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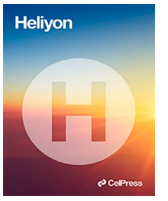

Research article

Alantolactone alleviates epithelial-mesenchymal transition by regulating the TGF-β/STAT3 signaling pathway in renal fibrosis

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ABSTRACT

Objective: The epithelial-to-mesenchymal transition (EMT) of renal tubular epithelial cells (RTECs) plays a crucial role in renal interstitial fibrosis and inflammation, which are key components of chronic kidney disease (CKD). Alantolactone, a selective inhibitor of signal transducer and activator of transcription 3 (STAT3), is used in Chinese herbal medicine. Despite its use, the effects of alnatolactone on EMT of RTECs has not been fully elucidated.
Methods: In this study, we investigated the potential of alantolactone to EMT *in vivo* and *in vitro*. Our experiments were performed using a unilateral ureteral obstruction (UUO) models and HK-2 cells, RTECs, treated with transforming growth factor (TGF-β).
Results: Alantolactone decreased tubular injury and reduced the expression of vimentin, a key EMT marker, while increasing E-cadherin expression in UUO kidneys. Similarly, in RTECs, alantolactone inhibited TGF-β-induced EMT and its markers. Furthermore, alantolactone attenuated UUO- and TGF-β-induced STAT3 phosphorylation both *in vivo* and *in vitro*, and inhibited the expression of TWIST, an EMT transcription factor, in both models.
Conclusion: Alantolactone improves EMT in RTECs by inhibiting STAT3 phosphorylation and Twist expression, suggesting its potential as a therapeutic agent for kidney fibrosis.

1. Introduction

The final progression of chronic kidney disease (CKD), leading to end-stage renal disease, is marked by renal interstitial fibrosis. This condition is characterized by epithelial-to-mesenchymal transition (EMT) of renal tubular epithelial (RTE) cells and the excessive accumulation of extracellular matrix (ECM) in the interstitial compartment. EMT involves the transformation of epithelial cells into fibroblasts through the action of profibrotic growth factors, which disrupt the junction of RTECs and are closely linked to kidney dysfunction [1–4].

Abbreviations: EMT, Epithelial-to-mesenchymal transition; CKD, chronic kidney disease; UUO, unilateral ureteral obstruction; RTECs, renal tubular epithelial cells; ECM, extracellular matrix; STAT3, Signal transducer and activator of transcription 3; TGF-β, transforming growth factor-beta; JAK/STAT, Janus kinase-signal transducer and activator of transcription; SMAD, small downstream mother against the decapentaplegic; UUO + Alantolactone, UUO with 5 mg/kg alantolactone treatment; H&E, hematoxylin and eosin.

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During EMT, epithelial cells exhibit morphological changes toward a mesenchymal phenotype, a decrease in epithelial marker such as E-cadherin, and an increase in interstitial markers such as N-cadherin and vimentin [4]. EMT-related transcription factors including Snail, TWIST, and Slug are responsible for regulating the expression of these epithelial and interstitial markers [4–6]. The morphological and functional transformations in RTECs during EMT contribute to renal injury, fibrosis and inflammation [4]. Transforming growth factor (TGF-β) is a key driver of EMT and plays a pivotal role in these pathological processes [4].

TGF-β is a pivotal factor involved in the induction of fibrosis [7] and its progression in different organ systems are related to various diseases, including renal fibrosis [8]. In the context of renal fibrosis, TGF-β, a profibrotic cytokine, is secreted by epithelial cells and other kidney cell types such as fibroblasts, mesangial cells, podocytes, and endothelial cells in response to various stimuli [9–11]. TGF-β drives renal interstitial fibrosis by promoting ECM production and EMT. This occurs through the activation various signaling pathway, including Janus kinase-signal transducer and activator of transcription (JAK/STAT), small downstream mother against the decapentaplegic (SMAD), Mitogen-activated protein kinase (MAPK), Phosphoinositide 3-kinase/Protein kinase B, Wnt/β-catenin pathways [12–14].

STAT3 plays a crucial role in fibrosis, cell differentiation, proliferation, survival and apoptosis [15,16], and is activated and phosphorylated by TGF-β [16,17]. Activated STAT3 has been detected in renal interstitial fibroblasts within fibrotic kidney, and the treatment of STAT3 inhibitors has been shown to reduce renal fibrosis [18,19], making them therapeutic agents. Additionally, STAT3 binds to the promoters of fibrotic target genes, including TWIST, and directly regulates their expression [20].

Alantolactone, a compound extreacted from *Inula* species and traditionally used in Chinese medicine for allergic contact dermatitis, has demonstrated antioxidant, antitumoral and anti-inflammatory properties [21–24]. Previous research has indicated that alalntolactone provides anti-inflammatory benefits in the kidneys of streptozotocin-induced diabetic mice [25], offers protection against neuronal cell death and memory loss in mice [26], and exhibits antifibrotic effects in pulmonary fibrosis [27]. Despite these finding, the role of alantolactone in the EMT of RTECs remains unexplored both *in vivo* or *in vitro*.

In this research, we examined the effects of alantolactone on STAT3 inhibition, the progression of renal interstitial fibrosis, EMT, and renal inflammation using unilateral ureteral obstruction (UUO) models and TGF-β-treated RTECs.

2. Materials and methods

2.1. Animals and UUO model

All experimental procedures received approval from the Animal Care and Use Committee of Daegu Gyeongbuk Institute of Science and Technology (DGIST-IACUC-19052105-01). The study utilized C57BL/6 mice, sourced from Koatech Technology Corporation (Pyeongtaek-si, Korea), which were randomly assigned to three distinct groups: Control (Sham, $n = 7$), unilateral ureteral obstruction (UUO, n=7), UUO with alantolactone (UUO + Alantolacone 5 mg/kg, n=7). Alantolactone (Carbosynth, Compton, Berkshire, UK) was prepared in a filtered solution containing 1.25 % DMSO, 2 % PEG 400, and 0.5 % Tween 20 in saline. This solution was administered daily by gavage at a volume of 250 μL per mouse, starting with a 5-day pretreatment phase. For a duration of 15 days, all groups received their respective oral treatments in equal volumes. The UUO surgical procedure was carried out as described in previous protocols [28].

2.2. Cell culture

HK-2 cell (ATCC, Manassas, VA, USA) was maintained in keratinocyte serum-free media (Gibco, Waltham, MA, USA), which was enriched with 50 μg/mL bovine pituitary extract and 5 ng/mL human recombinant epidermal growth factor. Cells were incubated in 5 % CO_2 at 37 °C in a humidity incubator. For experimental purposes, the cells were exposed to 5 ng/mL of TGF-β1 (R&D Systems, Minneapolis, MN, USA) for 24 h.

2.3. Histological analysis and immunohistochemical staining

Histological analyses were carried out hematoxylin and eosin (H&E, Sigma-Aldrich, Saint Louis, MO, USA) and immunohistochemical methods (Thermo Fisher Scientific, Waltham, MA, USA) on paraffin-embedded kidney sections (4 μm-thick). The sections were first deparaffinized in xylene and then rehydrated through a series of graded concentrations. For immunohistochemistry, the following antibodies were used: E-cadherin (1:200), N-cadherin (1:200) from Cell Signaling Technology (Danvers, MA, USA), and p-STAT3 (1:100) from Santa Cruz Biotechnology (Dallas, TX, USA). Detection was achieved with a DAB Staining Kit (Roche, Basel, Switzerland). After staining, the sections were dehydrated through sequential ethanol and xylene washes and then mounted onto glass slides for examination. Microscopic images at 200 × magnification were captured from sections of kidneys from seven different animals using a Leica Microscope (Leica Microsystem, Wetzlar, Land Hessen, Germany) equipped with Leica Application Suit V3.8 software (Leica Microsystems). Quantitative analysis of aniline blue (collagen, blue), Sirius Red (collagen fiber, red), and brown immunostaining were performed using computer-based morphometric tools.

2.4. Western blot analysis

HK-2 cells and kidney tissue were lysed using radioimmunoprecipitation assay lysis (RIPA) buffer (Thermo Fisher Scientific) supplemented with protease inhibitor (Roche), and phosphatase inhibitor (Thermo Fisher Scientific). Proteins were loaded and

separated in Mini-PROTEAN® Gels (Bio-Rad, Hercules, CA, USA) and transferred onto polyvinylidene fluoride (PVDF, Bio-Rad), then blocked using 5 % bovine serum albumin (Sigma-Aldrich). The membranes were incubated with the primary antibodies of anti-vimentin (1:1000), anti-E-cadherin (1:1000), anti-N-cadherin (1:1000) (Cell Signaling), anti-phospho-STAT3 (1:1000), anti-STAT3 (1:1000), anti-GAPDH (1:1000), anti-β-actin (1:1000), anti-Twist (1:1000), anti-Snail (1:1000), and anti-Slug (1:1000) (Santacruz), and then were reacted with secondary antibody (1:3500). Image bands were detected using ChemiDoc ™XRS+ (Bio-Rad) and quantified using ImageJ program 1.53e (National Institutes of Health, Bethesda, MD, USA).

2.5. Statistical analysis

The outcomes were expressed as the mean and standard error of the mean (SEM) from three separate experiments. For statistical comparisons, Student's t-test was performed. The statistical significance considered at a threshold of $P < 0.05$.

3. Results

3.1. Alantolactone attenuates renal damage and tubular EMT in UUO kidney

First, we explored the effects of alantolactone on renal tubular atrophy and EMT triggered by UUO. UUO-induced renal damage decreased significantly in the kidneys of alantolactone-treated mice (Fig. 1A-B). E-cadherin expression was significantly lower in UUO kidneys than in control kidneys, indicating that alantolactone inhibited the UUO-induced decrease in E-cadherin expression in UUO kidneys (Fig. 1A and C). Immunohistochemical staining and immunoblotting analysis showed that alantolactone effectively inhibited the UUO-mediated increase in vimentin expression in UUO Kidneys (Fig. 1A, D and 2A).

3.2. Alantolactone attenuates TGF-β-mediated EMT in RTECs

EMT of tubular epithelial cells is a hallmark of renal fibrosis [29]. Thus, we investigated the effects of alantolactone on the expression patterns of EMT markers in TGF-β-treated renal tubular HK-2 cells. As shown in Fig. 2B, alantolactone prevented the morphological changes induced by TGF-β in HK-2 cells. Furthermore, alantolactone countered TGF-β-induced decrease in E-cadherin

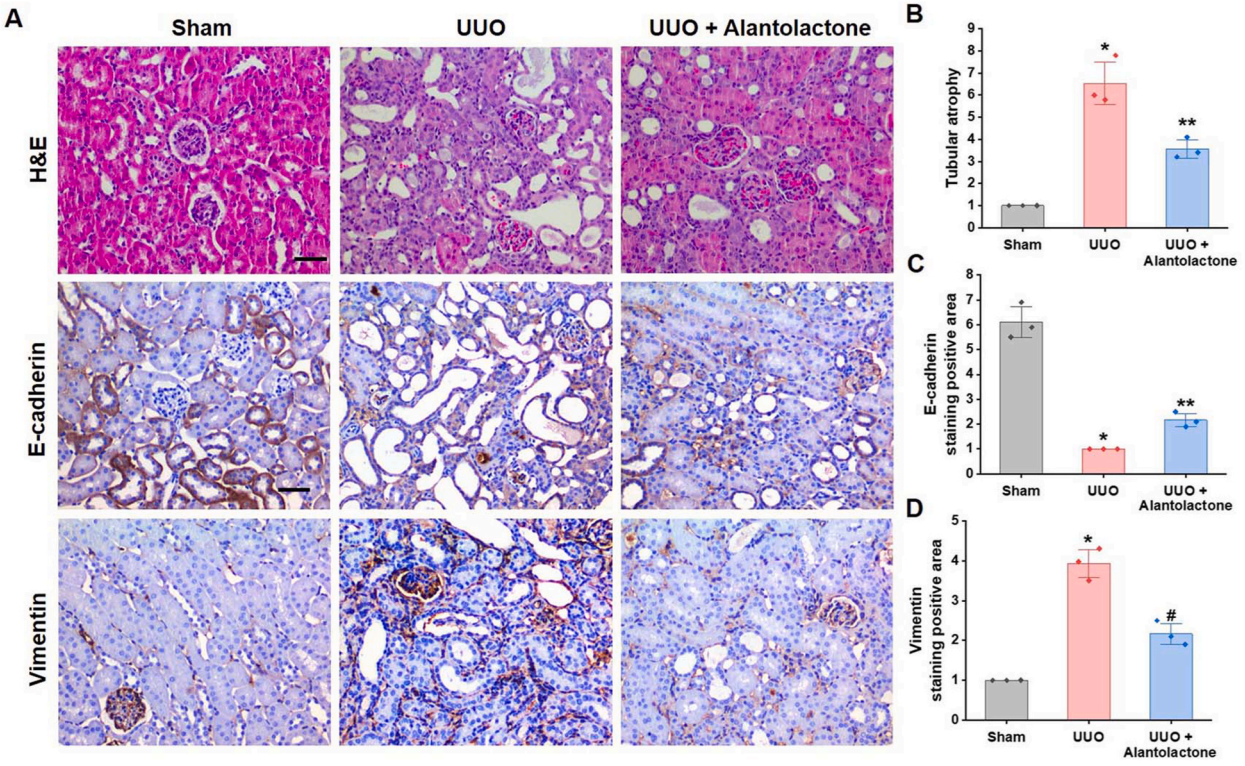

Fig. 1. Alantolactone attenuates renal damage and renal tubular EMT in UUO kidneys. Mice were administered a dosage of 5 mg/kg alantolactone via oral gavage for 5 days prior to UUO and continued for an additional 10 days post-UUO. (A) Kidney sections were stained with H&E and subjected to immunostaining for E-cadherin and vimentin, observed at 20 × magnification. (B–D) Quantification of atrophic tubules or positively-stained areas was conducted using computer-based morphometric analysis. All bar graph data were normalized to the control (n = 1) and presented as the fold increase relative to the control; *$P < 0.01$ versus control (Sham), **$P < 0.05$ and #$P < 0.01$ versus UUO (n = 7 for each group).

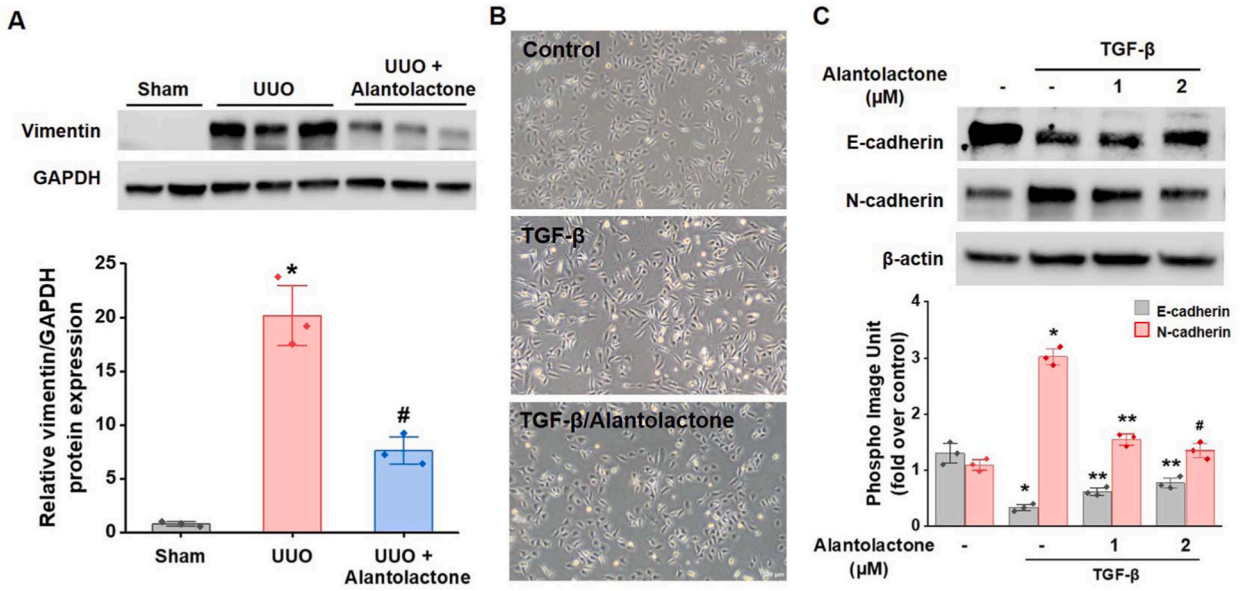

Fig. 2. Alantolactone regulates EMT factor expression *in vivo* and in *vitro*. Mice were administered a dosage of 5 mg/kg alantolactone via oral gavage for 5 days prior to UUO and continued for an additional 10 days post-UUO. (A) Representative Western blot showing vimentin expression in kidney lysates from control, UUO or UUO + alantolactone-treated kidneys. Vimentin levels in different groups were quantified by densitometry and normalized to GAPDH and the control (n = 1). All bar graph data were expressed as the fold increase relative to the control; $*P < 0.01$ versus control (Sham), and $\#P < 0.01$ versus UUO. HK-2 cells were treated with 5 ng/mL TGF-β for 24 h in the presence of alantolactone (0–2 μM). (B) Photomicrographs illustrating TGF-β-induced morphological alterations in HK-2 cells. (C) Western blot analysis of E-cadherin and N-cadherin protein levels in TGF-β-stimulated HK-2 cells. Quantification data are from three independent experiments and values are presented as the mean ± SEM; $*P < 0.01$ versus control (TGF-β (−)), and $**P < 0.05$ and $\#P < 0.01$ versus TGF-β.

protein levels and increase in N-cadherin protein levels in these cells (Fig. 2C)

3.3. Alantolactone ameliorated renal damage by inhibiting of STAT3 phosphorylation in both UUO kidneys and RTECs

STAT3 phosphorylation is crucial for differentiation of renal fibroblasts with phospho-STAT3 levels being upregulated in UUO kidneys and TGF-β-treated RTECs [16,19,30]. Therefore, we investigated how alantolactone modulates the STAT3 pathway, which is elevated in UUO kidneys and TGF-β-treated HK-2 cells. Immunohistochemical staining and immunoblotting analysis revealed that alantolactone substantially reduced UUO-mediated STAT3 phosphorylation in mouse UUO kidneys (Fig. 3A-C). Fig. 3D shows that alantolactone inhibited the activity of STAT3 molecules in HK-2 cells compared to Stattic, a selective STAT3 inhibitor. In HK-2 cells treated with TGF-β, STAT3 phosphorylation was elevated compared to untreated cells. However, alantolactone significantly inhibited TGF-β-induced STAT3 phosphorylation in these cells (Fig. 3E).

3.4. Alantolactone prevents the decrease in E-cadherin expression in renal fibrosis through the regulation of TWIST

Twist is a critical transcription factor and mediator of EMT [31]. In previous studies, ectopic expression of Twist not only suppressed E-cadherin expression but also induced the expression of mesenchymal markers like vimentin, N-cadherin, and fibronectin [32]. Finally, we examined whether alantolactone can affect the expression of EMT-related transcription factors in UUO kidneys and TGF-β-treated HK-2 cells. Immunoblotting analysis showed that alantolactone attenuated Twist expression in UUO-induced kidneys (Fig. 4A). Alantolactone significantly inhibited TGF-β-induced Twist expression and slightly reduced TGF-β-induced Snail expression in HK-2 cells. However, it did not affect the expression of other EMT transcription factors, such as Slug, in HK-2 cells (Fig. 4B).

4. Discussion

UUO kidneys exhibit characteristic features of obstructive nephropathy including tubular atrophy, tubulointerstitial fibrosis, EMT of tubular epithelial cell, macrophage infiltration, and proinflammatory cytokines [33–35]. EMT of RTECs is among the processes proposed to contribute to renal fibrosis development [36] Moreover, the expression levels of EMT-regulated proteins, such as E-cadherin, N-cadherin, and vimentin, are altered during the development of renal fibrosis and plays a significant role in UUO-induced renal interstitial fibrosis [5,6] In the present study, the UUO-induced decrease in E-cadherin expression was partially restored by alantolactone, whereas UUO-stimulated vimentin expression was inhibited by alantolactone in UUO kidneys. Furthermore, the TGF-β-induced mesenchymal phenotype, decreased E-cadherin expression, and increased N-cadherin expression were blocked by

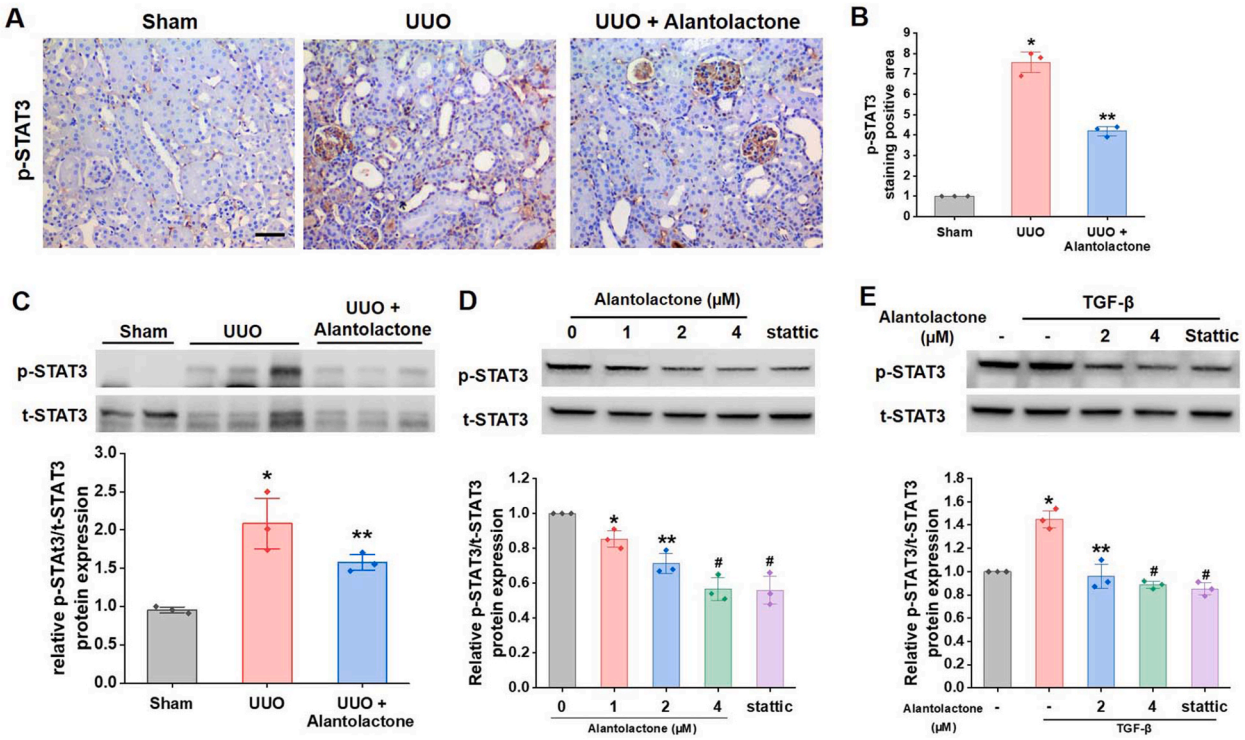

Fig. 3. Alantolactone prevents STAT3 phosphorylation in UUO kidneys and RTECs. Mice were administered a dosage of 5 mg/kg alantolactone via oral gavage for 5 days prior to UUO and continued for an additional 10 days post-UUO. (A–B) Kidney sections immunostained for phospho-STAT3 (p-STAT3) were observed at 20 × magnification and quantification of positively stained areas was done by computer-based morphometric analysis. All data were normalized to the control (n = 1) and expressed as the fold increase relative to the control. $^{*}P < 0.01$ versus control (Sham) and $^{**}P < 0.01$ versus UUO (n = 7 for each group). (C) Representative Western blot analysis of kidney lysates for p-STAT3 in control, UUO or UUO + alantolactone-treated kidneys. Expression levels of p-STAT3 in different groups were quantified by densitometry and normalized to t-STAT3, and compared to the control (n = 1). All data are expressed as the fold increase relative to the control (right). $^{*}P < 0.01$ versus control (Sham) and $^{**}P < 0.01$ versus UUO. (D-E) Western blot analysis of p-STAT3 protein expression in alantolactone-treated HK-2 cells. HK-2 cells were incubated with or without 5 ng/mL TGF-β for 24 h in the presence of alantolactone (0–4 μM) and the STAT3 inhibitor, Stattic (2 μM). Quantification of Western blot analysis; data are from three independent experiments and are shown as the mean ± standard error of the mean. (D) $^{*}P < 0.05$, $^{**}P < 0.01$ and $\#P < 0.001$ versus control (0 μM) (E) $^{*}P < 0.05$ versus control (TGF-β (−)), and $^{**}P < 0.01$ and $\#P < 0.001$ versus TGF-β.

alantolactone in HK-2 cells.

The activation and phosphorylation of STAT3 by TGF-β contributes to the development of renal fibrosis [17,18], and is observed in various fibrosis-related kidney diseases [37,38] as well as in a UUO model of experimentally-induced fibrotic kidneys [19,20]. Furthermore, Stat3 directly regulates various fibrotic target genes with a Stat3-binding site in the promoter, such as Twist and vimentin [39]. STAT3 acts as an important mediator between tubular and interstitial cells during CKD progression [40–42], and its inhibitors can be used an antifibrotic.

Chinese herb medicine has been used for thousands of years to treat various disorders, including renal fibrosis [43,44]. For example, one Chinese herbal medicine improved renal fibrosis and renal tubular EMT in a diabetic nephropathy rat model by inhibiting the JAK/STAT3 or Toll-like receptor 4/nuclear factor kappa B signaling pathways [45,46]. Recent reports show that ginsenoside, obtained from ginseng extracts, protects against UUO-induced renal fibrosis by inhibiting TGF-β/Smad signaling [47] and ameliorates EMT-related renal fibrosis by suppressing binding immunoglobulin protein/eukaryotic initiation factor 2/C/EBP homologous protein signaling [48].

Previously, we reported that alantolactone effectively inhibits the expression of renal fibrotic factors through downregulating TGF-β/Smad3 pathway, thereby ameliorating renal fibrosis. Additionally, our previous research briefly showed that alantolactone inhibits the phosphorylation of STAT3 in TGF-β-treated renal cells and UUO kidneys [49]. Several studies have shown that STAT3 activation induces the EMT of tubular epithelial cells in UUO [50] and that STAT3 knockdown protects kidney epithelial cells from angiotentin II-induced kidney damage [51]. In this research, we determined that alantolactone significantly decrease STAT3 phosphorylation in RTECs, similar to the selective STAT3 inhibitor Stattic. Furthermore, alantolactone effectively inhibited UUO- and TGF-β-induced STAT3 phosphorylation both *in vivo* and *in vitro*. These findings suggest that alantolactone may be play a role in suppressing EMT and fibrosis in obstructive nephropathy by inhibiting of STAT3 activation.

Twist and Snail are pivotal transcriptional regulators of EMT. Lovisa et al. reported that conditional knockout of Twist1 or Snail in proximal tubular epithelial cells led to the suppression of the EMT process and preservation of tubular epithelial cells integrity in

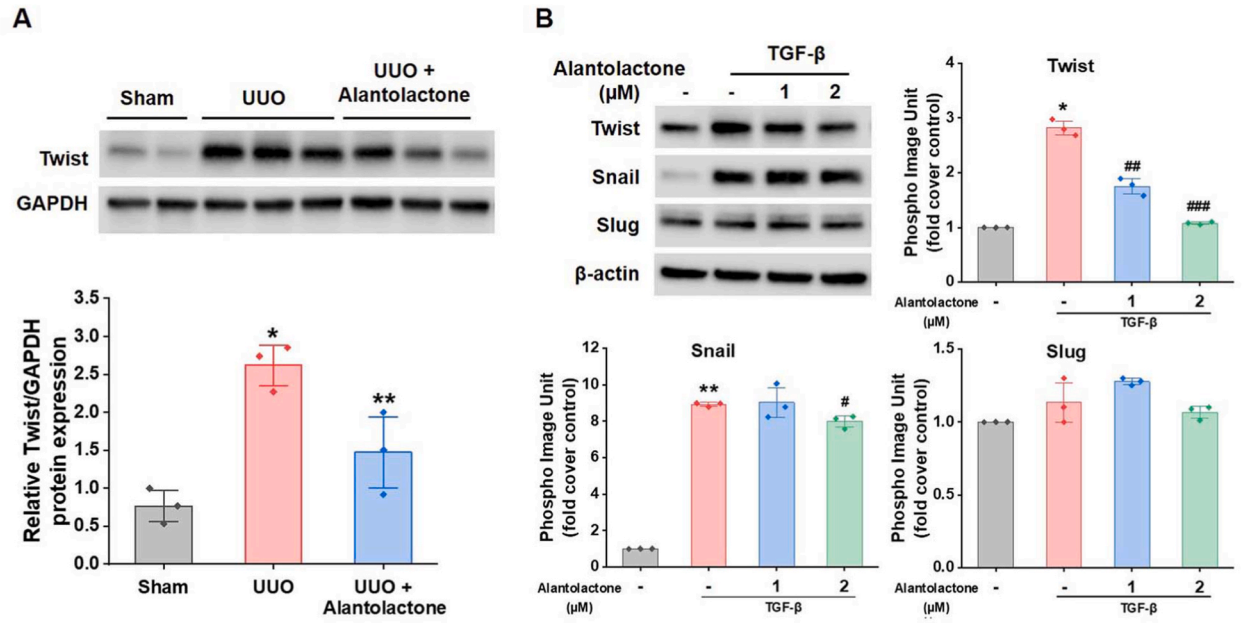

Fig. 4. Alantolactone prevents EMT related transcription factors, *in vivo* and *in vitro*. (A) Representative Western blot showing twist expression in kidney lysates from control, UUO or UUO + alantolactone-treated kidneys. Twist levels in different groups were quantified by densitometry and normalized to GAPDH and the control (n = 1). All bar graph data were expressed as the fold increase relative to the control. $*P < 0.01$ versus control (Sham) and $**P < 0.05$ versus UUO. (B) Western blot analysis of Twist, Snail and Slug protein levels in TGF-β-stimulated HK-2 cells. HK-2 cells were incubated with 5 ng/mL TGF-β for 24 h in the presence of alantolactone (0–2 μM). Quantification data are from three independent experiments and values are presented as the mean ± SEM. $*P < 0.01$ and $**P < 0.001$ versus control (TGF-β (−)), and $\#P < 0.05$, $\#\#P < 0.01$ and $\#\#\#P < 0.001$ versus TGF-β.

mouse models of UUO-induced renal fibrosis [52]. Our findings showed that alantolactone significantly inhibited UUO- and TGF-β-stimulated Twist expression *in vivo* and *in vitro*. Previous research has demonstrated that STAT3 mediates TGF-β-stimulated Twist1 expression, which contributes to cancer invasion [53] Furthermore, both the use of STAT3 inhibitor and silencing STAT3 can reduced the upregulation of Twist1 induced by TGF-β in RTECs [54]. In this study, we demonstrated that alantolactone simultaneously inhibits the activation of STAT3 and the increase in Twist in UUO kidneys and TGF-β-treated HK-2 cells. These findings suggest that alantolactone may prevent the progression of obstructive nephropathy by inhibiting the EMT of RTECs thorough the suppression of the STAT3/Twist pathway. However, further studies are needed to mechanistically prove.

5. Conclusion

The results of this study suggest that alantolactone ameliorates renal tubulointerstitial fibrosis and renal tubular EMT in UUO kidneys and TGF-β-treated renal tubular interstitial cells. Alantolactone inhibits UUO- and TGF-β-stimulated STAT3 phosphorylation and Twist expression *in vivo* and *in vitro*. Furthermore, alantolactone decreased the pro-inflammatory response in UUO kidneys. This study is the first to that alantolactone can reduce renal interstitial fibrosis and slow down the progression of renal tubular EMT in an animal model of obstructive nephropathy induced by UUO. Alantolactone not only effectively reduces kidney damage when administered orally in animal models but is also used as Chinese herbal medicine with a proven safety record. This indicates its potential as a promising candidate for therapeutic use in kidney disease.

Ethics statement

All animal experiments received approval from the Animal Care and Use Committee at Daegu Gyeongbuk Institute of Science and Technology (DGIST-IACUC-19052105-01).

Data availability

All data generated or analyzed during this study are presented within this published article.

CRediT authorship contribution statement

Yeo Jin Hwang: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation,

Formal analysis, Data curation. **Gwon-Soo Jung:** Writing – original draft, Methodology, Investigation, Formal analysis. **Kyeong-Min Lee:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36253.

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