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# Angiogenin regulates epithelial-mesenchymal transition of hepatocellular carcinoma through upregulation of HMGA2

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Angiogenin (ANG) is known to alter multiple cell behaviors by directly targeting downstream targets, but its role in hepatocellular carcinoma (HCC) remains to be elucidated. The expression of ANG in HCC cell lines was measured by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot. The effects of ANG expression on cell proliferation, cell migration, and hallmarks of epithelial-mesenchymal transition (EMT) process were also investigated. The relationship between ANG and high mobility group AT-hook 2 (HMGA2) was evaluated. ANG expression was increased in HCC cell lines. Downregulating of ANG inhibits proliferation, migration, and EMT of HCC cells. The direct regulation of ANG on HMGA2 was verified by luciferase activity reporter assay and western blot assay. Furthermore, overexpression of HMGA2 reversed the inhibitory effects of ANG downregulation on HCC cell behaviors. Our results illustrated the mechanism that ANG promote the EMT of HCC through targeting HMGA2.

## 1. Introduction

Hepatocellular carcinoma (HCC) also is reported to account for 75 %-85 % of all liver cancer cases and thus represents a heavy health burden to the world (Bray et al. 2018). The main risk factors for HCC are chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), and aflatoxin-contaminated foodstuffs (Arem et al. 2017). The 5-year survival of HCC patients remains poor even though surgical methods have significantly improved in recent years (Ho et al. 2018). However, the mechanisms underlying HCC progression remain largely unknown.

Epithelial-mesenchymal transition (EMT) has a crucial role in the initiation and progression of HCC (Giannelli et al. 2016). The EMT process is characterized as increased cell contractility and actin stress fibre formation mediated by proteins including moesin and therefore the epithelial cell was converted to the mesenchymal phenotype (Lamouille et al. 2014). The process of EMT is accompanied by multiple gene expression abnormalities (Lamouille et al. 2014). Downregulation of epithelial cadherin (E-cadherin) and β-catenin, and upregulation of neural cadherin (N-cadherin) and vimentin are hallmarks for the EMT process (Lamouille et al. 2014).

The progression of cancer is accompanied by the formation of novel blood vessels (Pluda et al. 1997). Angiogenin (ANG), a 14 kDa protein, is a member of the ribonuclease A superfamily (RNASE5) (Fett et al. 1985). It was proven that ANG is crucial for angiogenesis and neovascularization (Fett et al. 1985). To date, ANG has been shown to be upregulated in multiple human cancers such as colorectal cancer, renal cell carcinoma, and squamous cell lung carcinoma (Bruno et al. 2018; Kandori et al. 2018; Xu et al. 2016). ANG was reported to be involved in a wide range of biological functions including rRNA production, cell survival, proliferation, migration, and tumor angiogenesis (Bruno et al. 2018; Kandori et al. 2018; Xu et al. 2002; Xu et al. 2016). ANG could also activate multiple cancer-related signaling pathways including the nuclear factor-kappa B pathway, the phosphoinositide 3-kinase/Serine/threonine kinase/mechanistic target of rapamycin pathway, and the ERK1/2 pathway (Miyake et al. 2015; Peng et al. 2014; Xia et al. 2015). So far, whether ANG could regulate the EMT of HCC cells and the biological mechanism remains not fully understood.

Here, we investigated the role of ANG on cell proliferation and migration of HCC cells. Furthermore, we examined whether high mobility group AT-hook 2 (HMGA2) could be targeted by ANG. Moreover, we investigated whether ANG was an inducer of EMT in HCC and enhances the metastatic potential.

## 2. Investigations and results

### 2.1. ANG expression was increased in HCC cells

The methods of qRT-PCR and western blot were conducted to measure ANG expression in HCC cell lines and L02 cell line. qRT-PCR showed that the mRNA expression level of ANG was significantly higher in HCC cell lines than in the L02 cell line (Fig. 1A). Similarly, western blot results showed higher ANG protein levels in HCC cell lines compared to the L02 cell line (Fig. 1B).

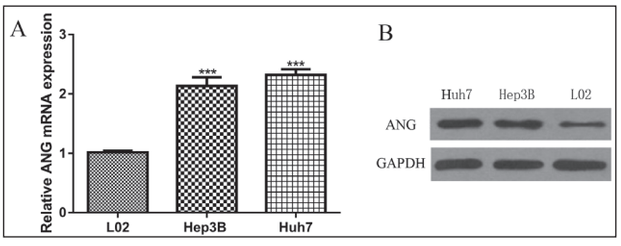


Fig. 1: Quantitative real-time polymerase chain reaction (qRT-PCR) and western blot were conducted to measure the A, mRNA, and B, protein levels of ANG in HCC cell lines and normal cell line L02. (***P<0.001) ANG: angiogenin; mRNA: message RNA; HCC: hepatocellular carcinoma.

### 2.2. ANG regulates HCC cell proliferation and migration in vitro

To investigate the role of ANG in HCC, we conducted loss-of-function experiments on HCC cells. qRT-PCR and western blot analysis results showed that ANG expression was significantly downregulated by si-ANG in both mRNA and protein levels,

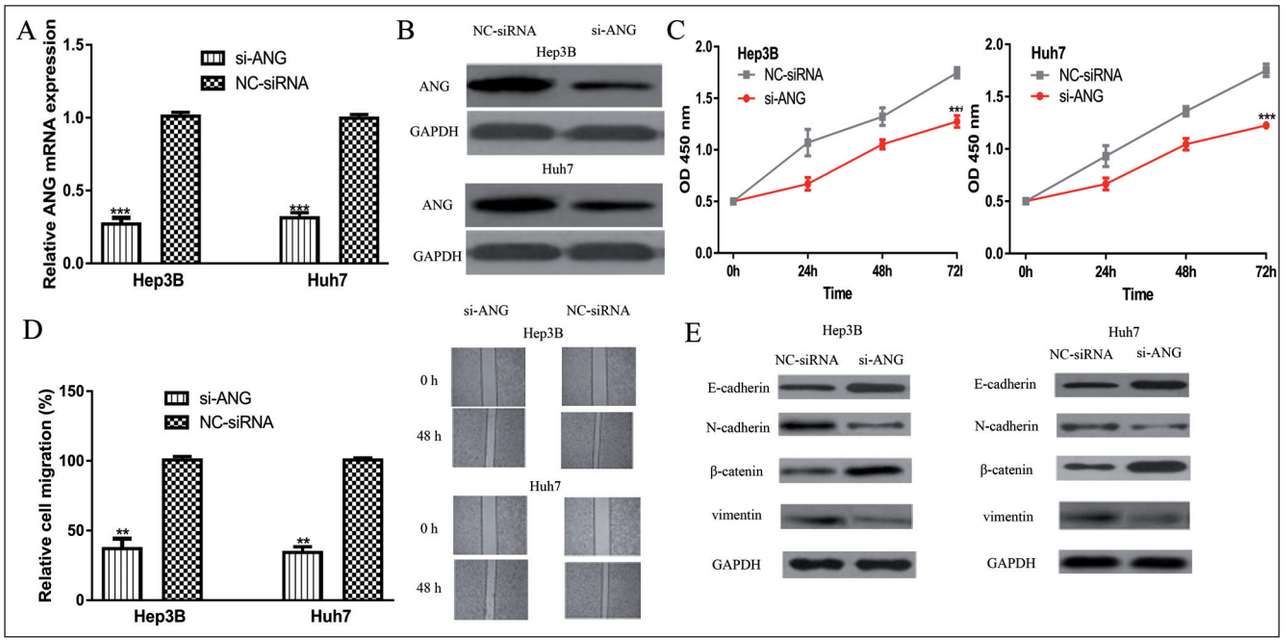


Fig. 2: Downregulation of ANG inhibits proliferation, migration, and EMT of HCC cells. A, ANG mRNA expression level, B, ANG protein expression level, C, Cell proliferation, D, Cell migration, and E, E-cadherin, vimentin, β-catenin, and N-cadherin expression level in HCC cells transfected with si-ANG or NC-siRNA. (***P<0.001) ANG: angiogenin; mRNA: message RNA; HCC: hepatocellular carcinoma; EMT: epithelial-mesenchymal transition; si-ANG: small interfering RNA targeting ANG; NC-siRNA: negative control siRNA; E-cadherin: E-cadherin; N-cadherin: neural cadherin.

which revealed the successful transfection of si-ANG (Fig. 2A and 2B). CCK-8 assay showed that cell proliferation rate was higher in the NC-siRNA group than in the si-ANG group (Fig. 2C). Using wound-healing assay, we found cell migration ignificantly inhibited by si-ANG compared with NC-siRNA (Fig. 2D).

### *2.3. ANG regulates the EMT of HCC cells*

We further investigated the effect of ANG on the EMT process of HCC cells. It was observed that si-ANG transfection increased the expression of E-cadherin and β-catenin but decreased the expression of N-cadherin and vimentin in both cell lines (Fig. 2E). These results demonstrated that ANG could promote EMT of HCC cells.

### *2.4. ANG directly regulates HMGA2 expression in HCC*

si-ANG transfection decreased the protein levels of HMGA2 (Fig. 3A). Luciferase activity reporter assay showed that downregulation of ANG decreased the luciferase activity of cells transfected with HMGA2-WT (Fig. 3B). Downregulation of ANG did not alter the luciferase activity of cells transfected with HMGA2-MT (Fig. 3B).

### *2.5. HMGA2 mediates the effects of ANG on HCC proliferation and migration*

We then investigated whether the effects of ANG on HCC proliferation and migration were mediated *via* HMGA2. Therefore, we transfected the si-ANG and pcDNA3.1-HMGA2 into HCC cells. The transfection of pcDNA3.1-HMGA2 increased the levels of HMGA2 and reversed the effect of si-ANG on HMGA2 expression (Fig. 4A and 4B). Furthermore, it was found that pcDNA3.1-HMGA2 increased proliferation and migration of HCC cells (Fig. 4C and 4D). Moreover, the inhibitory effects of si-ANG could be abolished by pcDNA3.1-HMGA2 transfection (Fig. 4C and 4D). Western blot assay showed increased N-cadherin and vimentin expression, while E-cadherin and β-catenin expression was decreased after pcDNA3.1-HMGA2 transfection (Fig. 4E).

## 3. Discussion

Cancer is still a global health burden and therefore researchers have tried to fight with it from different angle, identify the genes associated with cancer progression or seek for novel treatment

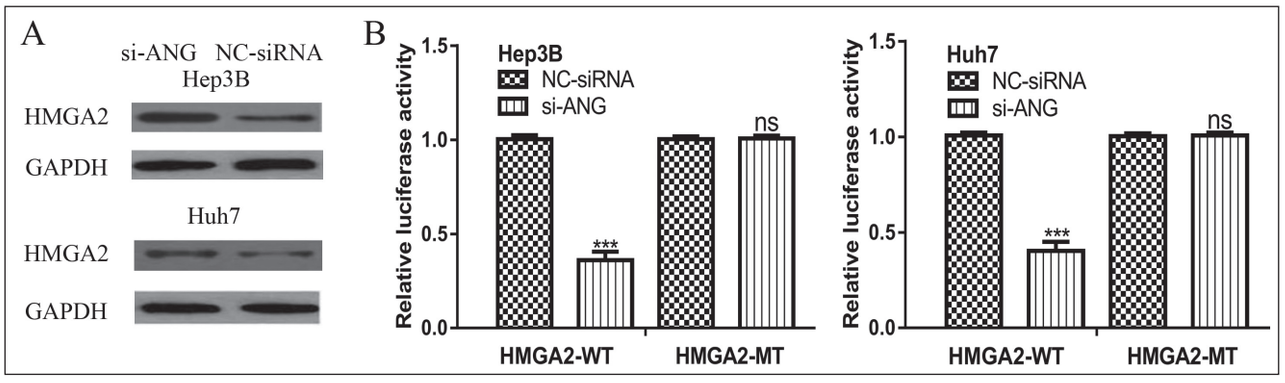


Fig. 3: HMGA2 was a target of ANG. A, HMGA2 protein expression in cells transfected with si-ANG or NC-siRNA. B, Luciferase activity was repressed by si-ANG in cells transfected with HMGA2-WT but not HMGA2-MT. (ns not significant, ***P<0.001) ANG: angiogenin; si-ANG: small interfering RNA targeting ANG; HMGA2: high mobility group AT-hook 2; WT: wild-type: MT: mutant.

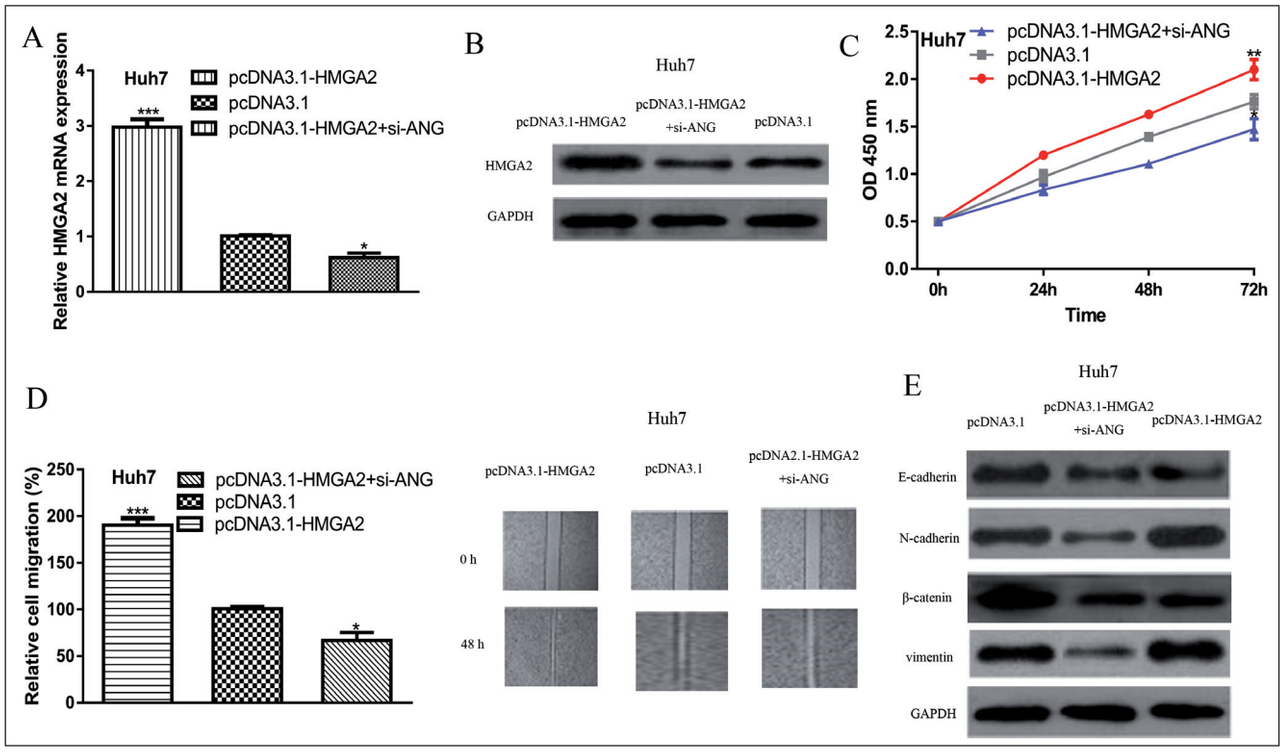


Fig. 4: HMGA2 was a mediator of ANG in regulating HCC cell proliferation, migration and EMT. A, HMGA2 mRNA expression level, B, HMGA2 protein expression level, C, Cell proliferation, D, Cell migration, and E, E-cadherin, vimentin, β-catenin, and N-cadherin expression level in HCC cells transfected with pcDNA3.1, pcDNA3.1-HMGA2, or pcDNA3.1-HMGA2 and si-ANG. (ns not significant, *P<0.05, ***P<0.001) ANG: angiogenin; si-ANG: small interfering RNA targeting ANG; HMGA2: high mobility group AT-hook 2; mRNA: message RNA; HCC: hepatocellular carcinoma; E-cadherin: E-cadherin; N-cadherin: neural cadherin.

measures to achieve targeted therapy (Tang et al. 2018a; Tang et al. 2018b; Zhao et al. 2018). ANG, a protein capable to regulate angiogenic, was reported to have other biological activities other than angiogenesis (Hisai et al. 2003). In this work, we demonstrated that ANG expression was significantly higher in HCC cell lines compared with the normal cell line L02 in both mRNA and protein level. We were further interested to investigate the biological role of ANG in HCC using loss-of-function experiments. The successful transfection of si-ANG was validated by qRT-PCR and western blot. Cells were divided into two groups: 1) si-ANG groups, cells transfected with si-ANG; and 2) NC-siRNA group, cells transfected with NC-siRNA. CCK-8 assay and wound-healing assay revealed that HCC cells transfected with si-ANG displayed significantly lower cell proliferation and cell migration rate compared with NC-siRNA. Meanwhile, we examined the effects of ANG on the marks of EMT process. Our results showed that downregulation of ANG inhibits the EMT progress as the expression of E-cadherin and β-catenin was increased but the expression of N-cadherin and vimentin was decreased. These results suggested that ANG could promote HCC cell proliferation, migration, and EMT *in vitro*.

HMGA2 was reported as an oncogene and to be involved in the development of malignant cancer (Hawsawi et al. 2018; Xing et al. 2018). HMGA2 expression was shown capable to the expression of a large number of target genes and signal pathways (Hawsawi et al. 2018). Interestingly, HMGA2 could target the MAPK pathway to regulate the EMT of prostate cancer (Hawsawi et al. 2018). Therefore, we presumed that AGN might regulate proliferation, migration, and EMT of HCC cells through regulating HMGA2 expression. Here, we found that downregulating ANG could influence the expression level of HMGA2 in HCC cells. Moreover, the luciferase activity reporter assay revealed that si-ANG transfection could downregulate the luciferase activity of cells transfected with HMGA2-WT. Functional experiments showed that overexpression of HMGA2 could reverse the inhibitory effects of si-ANG on HMGA2 expression levels, HCC cell proliferation, and cell migration *in vitro*. These results demonstrated that ANG plays a crucial role in tumor development of HCC *via* targeting HMGA2.

In conclusion, we described that ANG regulates HCC cell proliferation, migration, and EMT process through targeting the expression of HMGA2. Therefore, ANG has the potential to be developed as a therapy target for HCC. Our findings will advance our understanding of HCC progression biology.

## 4. Experimental

### *4.1. Cell culture*

HCC cell lines (Hep3B and Huh7) and normal cell line (L02) purchased from American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10 % fetal bovine serum (FBS, Invitrogen), 100 µg/ml streptomycin, and 100 units/ml penicillin (Beyotime, Haimen, Jiangsu, China) in an incubator maintained at 37 °C with 5 % of $CO_2$.

### *4.2. Cell transfection*

Small interfering RNA targeting ANG (si-ANG) and the corresponding negative controls (NC-siRNA) were purchased from GenePharma (Shanghai, China). Open reading frame of HMGA2 (pc-DNA3.1-HMGA2) and pcDNA3.1 were purchased from GenScript (Nanjing, Jiangsu, China). $2 \times 10^5$ cells were incubated overnight to achieve 50 %-60 % confluence, and Lipofectamine 2000 reagent (Invitrogen) was used for transfections according to the manufacturer's instructions.

### *4.3. Quantitative real-time polymerase chain reaction (qRT-PCR)*

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen). qRT-PCR was performed using SYBR Premix Ex Taq kit (TaKaRa, Dalian, Liaoning, China) at an ABI 7500 equipment (Applied Biosystems, Foster City, CA, USA). The primers used in this work were summarized in the Table. The qRT-PCR procedures were as follows: 95 °C for 30 s (1 cycle) and 95 °C for 5 s and 60 °C for 30 s (40 cycles). Relative expression levels of target genes were normalized to GAPDH and analyzed using $2^{-\Delta\Delta Ct}$ method.

**Table: Primers sequence used in this study**

| Genes | Sequences |
|---|---|
| ANG | Forward: 5'-GGACTTGTTCTGAGGCCGAG-3'<br>Reverse: 5'-CCAGCACGAAGACCAACAAC-3' |
| HMGA2 | Forward: 5'-AGCAGCAGCAAGAACCAACC-3'<br>Reverse: 5'-CCTGAGCAGGCTTCTTCTGA-3' |
| GAPDH | Forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3'<br>Reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' |

### *4.4. Western blot*

Total protein was extracted using RIPA cell lysis buffer (Beyotime). Bicinchoninic acid (BCA) Protein Assay Kit (Beyotime) was applied to analyze protein concentration. Then, protein samples were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocked with fat-free milk, the primary antibodies (anti-ANG: ab139947, anti-HMGA2: ab97276, anti-E-cadherin: ab40772, anti-vimentin: ab92547, anti-β-catenin: ab32572, anti-N-cadherin: ab18203, anti-GAPDH: ab181602, all from Abcam, Cambridge, MA, USA) were applied to incubate with the membranes at 4 °C for overnight. After washed with tris-buffered saline and Tween 20, the membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (ab6721, Abcam) at 37 °C for 1 h. Finally, the bands were visualized by BeyoECL kit (Beyotime) and analyzed with Image J 1.42 software (NIH, Bethesda, MD, USA).

### *4.5. Cell proliferation assay*

Cell Counting Kit-8 (CCK-8, Beyotime) was used to measure cell proliferation rate. 2,000 cells/well were incubated in 96-well plate and incubated for 0, 24, 48, and 72 h before adding 10 µl CCK-8 solution to the well. The plate was further incubated for 2 h before optical density measurement at 450 nm using a plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

### *4.6. Cell migration assay*

$1\times10^6$ cells were seeded into six-well plates and incubated 1 h with mitomycin. After creating a scratch at cell surface using pipette, the cells were washed with phosphate-buffered saline and incubated in serum-free DMEM. At 0 and 48 h, cells were observed and captured under a microscope to measure cell migration.

### *4.7. Luciferase assay*

The 1 to 1500 bp of the transcription start site (TSS) of HMGA2 gene was synthesized by GenScript and cloned into psiCHECK-2 basic vector (Promega, Madison, WI, USA) and named as HMGA2-WT. The HMGA2-MT was built by inserting the synthesized 1 to 846 bp and 948 to 1500 bp of TSS of HMGA2 gene into psiCHECK-2 basic vector (Promega). Cells were co-transfected with HMGA2-WT or HMGA2-MT and sh-ANG or NC-shRNA. Luciferase activity was measured at 48 h after transfection with renilla luciferase activity as internal control using the Dual-Luciferase Reporter Assay (Promega).

### *4.8. Statistical analysis*

Data are presented as mean±standard deviation and analyzed with SPSS v 19.0 (SPSS Inc., Chicago, IL, USA). Group differences were analyzed using either one-way ANOVA and Tukey post-hoc test or Student's t-test. P-value less than 0.05 was considered statistically significant.

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