Down-Regulated MAC30 Suppresses Lung Cancer Invasion and EMT by Inhibiting Wnt/β-Catenin Signaling Pathway

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Abstract

Backgrounds: Over-expressed meningioma-associated protein (MAC30) was proved to be a biomarker for worse prognosis in non–small cell lung cancer (NSCLC). However, the regulated mechanism of MAC30 in epithelial–mesenchymal transition (EMT) and lung cancer invasion is unknown.

Methods: Transformed growth factor (TGF-β) was used to induce EMT in A549 cells in vitro. MAC30 siRNA was transfected into cells to silence the gene expression. Real-Time PCR was prepared to assess the levels of MAC30 mRNA. Methyl thiazolyl tetrazolium (MTT) and Transwell invasion assays were performed to study the proliferation and invasion of A549 cells. Expression of MAC30, EMT-related proteins, Wnt/β-catenin signal and its downstream factors were explored by ELISA.

Results: We found enhanced MAC30 expression in A549 cells. MAC30 Knockdown inhibited TGF-β-induced lung cell proliferation and invasion. Furthermore, elevated levels of mesenchymal markers (N-cadherin, vimentin) and decreased levels of epithelial markers E-cadherin in A549 cells with TGF-β incubation were reversed by MAC30 siRNA. Finally, MAC30 knockdown significantly suppressed TGF-β-upregulated protein levels of Wnt/ β-catenin signaling and its downstream genes (surviving, c-myc and cyclin D1).

Conclusions: We firstly confirm that MAC30 knockdown limits lung cancer growth and EMT through inhibiting the activation of Wnt/β-catenin pathway.

Introduction

Lung cancer, the most frequently diagnosed cancer, remains a leading contributor to cancer-related death across the globe. In particular, Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases, mainly including adenocarcinoma and squamous cell carcinoma (1). Traditionally, platinum-based chemotherapy is accepted as the main clinical treatment for lung cancer, although with limited less than 1-year median overall survival (2). Unfortunately, lung cancer patients received chemotherapy always have toxic side effects, such as nausea and vomiting, sore mouth, diarrhea, hepatotoxicity and immunosuppression, leading to discontinuation of clinical treatments (3). With an improved understanding of the oncogenic aberrations, lung cancer is sub-classified by driver gene mutation subset. Accordingly, targeted therapy due to its low toxicity, specificity and efficiency, widely enters into the management of NSCLC (4). To data, recent advances of tumor-targeted therapy have occurred in clinical therapy, most of advanced NSCLC patients still uncured with a 5-year overall survival rate of less than 15% (5). It’s not fully elucidated in the molecular mechanism of lung cancer. Therefore, a deep identifying of genic association with the growth of cells keeps a critical need in fully understanding of lung cancer.

It’s widely accepted that epithelial–mesenchymal transition (EMT) plays a role event in lung cancer initiation and progression (6). EMT, a complex molecular metastasis, is characterized by loss of epithelial cell adhesion protein E-cadherin and cytokeratins and gain of mesenchymal-associated molecules N-

cadherin and vimentin. EMT is recognized as a research hotspot in order to be regulated and used as anti-metastasis therapies. Previous studies reported that activation of Wnt/β-catenin signaling enhanced proliferation of lung cancer cells (7). A recent study has confirmed that blocking Wnt/β-catenin activation significantly suppressed cellular proliferation, migration, and invasion of lung cancer A549 cell (8). Zhang et al., further found that the inhibitor of Wnt/β-catenin signaling could reversed EMT process, together with decreasing EMT-related transcription factors (9). As well known, transformed growth factor (TGF-β) to epithelial cells is a convenient way to cause EMT (10). However, the mechanisms of EMT activation remain unclear, a deeper understanding in metastasis of lung cancer is essential.

Meningioma-associated protein (MAC30) locating at 17q11.2 was confirmed as a member of the insulin-like growth factor-binding protein family. Besides regulating cholesterol and lipid metabolism, MAC30 was originally identified as an increased expression gene in human meingiomas (11). Indeed, over-expressed level of MAC30 protein acting as a tumor promoter was found in oral, breast, colon, gastric, and esophageal cancers (12–14). Otherwise, down-expressed MAC30 may act as a suppressor in pancreatic and renal cancers (15). Our previous studies have reported that NSCLC with elevated MAC30 levels resisted to platinum-based chemotherapy and exhibited worse survival (16). We already confirmed that MAC30 was a valuable independent predictable factor for poor tumor differentiation and short survival in lung cancer patients (17). Although Ma et al., recently reported that down-regulation of MAC30 got an inhibited effect on invasion and EMT by suppressing Wnt/β-catenin signaling in breast cancer cells (18). The molecular effects of MAC30 on EMT and signaling in lung cancer are still unknown.

In this study, we firstly investigate the growth characteristic and EMT in A549 cells with MAC30 gene silencing, in order to explore the precise mechanisms of MAC30-regulated EMT via Wnt/β-catenin signaling in lung cancer.

Materials And Methods

Cell culture and reagents

Human lung cancer cell line (A549) and normal lung epithelial cells (BEAS-2B) were purchased from Type Culture Collection (Academy of Science, China). The A549 cells were plated in six-well plates and grown until 70% to 80 confluences. The cells were grown in Dulbecco's modified Eagle's medium (Gibco, USA) maintained with 10% fetal bovine serum (Gibco, USA). A549 cells at 4 to 6 passages were used in the experiments. Briefly, cells were treated with TGF-β1 (5 ng/ml) for 48 h. In transfected experiments, cells treated with siRNA for MAC30 and siRNA-negative control (SantaCruz, USA) were performed according to the manufacturer's instructions. After 48 hours of transfection, the efficiency of knockdown was analyzed by ELISA and qPCR. Whole-cell lysates were prepared for protein and mRNA collection.

Proliferation assay

Cells at $1*10^4$ density were seed into 96-well culture plates. Cell proliferation assay was carried out with a Cell Methyl thiazolyl tetrazolium (MTT) Kit (Toscience, China) according to the manufacturer's

instructions. Experiments were repeated in triplicate and results were analyzed by paired t-test.

Transwell invasion assay

In order to assess the ability of invasion, transwell invasion assay were peformed as previously described [9]. Cells (5×104/well) were planted into 24-well Transwell chamber that were coated with Matrigel (BD Bioscience, USA) according to the manufacturer's protocols. Briefly, cells in the upper chamber were suspended into serum-free medium, while cells in the lower chamber were seeded into medium with 10% Fetal Bovine Serum (FBS). According to the manuscript of transwell invasion assay kit (R&D, USA), the absorbance in the wash solution was then detected at a wavelength of 540 nm to quantify the number of cells formed. All assays were independently repeated three times.

Real-Time polymerase chain reaction (Real-Time PCR)

In order to determine the mRNA levels of MAC30, total RNA from cells was extracted using TRIzol reagent (Invitrogen, Inc). Real-time PCR was taken out as described previously[16]. Sequences of specific primers were prepared as MAC30: sense: 5'- GGCAGCAGAGGAGTAGCTTGA-3', antisense: 5'-GCTTGCTGGCGCTAAAAGG-3' and GAPDH: sense: 5'-CATGGCCTTCCGTGTTCCTA-3', antisense:5'-GCGGCACGTCAGATCCA-3'. The reactions were undertaken at 95°C for 30 seconds, then 35 cycles of 95°C for 20 seconds, 55°C for 15 seconds, and 72°C for 20 seconds, and a final extension at 72°C for 10 minutes. Specificity of the PCR product was verified by examination of the dissociation reaction plots. Data were normalized to GAPDH.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA kits for MAC30, E-cadherin, N-cadherin, c-myc, β-catenin, survivin and GAPDH were purchased from Abcam in USA. And kits for Wnt-1 and Wnt-2 were from SantaCruz in USA. The experiment was performed three times acrrording to the manufacturer's recommendations.

Statistical analysis

These data were presented as the Mean ± standard error deviation. Differences between groups were calculated by one-way ANOVA analysis or Student's t-test with SPSS software. A value of $P < 0.05$ was considered statistically significant. When applicable, the values were normalized to a value of 1 as the corresponding controls.

Result

MAC30 is overexpressed in the human A549 cells

In order to investigate the molecular roles of MAC30 in regulating lung cancer cell invasion, it's firstly necessary to identify the MAC30 levels in A549 cells. In this study, comparison of MAC30 mRNA and protein in lung normal cells and cancer A549 cells was assessed. The results from Real-Time PCR

obviously showed the elevated expression of MAC30 mRNA in A549 cells ($p<0.05$, Fig.1A). Furthermore, we used ELISA to find a comparatively higher level of MAC30 protein in A549 cells ($p<0.05$, Fig.1B). The date confirmed the overexpression of MAC30 at mRNA and protein levels.

The biological roles of MAC30 knockdown on the proliferation and invasion of A549 cells

To understand the biological regulation of MAC30 on lung cancer, we evaluated the proliferative and invasive capacity of A549 cells transfected with MAC30 siRNA. The results from proliferation assay showed that knockdown of MAC30 significantly decreased TGF-β-induced unnormal proliferation in A549 cells ($p<0.05$, Fig.2A). The invasion function of A549 cells incubated with TGF-β via transwell assays was also remarkably suppressed in cells with MAC30 siRNA ($p<0.05$, Fig.2B). This information suggested that MAC30 may come into playing effects in mediating proliferation and invasion of A549 cells.

MAC30 knockdown inhibits EMT of A549 cells.

A previous study reported the close relationship between EMT and lung cancer metastasis (19). We investigated the expression of EMT-associated proteins through ELISA. With the previous study (20), we found that TGF-β elevated the expression of mesenchymal markers N-cadherin and vimentin at protein levels and decreased the levels of epithelial marker E-cadherin protein ($p<0.05$, Fig.3). This data confirmed the promoting roles of TGF-β on EMT in A549 cells. In order to investigate the regulated effect of MAC30 on EMT of lung cancer cells, we used MAC30 siRNA to make the target gene knockdown models in A549 cells. Indeed, we found that decreased MAC30 in A549 cells obviously weakened TGF-β-caused EMT in A549 cells, as maintaining the suitable levels of N-cadherin, vimentin and E-cadherin proteins ($p<0.05$, Fig.3). The information suggested that MAC30 knockdown may act as an inhibitor in EMT process of A549 cells.

MAC30 knockdown inhibits Wnt/β-catenin pathway in A549 cells

Aberrant activation Wnt/β-catenin signaling improved A549 cell proliferation, invasion and EMT, which accelerated lung cancer progression (9). We found that TGF-β significantly increased the expression of Wnt1, Wnt2, β-catenin protein levels in A549 cells, including survivin, c-myc and cyclin D1 as the downstream targets of Wnt/β-catenin signaling ($p<0.05$, Fig.4). To confirm whether MAC30 affected EMT by regulating Wnt/β-catenin signaling, we treated with A549 cells with MAC30 siRNA. ELISA revealed that the elevated levels of Wnt1 and Wnt2 were suppressed in TGF-β-activated A549 cells with MAC30 siRNA ($p<0.05$, Fig.4). Furthermore, the increasing levels of β-catenin were also inhibited. Next, the knockdown of MAC30 also blocked the TGF-β-enhanced expression of survivin, c-myc and Cyclin D1 proteins in A549 cells. Our data showed that knockdown MAC30-suppressed EMT and cellular proliferation and invasion were regulated through eliminating Wnt/β-catenin signaling activation.

Discussion

Previously, we already revealed for the first time that MAC30 overexpression predict a worse tumor differentiated stage and prognosis in NSCLC receiving adjuvant chemotherapy (16). Subsequently, we also further confirmed that MAC30 in pleural effusion could be a potential prognostic marker in NSCLC with malignant pleural effusion (17). The present study mainly found that knockdown MAC30 suppressed the activation of Wnt/β-catenin signaling, leading to the inhibition of lung cancer invasion, proliferation and EMT. This study provided biological function of MAC30 gene on molecular mechanism in lung cancer.

As a newly identified protein, the roles of MAC30 on cancer metabolism are unclear. As a tumor suppressor, MAC30 was low expressed in pancreatic and renal cancers (15), while as a promoter was found in oral, breast, colon, gastric, and esophageal cancers (12–14). We firstly confirmed the stronger levels of MAC30 in lung cancer A549 cells than those in normal lung epithelial cells. It may hint the anti-lung cancer roles of MAC30, which were consistent with our previous research (16,17).

In cancer microenvironment, normal lung epithelial cells underwent EMT leading to enhanced cellular adhesion, apical-basal polarity and motility, which induced the increased capacity of invasion and proliferation (18–20). Once cancer cells experienced EMT have metastasized, it's easier to migrate to neighboring or distant organs (21–22), with more difficult treatment and more serious survival (23). To data, the stimulated EMT has been accepted as the favored explanation of distant promoter in lung cancer (19). EMT is recognized as a research hotspot in order to be regulated and reversed as anti-metastasis therapy. Among several factors causing EMT,TGF-β was confirmed as a major inducer in cancer progression (24). In our present study, we prepared molecular markers characteristic of A549 cells to confirm the transitioning of EMT. EMT is characterized by loss of epithelial cell adhesion protein E-cadherin and cytokeratin and gain of mesenchymal-associated molecules N-cadherin and vimentin (25). In our present study the decreased expression of E-cadherin, also with increased Vimentin and N-cadherin protein levels suggested the TGF-β-induced EMT in A549 cells. Indeed, Loss of E-cadherin was recognized as a necessary step to weaken cell-to-cell adhesion and to heighten cellular migration. Over-expressed Vimentin was identified to likely facilitate the transition of cells to a more elongated phenotype, resulting into accelerated tumor growth and invasion (26). The previous studies reported that N-cadherin expression was linked to oncogenicity (27,28). Luis et al., confirmed the pro-oncogenic roles of N-cadherin in lung cancer cells (29). In our study, we also confirmed that A549 cells became more proliferation and invasion Through TGF-β-induced EMT. As a concrete manifestation, MAC30 knockdown successfully reversed the change of molecular markers as ubiquitous features of TGF-β-caused EMT in A549 cells. Subsequently, MAC30 knockdown in style blocked the unexpected capacity of proliferation and invasion in TGF-β-activated A549 cells. To our known, we firstly verified the correction effects of MAC30 knockdown on TGF-β-induced EMT in lung cancer cells. The anticancer roles of MAC30 knockdown on repressing cancer cellular liveness were anticipated to be used in tumor treatment, based on the deeply molecular mechanism understanding and clinical trials.

Previous studies have shown that EMT in lung cancer could be activated by Wnt/β-catenin pathway (30). Wnt/β-catenin signaling took part in regulation of cell proliferation, migration, and invasion. Aberrant

Wnt/β-catenin signaling activation contributed to lung cancer initiation, progression, and metastasis (31). Sun et al., found that Myeloid cell-derived LL-37-induced β-catenin phosphorylation activiated Wnt/β-catenin signaling, which promoted lung cancer growth (7). Meanwhile, it's significant interested that inhibited β-catenin-induced activation of Wnt/β-catenin signaling blocked EMT and lung cancer invasion (9,32).

As the key downstream targets of Wnt/β-catenin pathway, c-myc and CyclinD1 always showed stronger levels on proteins in cancers, including lung cancer. Activated Wnt/β-catenin signaling promted the transcription of c-myc and Cyclin D1 proteins involved in cell proliferation (33). It was reported that Wnt1 and Wnt2 as Wnt family members were overexpressed in NSCLC cell lines, which promoter the development of lung cancer (34). The previous evidence provided important information that c-myc controlled cancer cell proliferation, and c-myc was associated with unfavorable survival of lung cancer patients (35). A vitro study confirmed that overexpression of Cyclin D1 promoted lung cancer cell proliferation and invasion (36). Cyclin D1 also was identified to activate the occurrence of EMT. The latest research suggested that cir-ITCH display the antitumor roles in lung cancer by suppressed the mRNA expression of c-myc and Cyclin D1 (8). As an important downstream target of Wnt/ β-catenin pathway, survivin was proved to be a promoter on cellular migration, invasion, and metastatic progress (37). More data concealed that the pivotal roles of survivin on cell survival in cancer cellular network (38). The previous paper reported an effect of activated Wnt-induced EMT in lung carcinoma cell lines (30). Indeed, activated Wnt signaling increased the downstream factor expression of vimentin as a mesenchymal marker and EMT promoter. Importantly, β-catenin participates in the process of cell proliferation, dedifferentiation, inhibition of apoptosis, and cancer progression via regulating downstream effectors. Not only that, β-catenin as a mesenchymal marker was proved to induce EMT (39). It's widely accepted that TGF-β as a regulator of EMT in several human cancers is widely acts as a facilitator of EMT in vitro (10). Gavert et al., found that TGF-β successfully induced EMT in A549 cells, largely in the form of increased expression of Wnt/β-catenin (40). Herein we identified the induction of TGF-β to A549 cells in vitro, reflecting as increased expression of mesenchymal markers (vimentin, N-cadherin) and decreased expression of epithelial markers (E-cadherin). Mechanically, we showed that elevated levels of Wnt1、Wnt2 and β-catenin proteins were confirmed in A549 cells with TGF-β incubation. Not surprisingly, TGF-β enhanced the expression of c-myc, Cyclin D1 and survivin, as the following targets of Wnt/β-catenin signaling. The data identified that TGF-β deteriorated the EMT and lung cancer cell metastasis through the Wnt/β-catenin signaling activation. A recent paper reported the regulation role of MAC30 siRNA on EMT in breast cancer cells (18). More importantly, we found that knockdown MAC30 in A549 cells blocked the EMT process, following with activiation of Wnt/β-catenin signaling. And the intensive levels of c-myc, Cyclin D1 amd survivin were also reversed. The following response triumphantly eliminated the undesirable cellular invasion and proliferation. In this study, we firstly confirmed knockdown MAC30 inhibited EMT via Wnt/β-catenin signaling, resulting into maintain the normal cellular growth.

Conclusion

Our data are the first to report that MAC30 knockdown weakened the EMT initiation through regulating Wnt/β-catenin and its downstream genes, maintained the A549 cell biological behavior. Therefore, our information reminded that MAC30 may represent a therapeutic target for NSCLC metastasis.

Abbreviations

NSCLC, Non-small cell lung cancer; EMT, epithelial–mesenchymal transition; TGF-β-1, transforming growth factorβ-1; MAC30, Meningioma-associated protein; MTT, Methyl thiazolyl tetrazolium; FBS, Fetal Bovine Serum; RT-PCR, Real-Time-polymerase chain reaction; ELISA, Enzyme Linked Immunosorbent Assay.

Declarations

Ethics approval and consent to participate

This study was approved from the institutional review boards (IRBs) at the Yixing People's Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Jingzhu Zhou and Hui Ding wrote the manuscript. Jingzhu Zhou and Jixiu Hai carried out the study. Ruhua Chen and Yan Fen prepared the samples. All authors read and accepted the manuscript.

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Availability of Data and Materials

The authors ensure the availability of supporting data and materials.

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Figures

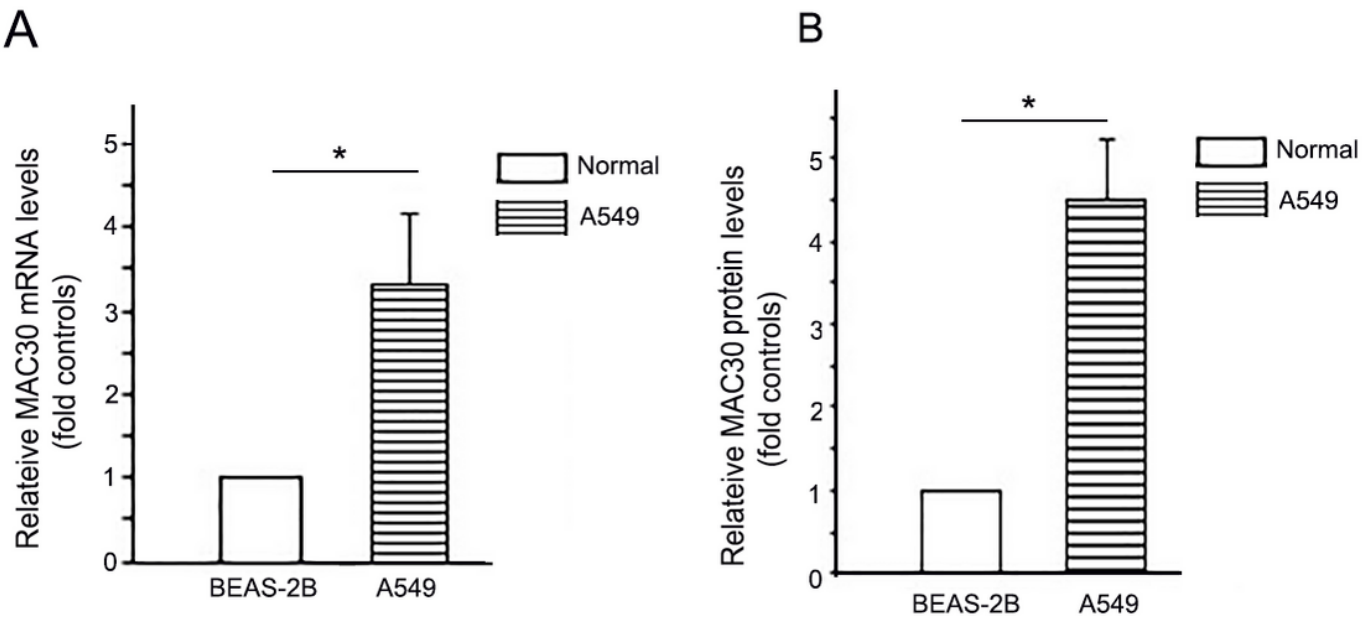

Figure 1

Overexpression of MAC30 gene in lung cancer line and lung epithelial cells. (A) Compared in BEAS-2B cells, MAC30 mRNA was at a higher level in A549 cells by Real-Time PCR. (B) The expression of MAC30 protein was comparatively more inA549 cells than in BEAS-2B cells. Data are shown as mean ± SE of three independent experiments. * $P < 0.05$.

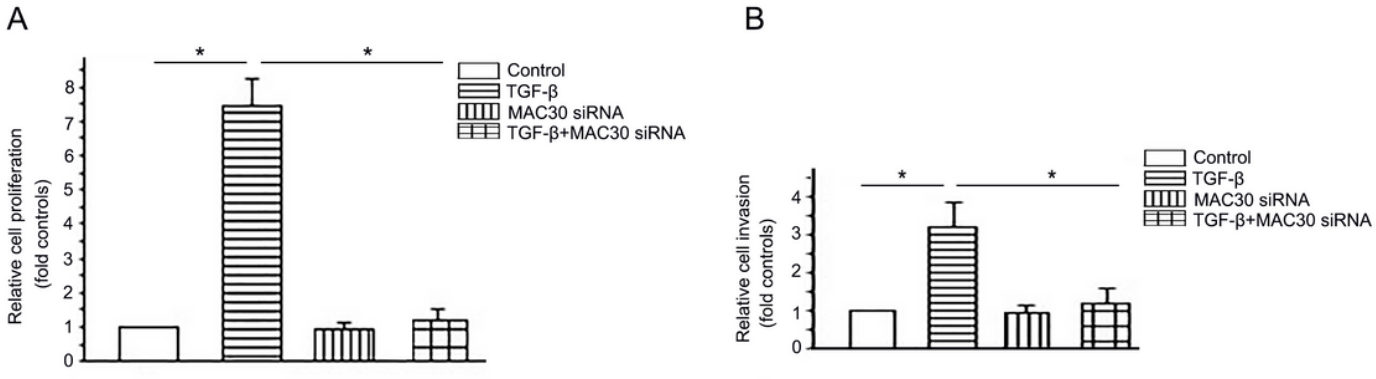

Figure 2

MAC30 siRNA treated A549 cells had an ability to suppress TGF-β1-induced proliferation and invasion. (A) MTT showed that MAC30 siRNA in A549 cells showed an inhibition of TGF-β1-increased proliferation. (B) The invasion of cells was assessed by a transwell assay. MAC30 knockdown significantly inhibited TGF-β1-induced invasion of A549 cells. Data are shown as mean ± SE of three independent experiments. * P < 0.05.

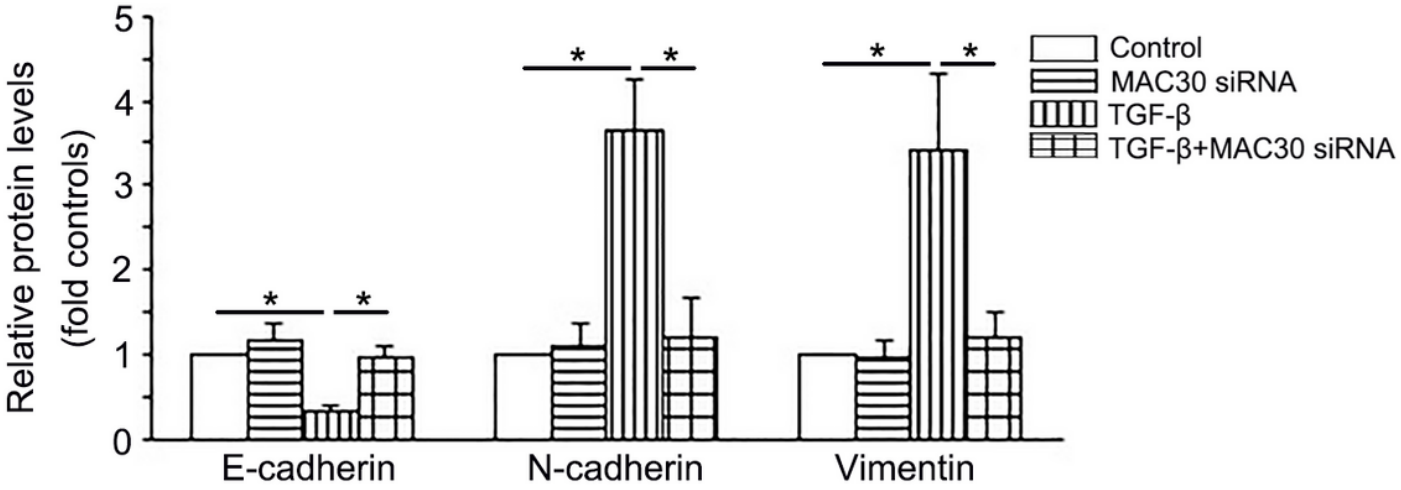

Figure 3

The effect of MAC30 siRNA on TGF-β1-induced EMT in A549 cells. ELISA showed that the increased levels of mesenchymal markers (N-cadherin, vimentin) and the decreased expression of epithelial marker (E-cadherin) in TGF-β1-treated A549 cells were reversed by MAC30 siRNA transfection. Data are shown as mean ± SE of three independent experiments. * P < 0.05.

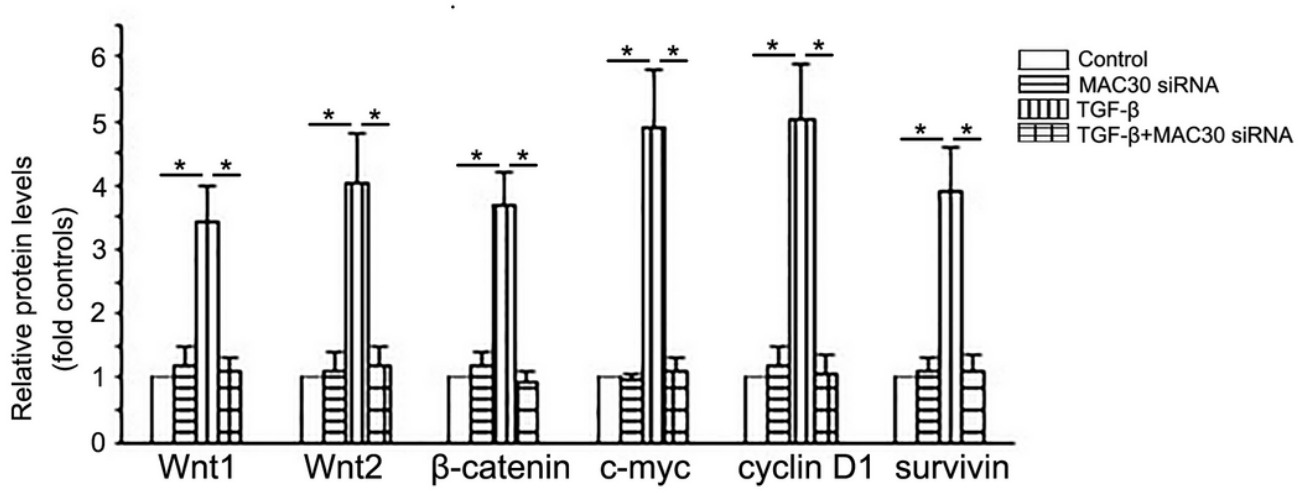

Figure 4

MAC30 siRNA in A549 cells inhibited Wnt/β-catenin signaling pathways and its downstream factors. TGF-β1 increased the protein levels of Wnt1, Wnt2, β-catenin, c-myc, cyclin D1 and surviving detected by

ELISA in A549 cells. And the protein levels were decreased in MAC30-knockdown A549 cells than the controls. Results are representative of three different experiments and data are expressed as mean ± SE. * $P < 0.05$.