Abstract. The present study aimed to determine the effect of Twist downregulation on the proliferation, apoptosis and migration of human endometrial carcinoma Ishikawa cells. Endogenous expression of the Twist transcription factor was knocked down by delivery of Twist-targeting small interfering RNA (siRNA). Changes in the expression of epithelial-mesenchymal transition biomarkers, namely epithelial (E)-cadherin, neural (N)-cadherin and Twist, were determined by western blot analysis. Cell cycle distribution and apoptosis were evaluated by flow cytometry. Cell proliferation and migration were analyzed using cell-counting and wound-healing assays, respectively. Transfection with Twist siRNA led to a significant reduction in the expression of Twist and N-cadherin (P<0.05), while significantly increasing the expression of E-cadherin, relative to negative control transfectants (all P<0.05). Proliferation was also significantly decreased in Ishikawa cells transfected with Twist siRNA (P<0.05), which was accompanied by an increased rate of apoptosis and cell cycle arrest at S-phase. In addition, Twist downregulation led to a significant reduction in cell migration (P<0.05). These data suggest that Twist serves a role in the regulation of cell proliferation and migration in Ishikawa cells and may represent a potential target for the treatment of human endometrial carcinoma.

Introduction

Endometrial carcinoma is one of the most prevalent gynecological malignancies (1). Despite advances in therapeutic approaches, many patients with endometrial carcinoma develop localized, recurrent and/or distant metastases (2). Therefore, it is important to identify the key regulators of endometrial carcinoma progression and metastasis to develop more effective therapies to treat endometrial carcinoma.

Epithelial-mesenchymal transition (EMT) is a crucial event in tumor invasion and metastasis, whereby epithelial cells lose their polarity and cell-cell contacts and undergo cytoskeletal remodeling (3). EMT is typically associated with a downregulation of epithelial (E)-cadherin and upregulation of neural (N)-cadherin (4), and decreases in E-cadherin expression are associated with a more infiltrative growth pattern in a variety of cancers, including non-small-cell lung cancer, prostate cancer and endometrial cancer (5-7). The transcription factor Twist has been identified as an inducer of EMT by its transcriptional repression of E-cadherin (8,9). Twist is also involved in tumor cell proliferation and survival (10) and it has been documented that Twist mediates aggressive phenotypes in human colorectal cancer cells, thus contributing to cell migration, invasion and chemoresistance (11). Twist also facilitates EMT and cell motility in breast cancer through the integrin β1 subunit-focal adhesion kinase/integrin-linked kinase signaling axis (12). Furthermore, during cervical carcinogenesis, Twist has been implicated in the induction of EMT by regulating the tumor growth factor-β/Smad3 signaling pathway (13). Therefore, Twist may be a promising target of anticancer therapies.

However, the biological functions of Twist in human endometrial carcinoma remain unknown. Through loss-of-function experiments, the present study aimed to elucidate the roles of Twist in the proliferation, migration, invasion and EMT of endometrial carcinoma cells.

Materials and methods

Cell culture. Human endometrial carcinoma Ishikawa cells were provided by Professor XiaoPing Wan (Department of Gynecology, Shanghai First People’s Hospital, Shanghai, China). Cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator at 37°C and 5% CO₂ for 48 h.
To the manufacturer's instructions, and cell proliferation was measured at 490 nm using an ELISA microplate reader (Bio-Rad Laboratories, Inc.).

Quantification of apoptosis by flow cytometry. Cell apoptosis was analyzed using an Annexin-V/propidium iodide (PI) assay. Briefly, cells were digested with 0.25% trypsin (Invitrogen; Thermo Fisher Scientific, Inc.), washed and resuspended in binding buffer. Annexin V-FITC and PI were then added (BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) and apoptotic cells were detected using a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) washed and suspended in staining buffer containing 1 µg/ml PI and 0.025 µg/ml Annexin V-fluorescein isothiocyanate. Double labeling was performed at room temperature for 10 min in the dark. Following incubation, the percentage of apoptotic cells was measured by flow cytometry FC500 (Beckman Coulter, Inc., CA, USA). The software used for analysis is Cytometer supplied by the instrument company.

Cell cycle analysis. Ishikawa cells (1x10⁶ cells/well) were plated with RPMI-1640 medium in 6-well plates (three wells per group) and treated with vehicle or honokiol (5 or 10 µM) for 48 h. For cell cycle assay, cells were washed twice with ice cold PBS, fixed in 70% ethanol at -20°C for 4 h and incubated with 50 µg/ml PI (Sigma-Aldrich; Merck KGaA). After fixation, cells were washed by phosphate buffer 2 times and then stained by 50 µg/ml PI in 500 µl PBS containing 100 µg/ml RNase A for 30 min. Cell cycle distribution was assessed by flow cytometry FC500 (Beckman Coulter, Inc., CA, USA) and analyzed by the software provided.

Wound healing assay. Ishikawa cells (1x10⁶ cells/well) were plated in 6-well plates and grown to 70-80% confluence with RPMI-1640 without fetal bovine serum for 24 h. Using a 200-μl pipette tip, a scratch wound was made through the cell monolayer. Cells were washed with PBS to remove any detached cells and incubated in RPMI-1640 with 10% fetal bovine serum at 37°C for 48 h. The distance between the two sides of the wound was measured with a light microscope before and after wound healing.

Statistical analysis. Data were expressed as the mean ± standard deviation and analyzed by one-way analysis of variance followed by a Tukey's multiple comparisons test. Statistical differences were determined using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) and P<0.05 was considered to indicate a statistically significant difference. All experiments have been replicated three times.

Results

To downregulate the expression of Twist in human endometrial carcinoma Ishikawa cells, three different Twist siRNAs were individually transfected into cells. Ishikawa cells without siRNA transfection were used as control group and cells with non-targeting control siRNA transfection were used as mock group to eliminate the effect of transfection treatment. As presented in Fig. 1, transfection with each of the Twist siRNAs lead to a marked reduction in the levels of Twist protein levels.
by 48 h post-transfection, relative to mock and control cells. As expected, expression of GAPDH was unaltered by Twist siRNA transfection. These results indicate that the RNA interference assay was specific and effective. Therefore, Ishikawa cells were transfected with a mixture of all three Twist siRNAs in subsequent experiments to determine the biological functions of Twist.

**Downregulation of Twist inhibits Ishikawa cell proliferation.** Results from the CCK-8 assay demonstrated that downregulation of Twist led to a significant reduction in the proliferation of Ishikawa cells, relative to mock cells transfected with non-targeting siRNA (P<0.05; Fig. 2). Flow cytometric analysis also indicated that knockdown of Twist significantly induced apoptosis (~4-fold) in Ishikawa cells compared with control and mock-transfected cells (P<0.05; Fig. 3A and B). In addition, Twist downregulation led to a marked decrease in the percentage of G0/G1 phase-cells and a concomitant increase in the percentage of S-phase cells, indicating an arrest of the cell cycle predominantly in the S-phase following Twist knockdown (Fig. 3C and D).

**Twist downregulation impairs Ishikawa cell migration.** The effects of Twist knockdown on the migration of Ishikawa cells were subsequently evaluated. In a wound-healing assay, it was observed that knockdown of Twist significantly attenuated the migration of Ishikawa cells, compared with control cells (P<0.05; Fig. 4).

**Twist downregulation affects the expression of EMT-related proteins.** The effects of Twist downregulation on the expression of EMT-related proteins were also evaluated. As depicted in Fig. 5, it was observed that Twist silencing significantly induced the expression of E-cadherin and significantly decreased the expression of N-cadherin, relative to control cells (both P<0.05).

**Discussion**

Previous results have indicated that Twist expression is correlated with the invasiveness of tumor cells and a poor prognosis in many solid cancers, including prostate cancer and hepatocellular carcinoma (8,14,15). In addition, Twist has the ability to trigger EMT in a variety of tumor cells, including prostate cancer and breast cancer (16). However, no previous results have identified a role of Twist in the invasion and metastasis of endometrial carcinoma. In the present study, it was determined that knockdown of Twist led to a significant upregulation in
E-cadherin and a significant downregulation in N-cadherin. A wound scratch assay also demonstrated that the migratory rate of Twist-knockdown cells was significantly lower than that of control cells. These results suggest the decreases in cell migration mediated by Twist silencing were associated with a reversion to an epithelial cell phenotype, resulting in an impaired migratory capacity in endometrial carcinoma cells.

Loss of E-cadherin expression is a critical component of EMT. A primary mechanism by which E-cadherin expression is inhibited is through the upregulation of its transcriptional repressors. Yang et al (8) documented that overexpression of Twist in human mammary epithelial cells inhibited the promoter activity of E-cadherin. In addition, Twist overexpression in mammary and prostate cancer cells leads to a decrease in E-cadherin expression, thus promoting EMT and cell migration (8,14). Consistent with these results, the current study identified a statistically significant inverse association between Twist and E-cadherin expression in endometrial carcinoma cells. Yang et al (8) also demonstrated that Twist may act as an EMT inducer, resulting in enhanced tumor invasion and metastasis in a model of breast cancer. Collectively, these results suggest that the delivery of Twist siRNA may be a potential therapeutic approach for the treatment of endometrial carcinoma.

Cell cycle control is the major regulatory mechanism of cell growth. In particular, activation of the tumor suppressor protein p53 serves a role in the regulation of cell cycle arrest and apoptosis (17). Many chemotherapeutic drugs and Chinese herbal medicines arrest the cell cycle and subsequently induce cell death (18,19). It has also been suggested that Twist has oncogenic properties. For instance, it was demonstrated that overexpression of Twist in rhabdomyosarcoma inhibits myc-induced apoptosis and interferes with the tumor suppressive effects of p53 (20). Furthermore, upregulation of Twist has been associated with malignant transformation in T-cell lymphoma (21) and forced expression of Twist may trigger resistance in human cancer cells against drugs that inhibit microtubule formation, including taxol and vincristine (14). Vichalkovski et al (22) demonstrated that phosphorylation of the transcription factor Twist-1 by protein kinase B at Ser42 inhibits the activity of p53 in response to DNA damage. Indeed, it has been demonstrated that Twist participates in development and progression of nasopharyngeal carcinoma and breast cancers (23,24). Therefore, the present study investigated the regulatory effect of Twist on endometrial carcinoma cells by delivering Twist siRNAs into Ishikawa cells. The growth of cells transfected with Twist siRNA was significantly decreased and subsequent assays indicated that the suppressive effects of Twist on cell proliferation occurred, at least in part, through disruption of the S/M transition.

In conclusion, the present results indicate that Twist affects the proliferation, migration and cell cycle distribution of endometrial carcinoma cells, all of which may be associated with its regulatory effects on EMT. These findings suggest that Twist is a potential target for the treatment of endometrial carcinoma. Further in vivo studies are now warranted to confirm the roles of Twist in endometrial carcinoma.
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References