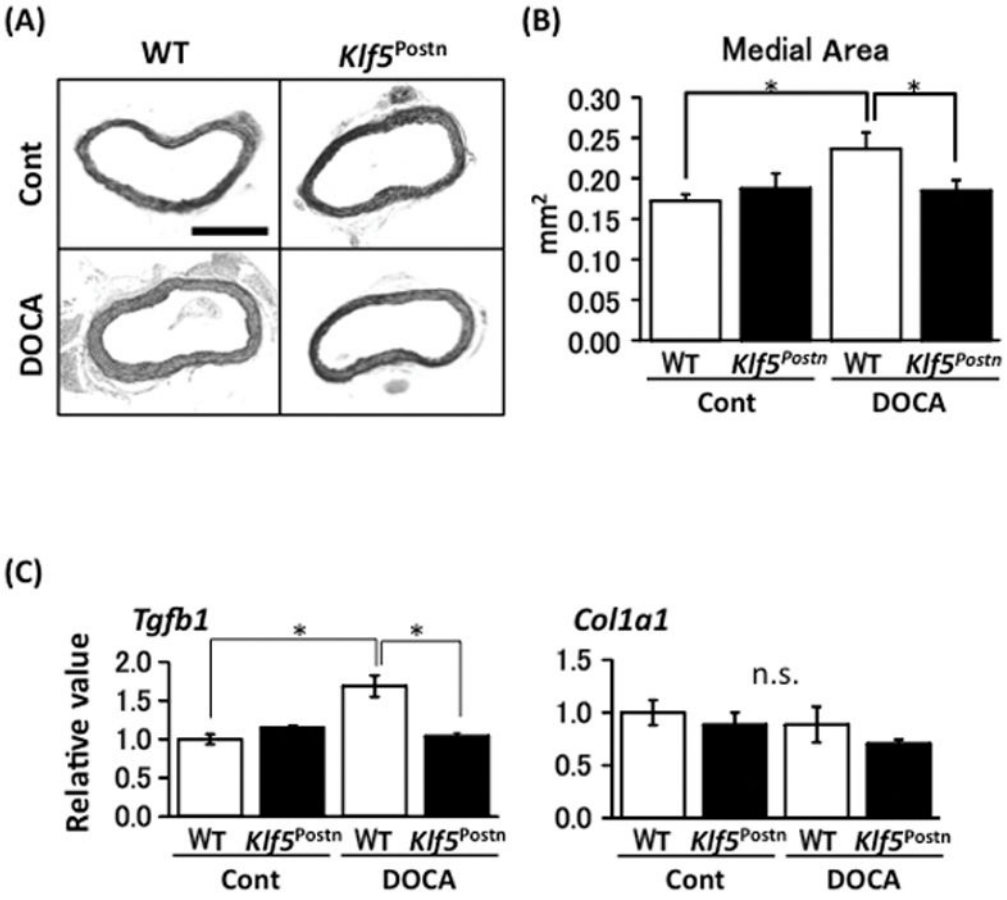

Figure 2.

Myofibroblasts localize in thickened aortic media after eight-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 *Col1a* in aorta. n = 5 per group. Values are mean ± SE. *P < 0.05.

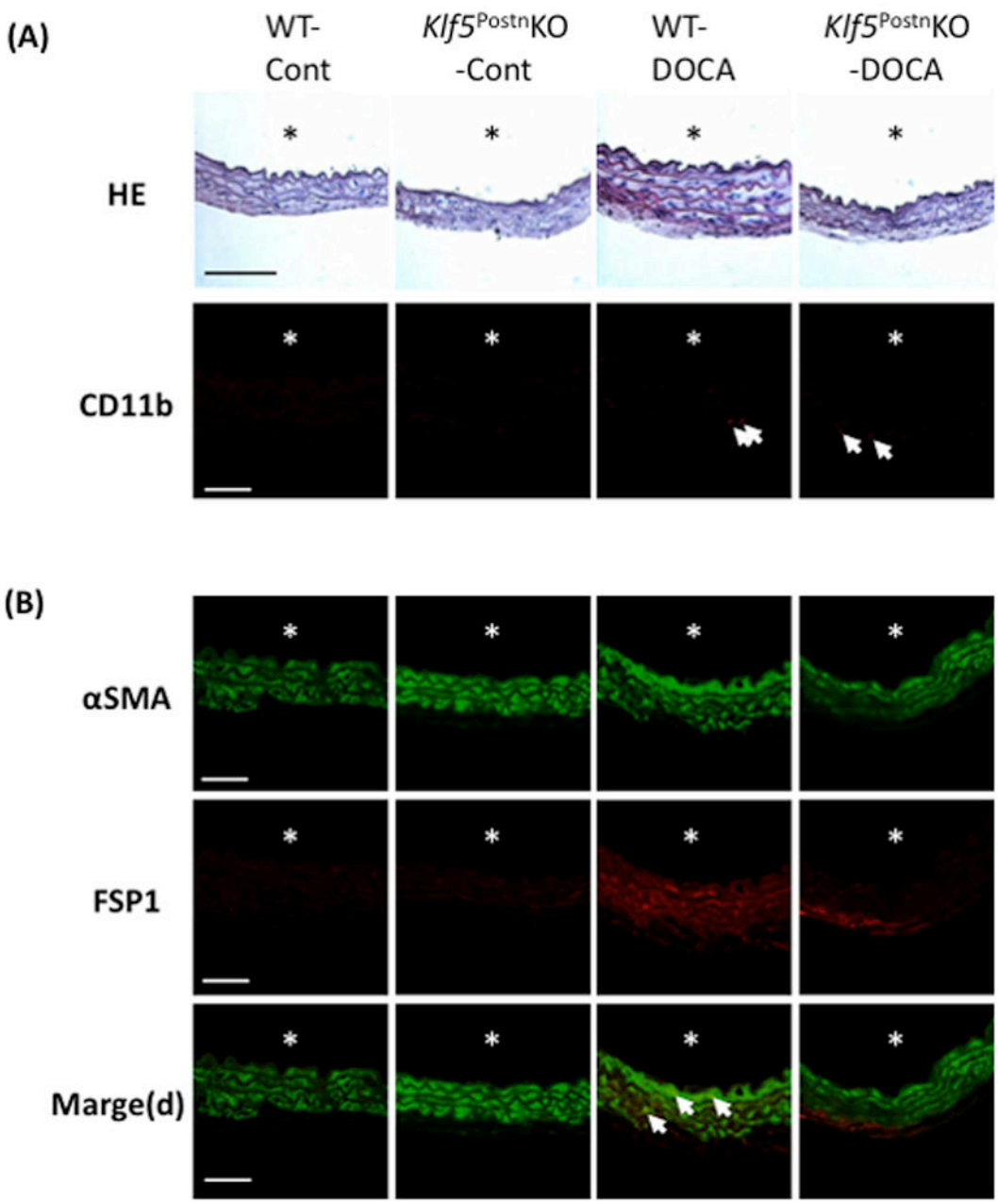

Figure 3.

DOCA-salt treatment induced myofibroblast transition in aortic media of WT, but not *Klf5*^{Postn}KO mice. (A) Representative EvG (Scale, ×400; bar, 100μm) and immunofluorescence staining (Scale, ×600; bar, 50μm) of aorta. Arrows point to CD11b^{+} cells. (B) Immunofluorescence staining of aorta revealed an increased number of αSMA^{+} FSP1^{+} cells in WT-DOCA-salt mice. Scale, ×600; Bar, 50μm. The asterisks indicate the lumen.