

HOXA13 exerts a beneficial effect in albumin-induced epithelial-mesenchymal transition via the glucocorticoid receptor signaling pathway in human renal tubular epithelial cells

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Abstract. Previous studies have suggested that albumin-induced renal tubular epithelial cell injury contributes to renal interstitial fibrosis. Epithelial-mesenchymal transition (EMT) is known to be a key mechanism in the pathogenesis and progression of renal interstitial fibrosis. Homeobox protein HOX-A13 (HOXA13) is a nuclear transcriptional factor that has been reported to be involved in renal fibrosis. However, the mechanism underlying the effect of HOXA13 in human serum albumin (HSA)-induced EMT in HKC renal tubular epithelial cells remains to be elucidated. Thus, the aim of the present study was to investigate the role of HOXA13 in HSA-induced EMT in HKC cells and the potential mechanism of the glucocorticoid receptor (GR) signaling pathway. The protein and mRNA expression levels of HOXA13, cytokeratin, and vimentin were determined by western blot analysis and reverse transcription-quantitative polymerase chain reaction in HKC cells, which were co-incubated with HSA at different concentrations or for different time periods. The results demonstrated that HOXA13 mRNA and protein expression decreased in a dose- and time-dependent manner when induced by HSA in HCK cells. The liposomal transfection experiment suggested that overexpression of HOXA13 activated the GR signal, which inhibits HSA-induced EMT. HOXA13 is involved in HSA-induced EMT in HKC cells and upregulation of HOXA13 exerts a beneficial effect in EMT, which may be associated with the GR signaling pathway.

Introduction

Interstitial fibrosis is a common pathway of progressive renal disease, leading to end-stage renal failure regardless of the etiology (1). Previous studies have indicated that the process of tubular epithelial-mesenchymal transition (EMT) contributes to the predominance of fibroblasts in idiopathic nephrotic syndrome (2). The pathological mechanism of EMT remains to be elucidated (3); however, recent studies have suggested that homeobox protein HOX-A13 (HOXA13) is involved in urogenital development and renal fibrosis (4). Furthermore, Williams *et al* (5) reported that HOXA13 exerts an inhibitory effect on transforming growth factor- β 1 (TGF- β 1) signaling, which has been widely demonstrated to induce EMT (6). Thus, HOXA13 is hypothesized to be involved in regulating EMT and renal fibrosis, however, the molecular mechanism underlying these effects remains to be elucidated.

Resistance to glucocorticoid (GC) treatment is a key clinical problem in multiple diseases, including idiopathic nephrotic syndrome (7). The majority of the effects of GC are mediated by the glucocorticoid receptor (GR). GR is known to inhibit the activity of a number of immune-regulating transcription factors (8). Abnormalities in the number and affinity of GRs have been demonstrated to be associated with peripheral blood mononuclear cell (PBMC) proliferation and cytokine secretion, which are important in the development of idiopathic nephrotic syndrome and renal failure (7,9,10). However, the effect of GR signaling on EMT has not yet, to the best of our knowledge, been reported. Thus, the aim of the present study was to investigate the potential function of HOXA13 in human serum albumin (HSA)-induced EMT and the GR signaling pathway in HKC human renal tubular epithelial cells.

Materials and methods

Reagents. Mouse anti- β -actin monoclonal antibody (cat no. 6008-1), rabbit anti-vimentin polyclonal antibody (cat no. 10366), rabbit anti-cytokeratin (CK) polyclonal antibodies (cat no. 10830) and rabbit anti-GR polyclonal antibody (cat no. 23978) were obtained from ProteinTech Group, Inc. (Chicago, IL, USA). Rabbit anti-HOXA13 polyclonal antibodies (cat no. sc-66922) were purchased from Santa Cruz Biotechnology,

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Inc. (Dallas, TX, USA). Rabbit anti-KAT3A/cAMP response element binding protein (CBP) polyclonal antibody (cat no. sc-ab119488) was obtained from Abcam (Cambridge, UK). The plasmids pGV230-eGFP-HOXA13 and pGV230-eGFP vector were provided by Shanghai Genechem Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and Fetal bovine serum (FBS) were obtained from Wuhan Procell Power Technology Co., Ltd. (Wuhan, China).

Cell culture and HSA treatment. HKC cells were obtained from the Nephropathy Laboratory at the Second Xiangya Hospital, Central South University (Changsha, China). HKC cells were cultured in DMEM supplemented with 10% FBS and maintained at 37°C in a humidified chamber of 5% CO₂. When the cells reached the appropriate confluence (50%), cells were seeded (1x10⁶ cells/ml) in 6-well plates. When they reached 80% confluence, cells were washed with sterile phosphate-buffered saline (PBS) and serum-starved for 24 h. One group of cells was treated with medium containing different HSA concentrations (0, 1, 5, 10, 20 and 30 mg/ml) in serum-free medium (SFM) for 48 h, another group cells were cultured in SFM containing 20 mg/ml HSA for 0, 12, 24, 48 and 72 h.

Cell viability studies. Adherent and floating cells were harvested every 12 h up to 72 h after exposure to either SFM alone (control) or HSA preparations (0-30 mg/ml). Cell viability was assessed using the trypan blue exclusion assay (Shanghai Biyuntian Biological Co., Ltd., Shanghai, China). The number of live and dead cells (that were stained blue) was counted, and the viability was expressed as the percentage of live cells within the total number of cells counted according to a previous report (6). Cells were visualized using an inverted biological microscope, DSZ-2000X Series; Nikon Corporation, Tokyo, Japan)

Plasmid transfection. Plasmid transfection was conducted with Lipofectamine 2000 according to the manufacturer's protocols (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were incubated for 48 h 37°C in a humidified chamber of 5% CO₂ and then subjected to the collection of cells, supernatants, proteins, and total RNA according to previous reports (4,6). The cells for plasmid transfection were subsequently exposed to DMEM containing 10% FBS and 20 mg/ml HSA for a further 48 h. Cells were divided into five groups: i) A control group; ii) a HSA group in which cells were treated with 20 mg/ml HSA and received non-transfected plasmid; iii) a HOXA13 transfection group in which cells were treated with 20 mg/ml HSA and received transfected plasmid pGV230-eGFP-HOXA13 (Shanghai Jikai Gene Technology Co., Ltd. Shanghai, China); iv) a negative control group in which cells were transfected with plasmid pGV230-eGFP vector and received 20 mg/ml HSA; and v) a CBP group in which cells were treated with 20 mg/ml HSA and transfected with plasmid pGV230-eGFP-HOXA13 and treated with 1 µg CBP. CBP, a GR inhibitor, was used to investigate the GR signaling pathway.

Western blot analysis. Western blot analysis was performed as described in previous studies (11,12). Briefly, 1x10⁶ cells from each group were collected and total protein was extracted, as

described previously (11,12). For each sample, 20 µl total protein was subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Kaiji Biotechnology., Shanghai, China) and then transferred to a nitrocellulose membrane (Kaiji Biotechnology.). Membranes were blocked in 5% fat-free dry milk (Biyuntian, Shanghai, China) and then incubated overnight at 4°C with anti-CK (at 1:200 dilution), anti-vimentin (at 1:1,000 dilution), anti-HOXA13 (at 1:200 dilution), anti-GR (at 1:1,000 dilution), anti-CBP (at 1:500 dilution) or anti-β-actin (at 1:4,000 dilution). Secondary antibody (goat anti-rabbit immunoglobulin G labeled with horseradish peroxidase; at 1:4,000 dilution) was added to membranes following washing with TBS-T (Shanghai Biyuntian Biological Co., Ltd.) and incubated for 1 h at room temperature as previously described (11,12). Following washing, Luminata Forte Western HRP substrate (EMD Millipore; Billerica, MA, USA) was added and allowed to develop for 2 min in a darkroom, and X-ray film (Gel Doc Efficient Zeitgeist, Bio-Rad, USA) was then exposed to the membranes. The β-actin signal was set as the internal reference. Relative intensities of protein bands were quantified using an image analysis system (Image Lab Software; version 4.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). CK, vimentin, and HOXA13 mRNA expression levels were detected by qPCR, according to a previous report (11). Approximately 1x10⁶ cells/ml from each group were collected from six-well plates for qPCR detection. The gene sequences were used to design primers and these were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.), as follows: Sense, 5'-ATCCACGAGCACAGTCCACAT-3' and antisense, 5'-AGGAGACGGTGACCAGGGTT-3' for CK; sense, 5'-TGACCGCTTCGCCAACTACAT-3' and antisense, 5'-TCCCGCATCTCCTCCTCGTA-3' for vimentin; sense, 5'-CACTCTGCCCCGACGTGGTCT-3 and antisense, 5'-CCG CCTCCGTTTGTCTTA-3' for HOXA13; and sense, 5'-CGA CAGGATGCAGAACGAGA-3' and antisense, 5'-AGTGAG GACCCTGGATGTGA-3' for β-actin.

Total RNA isolation and reverse transcription were conducted according to previous reports (13,14) and using SYBR Green PCR Mix (Takara Bio, Inc., Otsu, Japan). Double-distilled water used instead of a template served as a negative control. The number of β-actin transcripts was used as a reference of endogenous RNA, and the quantification of test genes for each sample was standardized relative to the number of β-actin transcripts. The 2^{-ΔΔC_q} cycle threshold formula was used to calculate the relative abundance of transcripts (13,14).

Statistical analysis. The SPSS software package (version 17.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Values are expressed as the mean ± standard deviation. Differences between groups were evaluated by one-way analysis of variance followed by Duncan's test by correction for multiple comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

Cell viability. The results demonstrated that incubation with HSA (0-30 mg/ml) treatment for 48 h reduced cell viability in

Table I. Effects of HOXA13 transfection on gene expression levels in HSA-induced epithelial-mesenchymal transition.

Gene	Control	HSA group	HOXA13 transfection	Negative control
HOXA13	1.00±0.05	0.27±0.01 ^a	0.95±0.06 ^b	0.33±0.01
Cytokeratin	1.00±0.05	0.34±0.01 ^a	0.77±0.03 ^b	0.29±0.01
Vimentin	1.00±0.06	2.65±0.12 ^a	1.07±0.06 ^b	2.90±0.13

^aP<0.05 vs. the control group; ^bP<0.05 vs. the negative control. HOXA13, homeobox protein HOX-A13; HSA, human serum albumin. Results are expressed as mean ± standard deviation.

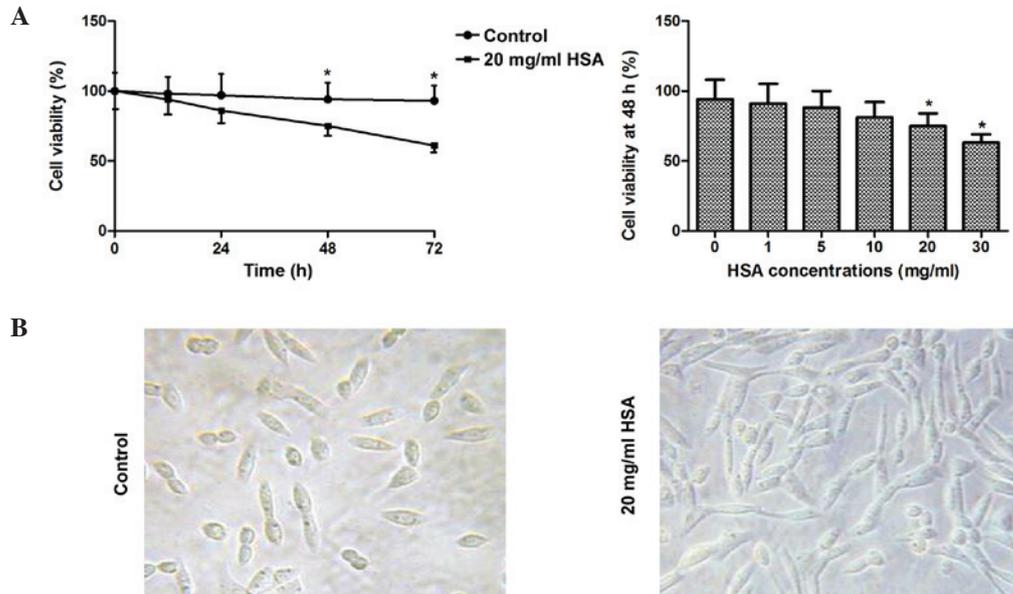


Figure 1. Effect of HSA treatment on cell viability and morphology of HKC cells. (A) Cell viability, human renal tubular epithelial cells were exposed to HSA overload. *P<0.05. (B) Morphologic analysis, cells were incubated for 48 h with 20 mg/ml HSA (magnification, x400). HSA, human serum albumin.

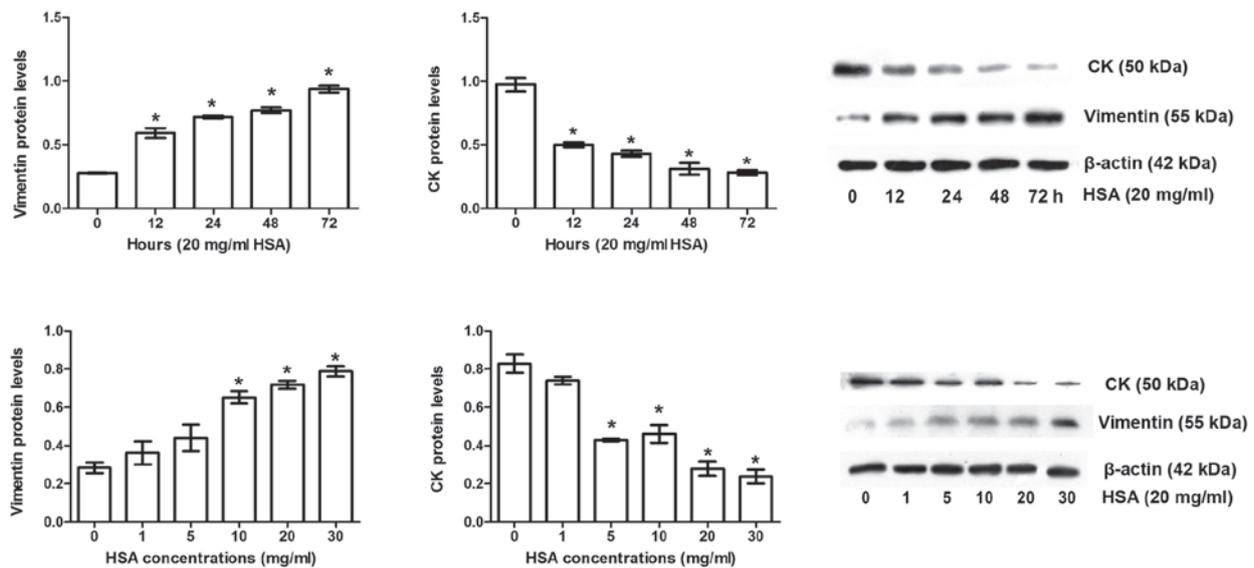


Figure 2. Effects of HSA on CK and vimentin protein expression levels in human renal tubular epithelial cells as analyzed by western blotting. *P<0.05, compared with control. HSA, human serum albumin; CK, cytokeratin.

a dose-dependent manner, and 20 and 30 mg/ml significantly decreased cell viability (P<0.05). Furthermore, incubation

with 20 mg/ml HSA significantly reduced cell viability at 48 and 72 h (P<0.05; Fig. 1). The results of cell morphology

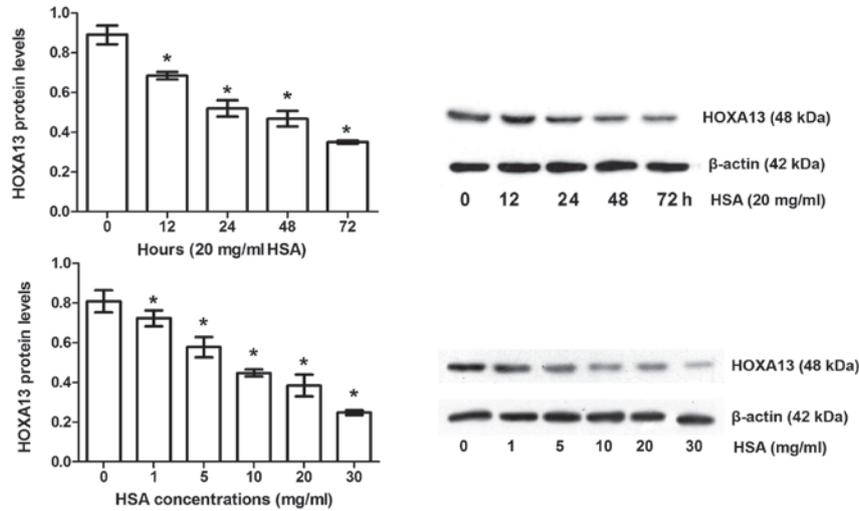


Figure 3. Effects of HSA on HOXA13 expression levels in human renal tubular epithelial cells as analyzed by western blotting. *P<0.05, compared with control. HSA, human serum albumin; HOXA13, homeobox protein HOX-A13.

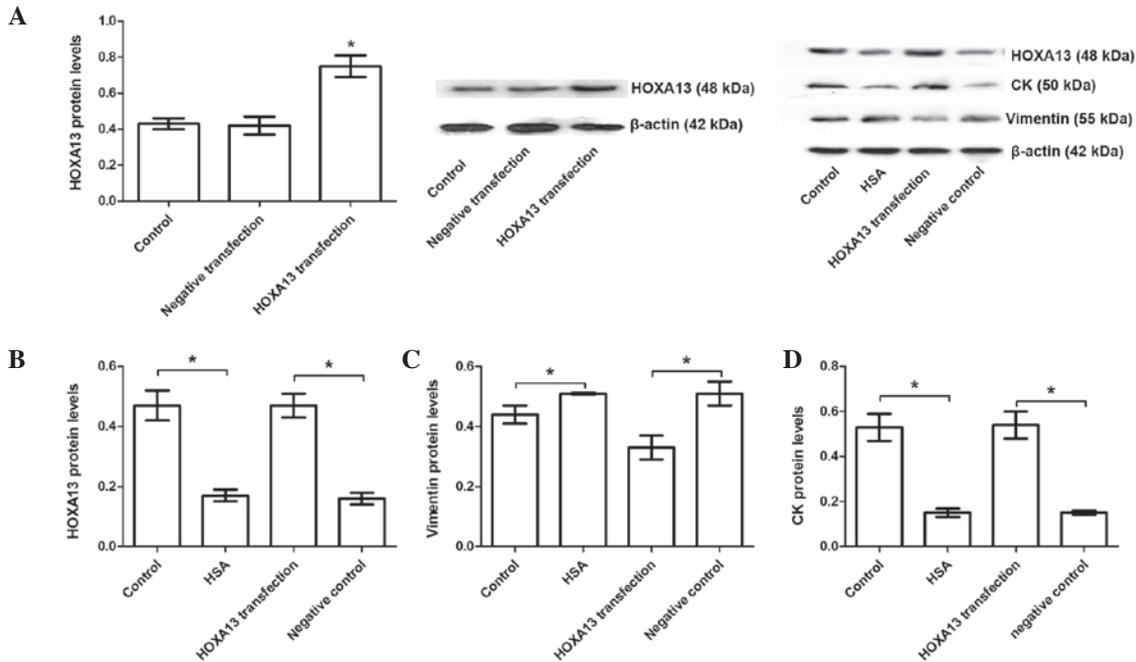


Figure 4. Effects of HOXA13 upregulation on CK and vimentin protein expression levels in human renal tubular epithelial cells as analyzed by western blotting analysis. *P<0.05. HOXA13, homeobox protein HOX-A13; CK, cytokeratin; HSA, human serum albumin. (A) and (B) HOXA13 protein levels. (C) Vimentin protein levels. (D) CK protein levels.

analysis demonstrated that cells treated with 20 mg/ml HSA for 48 h were spindle shaped and exhibited a fibroblast-like morphology compared with the control group (Fig. 1).

Albumin induces EMT in HKC cells. Vimentin (a mesenchymal cell marker) and CK (an epithelial cell marker) have been widely used to evaluate EMT (15). Western blotting data in the present study indicated that HSA significantly increased vimentin expression levels and lowered CK expression levels in a time- and dose-dependent manner (P<0.05; Fig. 2), indicating that albumin treatment induces EMT in HKC cells.

Expression of HOXA13 in albumin-induced EMT in HKC cells. Effects of albumin-induced EMT on HOXA13 expression are shown in Fig. 3 and Table I. HSA significantly inhibited HOXA13 in a time- and dose-dependent manner compared with the control group (P<0.05).

Albumin-induced EMT is restored by overexpression of HOXA13 in HKC cells. To investigate the protective role of HOXA13 in HSA-induced EMT, HKC cells were transfected with pGV230-eGFP-HOXA13 and pGV230-eGFP, and the results demonstrated that HOXA13 transfection significantly increased HOXA13 protein abundance compared with the

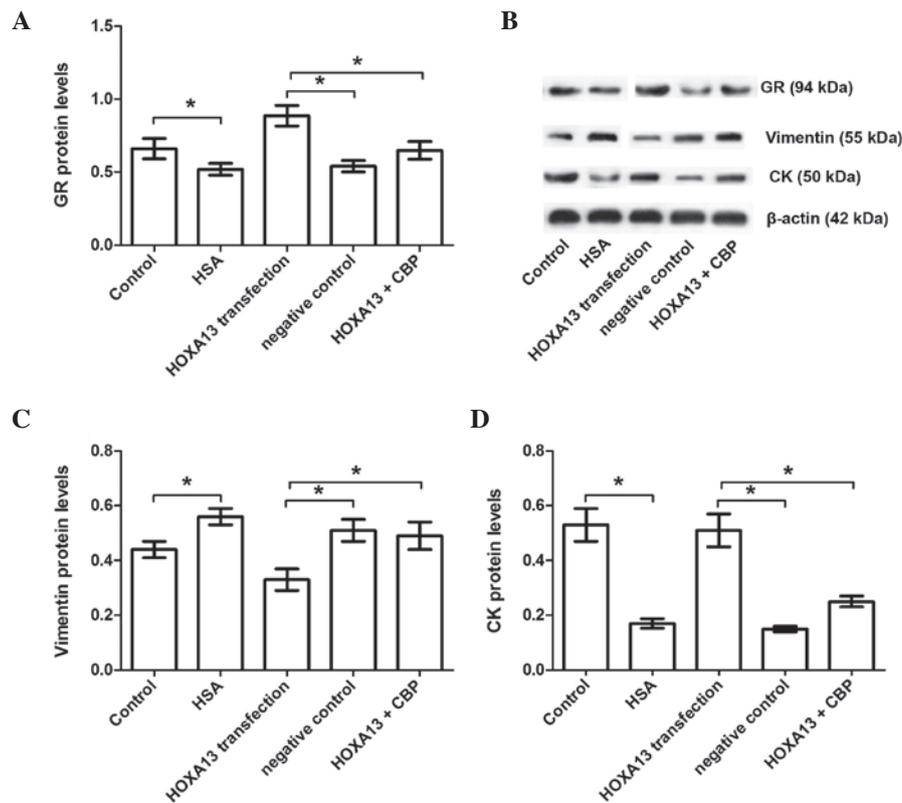


Figure 5. Western blot analysis of GR in human renal tubular epithelial cells with HOXA13 gene transfection. * $P < 0.05$. GR, glucocorticoid receptor; HSA, human serum albumin; HOXA13, homeobox protein HOX-A13; CBP, cAMP response element binding protein; CK, cytokeratin. (A) GR protein levels; (B) immunoblotting; (C) Vimentin protein levels; and (D) CK protein levels.

cells transfected with pGV230-eGFP vector ($P < 0.05$; Fig. 4A). HKC cells were transfected with pGV230-eGFP-HOXA13 and then treated with 20 mg/ml HSA, and the results demonstrated that HOXA13 transfection significantly upregulated HOXA13 expression compared with HSA-treated negative control group ($P < 0.05$; Fig. 4B). Furthermore, the effects of HOXA13 on albumin-induced upregulation of EMT were measured and the results demonstrated that upregulation of HOXA13 reversed albumin-induced EMT as suggested by downregulation of vimentin and upregulation of CK ($P < 0.05$; Figs. 4C and D). Furthermore, the results were validated by qPCR (Table I), indicating that HOXA13 has a protective role against HSA-induced EMT in HKC cells through mediating vimentin and CK levels.

Effects of HOXA13 on GR in albumin-induced EMT. The GR signaling pathway was also investigated in albumin-induced EMT using CBP, a GR inhibitor. GR expression levels in albumin-induced EMT were measured and the results demonstrated that HSA treatment significantly inhibited GR ($P < 0.05$; Fig. 5). HOXA13 transfection reversed the GR inhibition that resulted from HSA treatment; however, this effect was inhibited by CBP treatment ($P < 0.05$; Fig. 5). Furthermore, the present data also demonstrated that CBP inhibited the protective function of HOXA13 transfection as suggested by its ability to reduce vimentin and enhance CK expression ($P < 0.05$; Fig. 5). Thus, it was hypothesized that the protective function of HOXA13 transfection involves the GR signaling pathway.

Discussion

The present study demonstrated that albumin-induced EMT in HKC cells was characterized by downregulation of CK and upregulation of vimentin in a time- and dose-dependent manner. In addition, HOXA13, as a nuclear transcription factor, decreased CK and increased vimentin production, which contributed to the beneficial role in albumin-induced EMT of HKC cells. Furthermore, the present study suggests that the GR signaling pathway is involved in the protective function of HOXA13 in albumin-induced EMT.

Emerging evidence demonstrates that EMT is key in renal tubulointerstitial fibrosis (1,3). In the present study, a prolonged albumin overload model was used to mimic the effects of chronic proteinuria to induce EMT, the results demonstrated that there was a significant decrease in the viability of HKC cells exposed to HSA, and HSA treatment reduced the expression levels of the epithelial marker CK and increased the expression levels of the mesenchymal marker vimentin, suggesting that HSA induced EMT in HKC cells, which is consistent with the results of previous studies (16,17).

Members of the HOX family contain a highly conserved DNA sequence and encode the nuclear transcription regulatory proteins, which are important in the regulation of the differentiation and proliferation of adult tissue (18). The HOXA13 gene is important in mammalian embryonic development and is associated with limb formation and reproductive development (19). A previous study indicated that HOXA13 exerts a protective effect against renal fibrosis (4). Williams *et al* reported that HOXA13

inhibits TGF- β 1-mediated transcriptional activity, which has been demonstrated to induce EMT (5). In the current study, HSA was observed to induce EMT and significantly inhibit HOXA13 expression, and the reduced HOXA13 expression may serve as a marker for EMT. To further elucidate the role of HOXA13 in EMT, the expression of HOXA13 was upregulated and the results demonstrated that liposomal transfection with HOXA13 significantly reversed HSA-induced CK reduction and vimentin overexpression, suggesting a protective role of HOXA13 against EMT in HKC cells as a result of CK reduction and vimentin upregulation involved in EMT.

GR is known to inhibit the activity of a growing number of immune-regulating transcription factors (8). Abnormalities of number and affinity of GR have been demonstrated to be associated with PBMC proliferation and cytokine secretion, which are important in the development of idiopathic nephrotic syndrome and renal failure (7,9). The current study demonstrated that HSA-induced EMT significantly inhibited GR signaling, while upregulation of HOXA13 activated the GR signaling pathway. It has been demonstrated that CBP is a negative regulator of GR activity (20). Thus, CBP was used to clarify the underlying mechanism of GR in the protective role of HOXA13 in HSA-induced EMT and the results demonstrated that CBP treatment inactivated GR signaling and blocked the beneficial effect of HOXA13 in HSA-induced EMT (demonstrated by CK and vimentin expression levels).

In conclusion, albumin overload induces EMT in HKC cells and downregulates HOXA13 expression. Transfection experiments demonstrate that HOXA13 exerts a protective effect in HSA-induced EMT, which may involve the GR signaling pathway. The results of the present study suggested that HOXA13 may be a novel target for the therapy of renal interstitial fibrosis.

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