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Original Article

RNAi-mediated knockdown of RBPJ gene inhibits the growth of human breast cancer cells

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Abstract: The Notch signaling pathway has been implicated in tumor growth in a number of different human tissues, including the colon, liver, and breasts. Regulation of this pathway in breast tissue has been investigated through several different methods with limited success. This study explored the efficacy of short hairpin RNA (shRNA) in modulating Notch signaling through its downstream transcription factor RBPJ in the human breast cancer cell line MCF-7. In this cell line, shRNA delivery through a lentivirus vector effectively downregulated RBPJ expression and protein levels. Importantly, overall cell proliferation was significantly reduced, and there was an increase in p21 expression and decreases in the expression of CDK2, Hes1, bcl-2, and SKP2. Together, these data suggest that shRNA knockdown of RBPJ in the Notch signaling pathway could be a novel therapeutic approach to treating human breast cancer.

Keywords: shRNA, RBPJ, proliferation, MCF-7, breast cancer

Introduction

Breast cancer is a leading cause of cancer-related death in women the world over, particularly in the Western population. Each year in USA, 182,000 women are diagnosed with breast cancer and 43,300 die. One woman in eight either has or will develop breast cancer in her lifetime. If detected early, the five-year survival rate exceeds 95%. However, although the survival of the breast cancer patients has improved with the advent of chemotherapy, radiation therapy, as well as hormone therapy, the outcome still remains poor. Therefore, the development of alternative therapy strategies and discovery of more effective therapeutic targets is required and will greatly contribute to the treatment of breast cancer.

The Notch pathway has been reported to be highly active in a variety of human breast cancers [1], and a number of drugs have been reported to be more effective in treating breast cancer cells when used in conjunction with Notch inhibition [2-4], suggesting that Notch inhibition in combination with other drugs can be a promising route of therapy for breast cancer. This signaling pathway has been highly conserved in evolution and plays important roles during development. Notch signals regulate various physiological processes, including maintenance of stem cells, cell fate decisions, proliferation, differentiation and apoptosis [5-7]. In mammals, Notch signaling is initiated by the interaction of the Notch receptor with its ligands [8, 9]. Ligand binding ultimately results in proteolytic cleavage of the receptor by γ-secretase and release of the Notch intracellular domain (NICD), which interacts directly with its primary mediator RBPJ (Rbpsuh) which in turn modulates target gene expression.

The function of RBPJ, initially described in mammalians by Hsieh and Hayward [10], has been developed and modified as new information accumulated. The most commonly accepted model suggests that RBPJ is permanently bound to DNA in the nucleus, and activator and repressor complexes are assembled on the promoter. In the absence of Notch signals, RBPJ is associated with corepressors and represses Notch transcription. NICD displaces corepres-

sors from RBPJ, which allows for the recruitment of coactivators and thereby induces the activation of target genes like hairy and enhancer of split 1 (Hes-1), cyclin-dependent kinase 2 (CDK2), B-cell CLL/lymphoma 2 (bcl-2), ubiquitin ligase complex SCFSKP2 (SKP2) and cyclin-dependent kinase inhibitor 1 (p21) [11-15]. Recent studies, however, support the idea that RBPJ complexes could also be assembled partially in the nucleoplasm or cytoplasm, because RBPJ occupancy at the promoters was found to be increased when NICD was present [16-18]. Interestingly, RBPJ interacting and tubulin associated (RITA) protein has been shown to be involved in the export of RBPJ from nucleus to cytoplasm and therewith modulates the Notch signaling response [19, 20]. Thus, the amount of nuclear RBPJ might be another relevant factor that controls Notch transcriptional output.

Early studies targeted notch signaling through the use of γ-secretase inhibitors [2, 21, 22], but as there are more than 20 γ-secretase substrates which have biological functions in breast cancer [23] a more specific approach would be highly beneficial. After years of intensive investigation, there are still many uncertainties that prevent the successful exploitation of this target. To a large degree, these discrepancies arise from the complex nature of the Notch signaling pathway as well as from limitations of existing reagents. A recent report demonstrated that knockdown of RBPJ expression by RNA interference (RNAi) inhibited the anchorage-independent growth of rhabdomyosarcoma cells and the growth of xenografts in vivo [24], which suggested that interruption of RBPJ by RNAi could potentially be effective in other applications. Altogether, these findings suggest that the transcriptional regulator RBPJ, which plays a central role in canonical Notch signaling, can be a potential target for more specific manipulation of the Notch pathway.

In this study, we examined the impact of Notch inhibition by knocking down RBPJ expression with short hairpin RNA (shRNA) in MCF-7 cells. Our results revealed that downregulation of the RBPJ gene affected the cell cycle and significantly inhibited the proliferation of human breast cancer cells. It is essential to develop well-characterized and highly specific approaches that can be used to successfully interrupt Notch signaling in breast cancers, and these results suggest that modulation of RBPJ has potential as a novel therapeutic strategy for breast cancer treatment.

Materials and methods

Cell culture

HEK293T cells and human breast cancer cell line MCF-7 (American Type Culture Collection, Manassas, VA) were cultured in DMEM or RPMI-1640 (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin and streptomycin in a 5% CO_2 humidified atmosphere at 37°C.

Construction of RBPJ-shRNAs

cDNA was prepared using a RT-PCR kit (Takara, Shiga, Japan) and 1 μg of total RNA extracted from MCF-7 cells with TRIzol reagent (Invitrogen, Carlsbad, CA). The coding region of RBPJ transcripts was amplified with forward (5'-GGAAGATGGCGCCTGTTGTGACAG-3') and reverse 5'-GTTATCTCGAGTCAAGCGTAGTCTGGGACGGTATGGGTAGGACACCACGGTTGCTGTG-3') primers. The underlined sequence represents a HA tag. The amplicons were digested with XhoI and BamHI and subcloned into a pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) and the resulting construct was named pcDNA-RBPJ. The Dharmacon siDESIGN Center database (www.dharmacon.com/sidesign/siRNA) was used to design the shRNAs targeting RBPJ mRNA (NM_005349.3). Six shRNAs targeting the RBPJ coding region were selected based on ranking criteria of Reynolds [25]. All the shRNAs were cloned into pLKO.1 vector (Sigma, St. Louis, MO), with pLKO.1 used as the mock control.

Identification of effective RBPJ-shRNAs to knockdown RBPJ expression

HEK293T cells were seeded in six-well plates in complete medium (DMEM containing 10% FBS) one day prior to transfection. To generate the recombinant, the packaging cells HEK293T were transfected with 5-8 μg pVSV-G (Clontech, Mountain View, CA) and 15 μg of recombinant vectors using Lipofectamine®-2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The transfected cells were then harvested and whole cell lysates were extracted for western blotting using an anti-

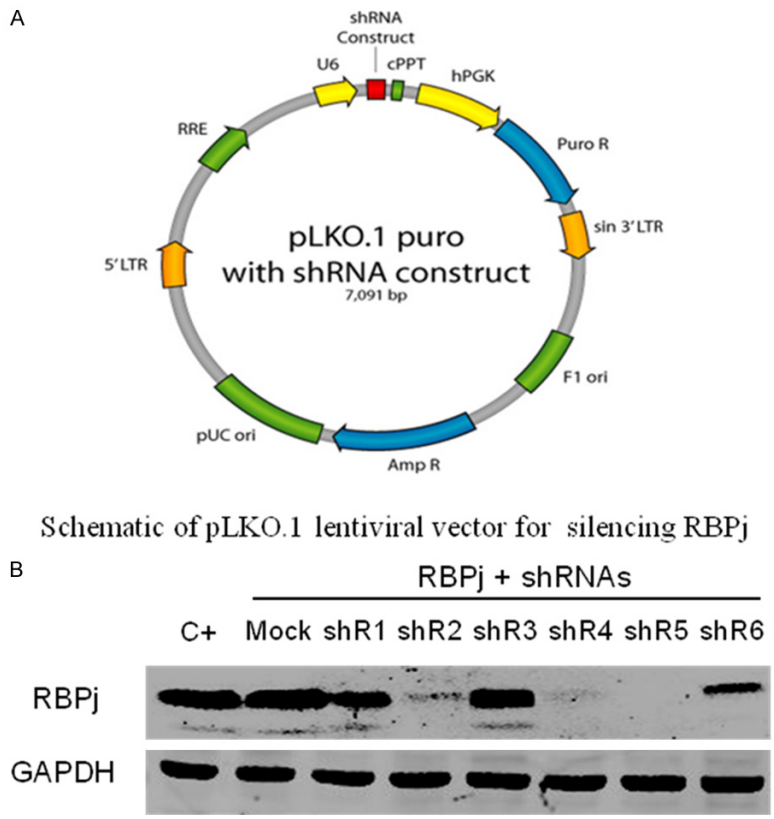

Figure 1. Identification of effective shRNAs to interfere with RBPJ expression. A. Schematic representation of pLKO.1 lentiviral vector for silencing RBPJ. B. Protein levels of RBPJ in HEK293T cells by western blot. C+, cells transfected with pcDNA-RBPJ plasmid; Mock, cells transfected with pLKO.1 vector; shR1 to shR6, shRNA constructs designed to target RBPJ based on mRNA sequence.

RBPJ antibody (Abcam, Cambridge, MA). The co-transfections were performed in duplicate for each RBPJ-shRNA construct.

Generation of RBPJ knockdown stable clones of MCF-7

To generate RBPJ-shRNA lentiviral particles, HEK293T cells were seeded in a 100 mm dish at 60,000 cells/cm^2 and co-transfected with 12 μg of RBPJ-shRNA and 6 μg each of packaging plasmids (REV, pMDL, and VSV-G) using Lipofectamine®-2000 (Invitrogen, Carlsbad, CA). The supernatant containing lentivirus particles was collected 48 h post-transfection and filtered through a 0.45 μm syringe filter. MCF-7 cells were seeded in 100 mm plates at 15,000 cells/cm^2 one day prior to lentiviral infection. The lentiviral particles were added along with 10 μg/ml polybrene (Sigma, St. Louis, MO) final concentration to the cell culture and incubated for 24 h. The resulting infected cells were then washed twice with fresh complete medium and cultured in the presence of puromycin to select the cells.

RT-PCR quantification analysis

Total RNA was extracted from MCF-7 cells with TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 1 μg of total RNA using a RT-PCR kit (Takara) and purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). RBPJ transcript was quantitated using LightCycler 1.5 Real-time PCR machine (Roche, Indianapolis, IN) as described previously [26]. PCR reactions contained 2 μl purified cDNA or standard plasmid, 4 mM $MgCl_2$, 0.5 μM each of primers, and 2 μl of FastStart DNA mastermix (Roche, Indianapolis, IN). Primers were designed to amplify the fragments between gene exons and are as follows: RBPJ (132 bp), sense 5'-CGCATTATTGGATGCAGATG-3' and antisense 5'-CAGGAAGCGCCATCATTTAT-3'; GAPDH (152 bp), sense 5'-AGAAGGCTGGG-GCTCATTTG-3' and antisense 5'-AGGGGCCA-TCCACAGTCTTC-3'. The real-time PCR results were presented as means from 3 independent experiments using the same cDNA preparation and normalized to GAPDH.

Western blot analysis

Proteins were extracted in a solution of RIPA and Halt™ Protease Inhibitor Cocktail (Thermo Scientific, Waltham, MA) from HEK293T or MCF-7 cells and subjected to SDS-PAGE. Quantification of total protein was carried out using BCA with BSA (Sigma, St. Louis, MO). The proteins (100 μg) were subjected to 12% SDS-PAGE. Separated proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and immune-blotted

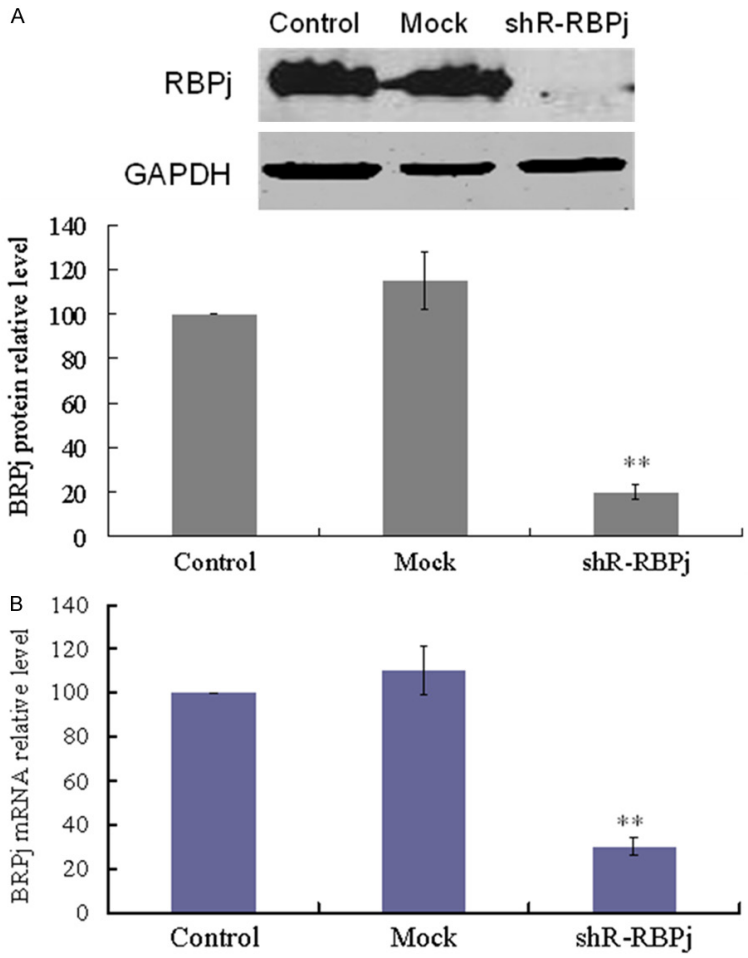

Figure 2. RBPJ expression in stably transduced MCF-7 cells. After 72 h culture, (A) western blot and BCA quantification of relative RBPJ protein levels in MCF-7 cells transduced with RBPJ-shRNA compared to GAPDH and (B) relative mRNA levels by RT-PCR compared to GAPDH were measured. Data represent the mean ± SD of duplicates based on three independent experiments. P<0.01.

with monoclonal mouse anti-human RBPJ (Santa Cruz, sc-136191), polyclonal rabbit anti-mouse/rat p21 (Abcam, ab2961), polyclonal rabbit anti-human Hes1 (Abcam, ab49170), polyclonal rabbit anti-human SKP2 (Cell Signaling, #4358) or polyclonal rabbit anti-human GAPDH (Sigma, G9545) antibodies. Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE) as described by the manufacturer.

Cell proliferation assay

A diphenyltetrazolium bromide (MTT) assay was performed to determine cell proliferation. Five thousand cells per well were seeded in a 96-well plate and grown for 24, 48, 72, and 96 h. The medium was then removed and washed with PBS, and 5 g/l of thiazolyl tetrazolium (Amersco, Indianapolis, IN) was added to each well. After 4 h of incubation, MTT was removed and 150 µl of dimethyl sulfoxide (Sigma, St. Louis, MO) was added. The viability of the cells was calculated from the absorption at 570/630 nm with an enzyme-linked immunosorbent assay reader.

Cell cycle analysis

Cells were rinsed twice with PBS. Cell pellets were cooled on ice and resuspended in a solution containing 100 µg/ml propidium iodide (Sigma, St. Louis, MO), 0.1% trinatriumcitrate-dihydrate, and 10% RNaseA (1 mg/ml) and then incubated at 37°C for 30 min. Cells were then analyzed by flow cytometry.

Statistical analysis

Data are shown as mean ± SD and subjected to one-way ANOVA with factors of treatment using the SPSS 13.0 for Windows. Comparisons between two groups were performed by an unpaired Student's t-test. $P<0.05$ (one-tailed) was considered statistically significant.

Results

RBPJ expression was effectively inhibited by shRNA constructs

Multiple shRNA constructs were created and then co-transfected with pcDNA-RBPJ into HEK293T cells to determine the efficacies of each in silencing RBPJ expression. **Figure 1A** provides a schematic representation of the pLKO.1 lentiviral vector used for silencing RBPJ. Western blotting was performed to measure RBPJ protein levels in the transfected cells using an antibody to RBPJ. Out of six constructs, three shRNAs resulted in substantial downregulation of RBPJ expression, with shR5 providing the most efficient silencing with up to a 93% reduction in protein levels (**Figure 1B**). Given

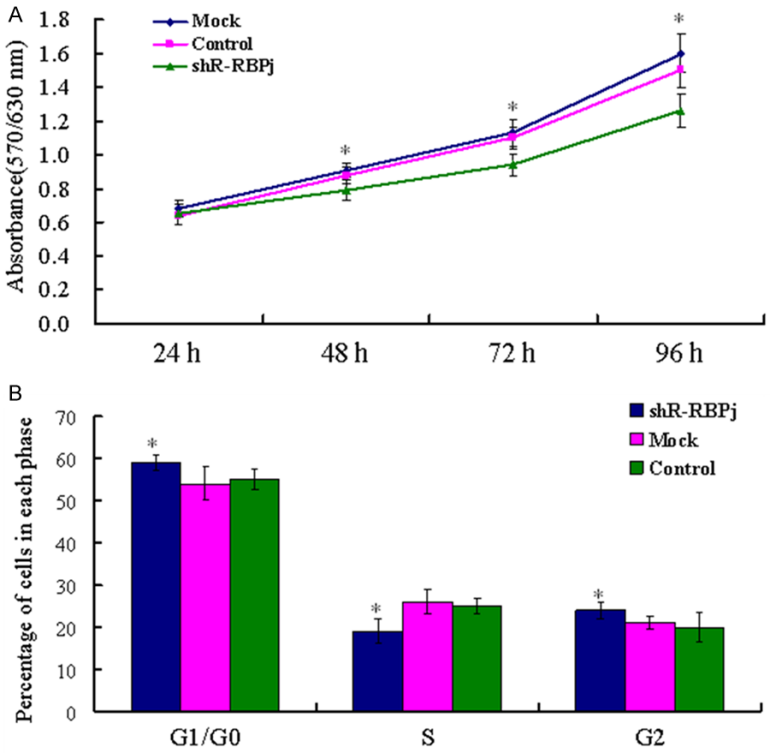

Figure 3. Interference of endogenous RBPJ inhibited MCF-7 proliferation. A. The proliferation of stably transduced MCF-7 was measured from 24 h to 96 h by MTT assay. B. Percentage of cells detected in each cell cycle phase (G1/G0, S, or G2) by flow cytometry at 72 h. Data represent the mean ± SD of duplicates based on three independent experiments. P<0.05.

these results, shR5 (RBPJ-shRNA), was chosen as the optimal shRNA to knock down RBPJ expression for the remainder of this study.

Endogenous RBPJ expression was knocked down by lentiviral RBPJ-shRNA in stably transduced MCF-7 cells

MCF-7 breast cancer cells were infected with RBPJ-shRNA and pLKO.1 lentivirus to generate stable clones which were then cultured in medium for 72 h before collection for protein and mRNA analysis. RBPJ protein levels were effectively reduced in the stably transduced cells by western blot compared to a GAPDH control (**Figure 2A**). Quantification of protein levels using the BCA assay also resulted in significantly lower levels in RBPJ-shRNA transduced cells compared to non-transduced (control) and pLKO.1 vector transduced cells (mock) (**Figure 2A**). Similar results were obtained through RT-PCR (**Figure 2B**), further confirming the successful knockdown of RBPJ expression by RBPJ-shRNA.

Reduction of endogenous RBPJ inhibited MCF-7 proliferation

To investigate whether RBPJ could be an effective therapeutic target for breast cancer, the impact of RBPJ-shRNA on cell growth was evaluated by the MTT cell proliferation assay. Cell proliferation was inhibited as early as 48 h, with an average reduction of 20% in RBPJ-shRNA transduced cells (**Figure 3A**). In further analysis, a cell cycle phase assay at 72 h revealed that the proportion of RBPJ-shRNA transduced cells in the S phase was significantly diminished compared to mock cells, going from 24.1% down to 18.6%, with a concomitant increase in both G0/G1 (52.3% vs. 58.1%) and G2 (20.1% vs. 23.8%) phases (**Figure 3B**). These results suggested that knockdown of endogenous RBPJ inhibited MCF-7 proliferation through inhibition of the S phase of the cell cycle.

Inhibition of MCF-7 proliferation is modulated by RBPJ target genes involved in S phase transition

To further clarify the mechanism of reduced cell growth by the knockdown of RBPJ, we examined the expression of factors downstream of the RBPJ gene in the Notch pathway by western blot. RBPJ-shRNA transduced cells exhibited a reduction in CDK2, Hes1, bcl-2, and SKP2 expression compared to control and mock cells while p21 expression was increased (**Figure 4**). CDK2 is a catalytic subunit of the cyclin-dependent kinase complex, whose activity is restricted to the G1-S phase of the cell cycle, and is essential for the G1-S transition [27]. The p21 protein binds to and inhibits the activity of cyclin-CDK2 or -CDK1 complexes, which suppresses the phosphorylation of Rb and functions as a regulator of cell cycle progression at G1 [28]. The ubiquitin-ligase com-

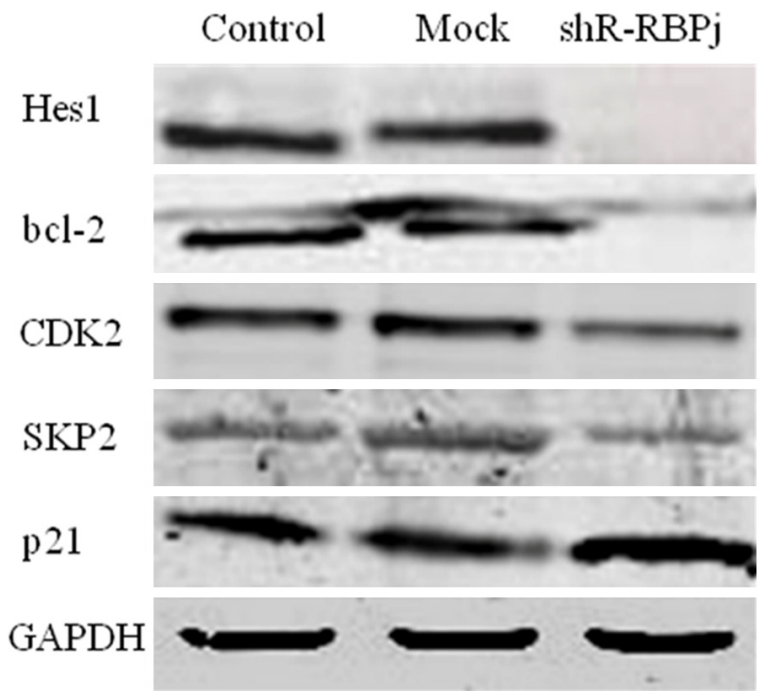

Figure 4. Knockdown of RBPJ inhibited MCF-7 proliferation through regulation of target genes. Protein levels of CDK2, Hes1, bcl-2, SKP2 and p21 expression by western blot.

plex SCFSKP2 (SKP2) is necessary for the degradation of p21 at the G1-S transition and during S phase in the cell cycle [29]. These results further support the idea that inhibition of RBPJ inhibited growth of MCF-7 cells through regulation of the cell cycle by inhibiting the transition from the G1 phase to the S phase.

Discussion

Recent reports have demonstrated that inhibition of Notch pathway by GSI, siRNA or γ-secretase inhibitors resulted in inhibited breast tumorigenesis [30-32]. GSIs originally used in Alzheimer's disease [33] are currently under clinical trials for the treatment of several tumors [34, 35]. However, GSIs proteolyze not only Notch receptor but also many other membrane proteins, including VEGFR, Ecadherin, CXCL16 and so on [36]. Thus, therapeutic approaches including treatment with GSIs or those γ-secretase inhibitors may affect other pathways resulting in unpredictable effects. On the other hand, RBPJ gene acts only downstream of the Notch pathway, and no evidence has been shown to demonstrate its function in other pathways. Therefore, we focused on the specific role of RBPJ in the Notch pathway to evaluate its effect as a potential therapeutic target for breast cancer.

In the present study, we developed a shRNA delivery strategy to achieve stable suppression of the RBPJ gene expression. RNAi is one of the most promising methods to specifically and efficiently silence gene expression at the mRNA level either by transfection of short interfering RNAs (siRNAs) or shRNAs from expression plasmids and viral vectors [37-40]. Delivering siRNA to target cells by physical transfection methods is of low and variable transfection efficiency and only suppresses gene expression transiently, limiting the application of siRNAs in long-term gene silencing [41]. Lentivirus vectors provide a more efficient, stable gene delivery tool in mammalian cells and transgenic animals [42, 43]. The lentiviral vector pLKO.1 is able to integrate into the host genome allowing for the stable expression of shRNA [44], which can drive high level expression of shRNA and, in turn, mediate highly successful gene silencing. Using this approach, we created a stable RBPJ-shRNA transfectant using recombinant lentivirus mediated gene transfer.

Our results demonstrated that the downregulation of RBPJ expression resulted in a 20% average reduction of cell growth in transfected MCF-7 cells. Furthermore, cell cycle analysis revealed that knockdown of RBPJ regulated the cell cycle and decreased MCF-7 cell proliferation. We also found that knockdown of RBPJ suppressed the expression of target genes CDK2, Hes1, bcl-2, and SKP2, but increased p21 expression. Given the roles of these genes in cell cycle progression, our results suggest that the knockdown of RBPJ inhibits breast cancer cell proliferation by regulation of the G1-S transition of the cell cycle. Together, these findings implicate the transcription of RBPJ through the Notch pathway in reducing breast cancer cell proliferation in MCF-7 cells. While this study was limited to effects in the MCF-7 cell line, the

results provide the potential for a novel approach in treating breast tumorigenesis. Further studies, such as applying the treatment in vivo to a mouse model of breast cancer or testing RBPJ-shRNA in different human breast cancer cell lines would be helpful in strengthening the link between RBPJ inhibition and proliferation in breast cancer cells. Additionally, the Notch pathway has been shown to play essential roles in many cancers [15, 45-47], suggesting that the direct inhibition of RBPJ may not only be limited to breast cancer, but can potentially be applied to other cancers as well.

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Disclosure of conflict of interest

None.

Abbreviations

shRNA, short hairpin RNA; NICD, Notch intracellular domain.

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