Knockdown of Rab25 expression by RNAi inhibits growth of human epithelial ovarian cancer cells in vitro and in vivo

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INTRODUCTION

Ovarian cancer is a leading cause of death among gynaecological malignancies. In spite of advances in surgery and chemotherapy, its mortality rate has remained relatively unchanged during the past several decades.1 Therefore, an understanding of the molecular mechanisms involved in ovarian cancer formation and progression should be helpful in developing more effective treatments for ovarian cancer.

Emerging evidence implicates alterations in the Rab small GTPases and their associated regulatory proteins and effectors in multiple human diseases including cancer.2 Rab proteins are Ras-like small GTPases, which are involved in regulating various aspects of the membrane trafficking process.3 More than 60 Rabs are present in mammalian cells.4 Studies have recently shown that Rab25, located at chromosome 1q22, is amplified at the DNA level and over-expressed at the RNA level in ovarian and breast cancer. These changes correlated with a worse outcome in both diseases. In addition, enforced expression of Rab25 in both breast and ovarian cancer cells decreased apoptosis and increased proliferation and aggressiveness in vivo, potentially explaining the worse prognosis.5 A better understanding of genetic alterations as well as the physiological and pathophysiological roles of Rab GTPases may open new opportunities for therapeutic intervention and better outcomes.

Technologies for gene knockout, antisense oligonucleotides and ribozyme have been frequently used to explore new functions of genes, but their low frequencies have limited their applications.6 Fortunately, the emergence of gene ablation technologies based upon the RNA interference (RNAi) phenomenon has provided new opportunities for experimental biology.7 8 Particularly, a generation of vectors directing the synthesis of short hairpin RNAs (shRNAs) that are processed to form small interfering RNAs (siRNAs), enables persistent suppression of endogenous gene expression.9 10 RNA interference (RNAi) can result in sequence-specific gene silencing. This phenomenon is now being exploited as a powerful tool for reverse genetics, and shows great promise for therapeutic applications.11

In the present study, we used RNAi technology to knock down the expression of the Rab25 gene in human ovarian epithelial cancer cells and to examine their effect in human ovarian cancer cells in vitro and on tumour growth in vivo, which may establish candidate status of Rab25-mediated signalling for studies in targeted ovarian cancer therapy.

Summary

Aims: Ovarian cancer is the leading cause of cancer death among gynaecological malignancies. Elevated expression of Rab25 has been seen in this malignancy. To better understand its role in maintaining the malignant phenotype, we used RNA interference (RNAi) directed against Rab25 in our study. RNAi provides a new, reliable method to investigate gene function and has the potential for gene therapy. The aim of the study was to examine the anti-tumour effects elicited by a decrease in the level of Rab25 by RNAi and its possible mechanism of action.

Methods: According to the Rab25 mRNA sequence in Genbank, a pair of 64 nt oligonucleotides were designed and synthesised, each containing the sites of restriction endonuclease at both ends. Oligonucleotides were annealed and ligated with linearised pSUPER by ligase. The recombinants (named pSUPER/Rab25 siRNA) were finally sequenced and identified by enzyme cutting and sequencing. The human ovarian cell line A2780 was grown without transfection, transfection with empty vector and with pSUPER/Rab25 siRNA with electroporation. The inhibitory effect was examined by RT-PCR, MTT, FCM and tumour growth of athymic nude mice.

Results: Rab25 siRNA expression vector was successfully constructed and identified by double endonuclease digestion. Sequence analysis of inserted fragment revealed the same sequence as synthesised siRNA oligonucleotides. Cells transfected with Rab25 siRNA can specifically knock down the transcription of Rab25, exhibiting cells with slower proliferation, increased apoptosis, and decreased tumour growth.

Conclusions: Rab25 siRNA expression vector has been successfully constructed, and it could inhibit the tumour growth both in vitro and in vivo. Our data suggest that the Rab25 signalling pathway plays a role in the regulation of cell proliferation and apoptosis in ovarian cancer cells, which indicates that the Rab25 gene plays a definite role in the development and aggressiveness of human ovarian cancer and should be further elucidated as a possible therapeutic target of ovarian cancer.

Key words: RNA interference, siRNA, Rab25, ovarian cancer, epithelial cell.
MATERIALS AND METHODS

siRNA design

Three pairs of siRNA were designed according to the human Rab25 sequence in Genbank (BC 009831; Qiagen, Germany) and selected optimal sites of interfering. As shown in Table 1, each pair contained a unique 19-nt double-stranded human Rab25 sequence that was presented as an inverted complementary repeat and separated by a loop of 9 nt spacer. For example:

S1: 5' GAT CCCC=[N19]TTCAAGA GA[as-N19]TTTTGGAAA 3',
S2: 3' GGG=[as-N19]AAGTTCTCT=[N19]AAACACTTTTGC 5'

Plasmid construction, transformation and extraction

Oligonucleotides sequence of Rab25 siRNA was chemically synthesised by TakaRa (Japan). Oligonucleotides were annealed in a buffer containing 10 mM Tris (pH 8.0), 10 mM NaCl and 1 mM EDTA at 95°C. The newly synthesised DNA was amplified by PCR. The reaction mixture contained 2 μL cDNA template, 1.5 mM MgCl2, 2.5 U Tag polymerase, and 0.5 μM GAPDH was used as an internal control. Amplification cycles were: 94°C for 3 min, then 33 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min, followed by 72°C for 15 min. Aliquots of PCR product were electrophoresed on 1.5% agarose gels and PCR fragments were visualised by etidium bromide (EB) staining. All experiments were repeated on at least three occasions.

Cell proliferation. MTT assay and flow cytometric analysis

We plated cells at a density of 1 × 10^5 cells/35-mm dish. Cells were then cultured in the presence of 10% FBS for 8 days. We harvested and counted total cells.

Flow cytometry (Beckman-Coulter, USA) analysis was used to determine apoptosis and cell cycle of the cells. In brief, A2780 cells were transfected with Rab25 siRNA or without Rab25 siRNA; cells (5 × 10^4–1 × 10^5) were harvested, washed twice with PBS, and resuspended in 100 μL PBS and stained with propidium iodide (PI, Sigma) for apoptosis and cell cycle analysis.

RESULTS

Plasmid identification

We designed and synthesised three Rab25 siRNA sequences, and ligated the sequences into pSUPER vector. The recombinant plasmid (named pSUPER/Rab25 siRNA) was finally sequenced and identified by enzyme cutting and sequencing. The result of sequencing confirmed that siRNA had been ligated to the vector. The transfected vector extracted was digested with EcoRI and Hind III, then checked with 1.5% agarose gel electrophoresis. The length of the fragments of 291 kb (recombined plasmid) and 227 kb (empty plasmid) showed the success of construction as expected (Fig. 1).
The knock-down efficiencies of pSUPER/Rab25 siRNA in A2780 cells were first evaluated using RT-PCR. After transfection, relative Rab25 levels were normalised against mRNA levels of an internal control gene, GAPDH, performed in the same run. As shown in Fig. 2, cells transfected with pSUPER/Rab25 siRNA showed a significantly reduced transcription of Rab25 mRNA when compared with empty vector and cells without transfectants, respectively. In 1–4 months, the suppression of pSUPER/Rab25 siRNA 3 in the cultured transfection cells was more powerful than the others.

Further examination was carried out with MTT and flow cytometry to observe the effect of decreasing Rab25 levels.

**Slower proliferation and increased apoptosis in Rab25 silenced cells**

Under 10% fetal bovine serum (FBS) conditions, cells with transfected pSUPER/Rab25 siRNA 3 decreased cell numbers stably (Table 2).

We examined the viability of Rab25 specific siRNA transfectants. The results showed that RNAi directed against Rab25 significantly decreased the growth rate of A2780 cells. As shown in Fig. 3, pSUPER/Rab25 siRNA transfected A2780 cells began to show a decreased viability at 48 hours after transfection and the decline slowed down and became mild at 72 hours. The two control cell lines grew rigorously with the cell number increased at 48 hours and the increase proceeding until 72 hours (Fig. 3). The knock-down of Rab25 in A2780 cells could significantly inhibit the growth of tumour cells in vitro.

Apoptosis analysis indicated that the number of apoptotic cells increased in A2780 cells transfected with pSUPER/Rab25 siRNA compared with the controls (Fig. 4). pSUPER/Rab25 siRNA transfected A2780 cells were most prone to apoptosis with an apoptotic rate of 12.3%, higher than the rate of 0.9% in the A2780 control cells. Thus, treatment with siRNA against Rab25 could activate apoptotic pathways.

Different cell cycle phases (G1, S or G2/M phase) are characterised by different DNA contents. Table 3 shows differences in cell cycle phases in A2780 cells transfected with or without Rab25 siRNA. It was clear that pSUPER/Rab25 siRNA transfection induced an increase in the number of cells in G1 phase and a decrease in the number of cells in S phase compared with the control (Table 3; Fig 5).

**Decreases in transformation efficiency in vitro and tumour growth in vivo**

Injections of A2780 cells in nude mice demonstrated relevant tumour biology. In this study, tumour growth was inhibited by treatment with Rab25 siRNA. Nude mice were randomly selected for treatment with Rab25 siRNA which would affect tumour growth in vivo. A2780 cells transfected with or without the empty vector produced

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**Table 2** Decrease in cell proliferation by Rab25 siRNA

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<th>Empty vector</th>
<th>Rab25 siRNA</th>
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<tr>
<td>A2780</td>
<td>103.5 ± 7.5</td>
<td>106.9 ± 7.4</td>
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<tr>
<td></td>
<td>38.5 ± 3.5*</td>
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</table>

*p<0.01.

Cell counts were performed on day 8 in stable cell lines. Numbers represent cell number × 10,000 cells/mL. Each sample is presented ± SEM of three replicates from one of three representative experiments. Results were confirmed with multiple subclones of each cell line. Clearly, cells transfected with Rab25 siRNA showed significantly decreased cell proliferation and cell counts were significantly less than in the controls.

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![Fig. 1](image1.png) **Identification of interfering vector by enzyme cutting.** 1, DNA marker; 2, pSUPER/Rab25 siRNA3, 3, empty vector.

![Fig. 2](image2.png) **RT-PCR analysis.** (A) Rab25 and (B) GAPDH. RT-PCR analysis showed a lower mRNA level in A2780 cells transfected with Rab25 siRNA. M, marker; 1, A2780; 2, empty vector; 3, Rab25 siRNA3.
tumours 25 mm in diameter within 4 weeks of their subcutaneous injection into nude mice, while A2780 cells transfected with Rab25 siRNA showed only minimal tumour growth at 4 weeks (Fig. 6). Furthermore, Rab25 siRNA significantly inhibited production of A2780 in the tumours. Therefore, transfected Rab25 siRNA is a significantly novel way to inhibit tumour growth in vivo.

DISCUSSION
As far as we know, cancer is caused by abnormalities in DNA sequence, copy number, rearrangements, or expression. The accumulation of multiple changes in critical genes within a single cell is required to escape from normal controls on cell growth and proliferation, allowing development into a clinically evident tumour. Cancer cells often show abnormal signal transduction pathways, leading to proliferation in response to external signals. Oncogene over-expression is a common phenomenon in the development and progression of many human cancers. Therefore, oncogenes provide a potential target for cancer gene therapy.

We know that Ras proteins are oncogenes and Rab proteins are Ras-like small GTPases, which have crucial roles in vesicle trafficking, signal transduction and receptor recycling, which in turn regulate normal cellular activity. Approximately 60 human Rab genes are encoded by the human genome. However, the complexity of the Rab protein family might be even greater as there is evidence that alternative splicing of Rab mRNAs results in the production of functionally distinct isoforms. Recent studies have shown multiple links between Rab GTPase dysfunction and associated regulatory proteins in human diseases, including cancer.

Most Rab proteins are constitutively expressed in all mammalian cells, although several Rab proteins have been shown to be differentially expressed in epithelia and neurons, perhaps fulfilling specialised transport functions of these polarised cells. Therefore, some Rab protein functions may be ubiquitous. Rab5 has been localised to the early endosomal fraction in a number of cell systems. Other Rab proteins may have different functions in...
different cellular systems. Thus, while Rab2 is associated with the intermediate endoplasmic reticular-Golgi compartment in Madin–Darby canine kidney (MDCK) cells, it is associated with the H,K-ATPase-containing tubulovesicles in gastric parietal cells. In addition to the ubiquitous small GTP-binding proteins, some demonstrate tissue-specific distribution, including Rab3A, Rab3D, and Rab15. Rab17 is expressed only in the kidney, intestines, and liver, and its distribution suggests a possible role in transcytosis.

<table>
<thead>
<tr>
<th>Group</th>
<th>G1 %</th>
<th>S %</th>
<th>G2/M %</th>
</tr>
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<tbody>
<tr>
<td>A2780</td>
<td>50.2</td>
<td>36.3</td>
<td>13.5</td>
</tr>
<tr>
<td>Empty vector</td>
<td>48.4</td>
<td>37.7</td>
<td>14.0</td>
</tr>
<tr>
<td>Rab25 siRNA</td>
<td>88.6</td>
<td>8.1</td>
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The cells with Rab25 gene silencing revealed an increase in the percentage of G1 phase and a decrease in S phase.

Rab25, which shares 68% sequence identity with Rab11a, is expressed exclusively in epithelial cells, suggesting it regulates aspects of vesicular transport that are unique to epithelia. The sequence of the GTP-binding site of Rab25 is unique among Rab proteins, in that the P-3 domain consensus sequence WDTAGQE contains a leucine residue in place of the conserved glutamine. An equivalent substitution in Ras creates a dominant-active GTPase. Casanova et al. have found that Rab25 over-expression alters transcytosis and apical recycling, and Cheng et al. have reported that Rab25 controls tumour progression, aggressiveness, and potentially chemosensitivity, and is amplified at the DNA level and over-expressed at the RNA level in ovarian cancer. Rab25 over-expression markedly increases cancer cell proliferation, preventing apoptosis. Schaner et al. reported similar results and also found that ovarian cancer patients with elevated Rab25 either failed to enter a disease-free state following surgery and chemotherapy, or exhibited short durations of disease-free survival. Therefore, specific down-regulation of Rab25 might be a potential therapeutic strategy against human ovarian cancer.

TABLE 3 Cell cycle phase distribution of A2780 cells after transfection of Rab25 siRNA

![Fig. 5](image_url)
Techniques based upon RNAi principle\textsuperscript{15} have become powerful and indispensable tools in the molecular toolkit, being honoured as ‘the breakthrough of 2002’.\textsuperscript{35,36}

By means of the RNAi method, cellular growth assays in vitro were used to determine the functional consequences of RNAi-mediated decreases of Rab25 in established ovarian carcinoma cells. Our results demonstrate that RNAi can effectively down-regulate Rab25 over-expression with great specificity and that the plasmids endogenously expressing siRNA can successfully deplete Rab25 expression in A2780 cells after transfection. Furthermore, the tumour inhibition effects persist after transfection as shown by experiments in vitro and in vivo. Therefore, it is not surprising that we have shown that reduction of the Rab25 level by Rab25 siRNA can significantly inhibit the growth rate of cancer cells.

Many anticancer agents induce apoptosis. Our data also suggest that knock-down of Rab25 by Rab25 siRNA in A2780 cells can increase the sensitivity of these cells to apoptosis. This was probably one of the reasons for the anti-tumour effect. Moreover, in cell cycle, increases in G1 and decreases in S further confirm a role for Rab25 in regulating cellular apoptosis. A recent study has provided direct evidence that multiple signalling molecules including AKT, extracellular signal-regulated kinase 1/2, and p38 mitogen-activated protein kinase, associate on endocytic vesicles and mediate their function by facilitating translocation of the vesicles to the nucleus, implicating Rab GTPases in conducting cell survival signals.\textsuperscript{32} However, the pathways that Rab25 controls and/or that are involved in the observed apoptosis need further study.

All of the nude mice injected with Rab25 transfected cells developed subcutaneous tumours. However, mice injected with Rab25 transfected A2780 cells developed obviously smaller tumours than the controls, and the controls developed larger tumours in a shorter time. This further demonstrates that Rab25 plays a master role in tumour progression and aggressiveness. These studies have marked a new target in the genetic manipulation of ovarian cancer development by allowing Rab25 to be down-regulated by RNAi.

In conclusion, Rab25 siRNA substantially reduces the expression of Rab25 mRNA, slows cell proliferation, increases apoptosis, suppresses transformation, and inhibits tumour growth, demonstrating that Rab25 siRNA silencing is a powerful approach for dissecting oncogenic signals in human ovarian cancer. Our data implicate Rab25 in the aggressiveness of ovarian carcinomas suggesting that Rab25 can be used to predict patient outcome and provide a novel therapeutic target. These studies also implicate Rab25 and, by extension, the Rab GTPase family in tumour aggressiveness. This adds the genes encoding the Rab family to other members of the Ras oncogene superfamily as tumorigenic mediators.

References


