CHAPTER 2

RESEARCH METHODOLOGY

I. Materials

1. siRNA

The siRNAs, as shown in table 3, were purchased from Invitrogen (USA).

2. Chemicals

All chemicals and solvents (analytical grade) used in this thesis were purchased from Life Technologies, USA; Sigma, USA; PIERCE, USA; Amersham Biosciences, UK; QIAGEN, Germany; and BIO-RAD, USA.

3. Antibodies

All antibodies used in this thesis were purchased from Santa Cruz Biotechnology, Inc., USA and Amershame Bioscience, UK.

Table 3. The sequences of siRNA.

Name	Region	siRNA
WT1-HSS111388	exon 7	5'-AAAUAUCUCUUAUUGCAGCCUGGGU-3'
WT1-HSS111389	exon 8	5'-UUCACAGUCCUUGAAGUCACACUGG-3'
WT1-HSS111390	exon 8	5'-UUUCACACCUGUAUGUCUCCUUUGG-3'

II. Methods

1. Cell culture

MCF-7 Breast cancer cell line was kindly provided from Assoc. Prof. Dr.Arunporn Itharat (Faculty of Pharmacology, Prince of Songkla University, Thailand), MDA-MB-468 breast cancer cell line, PC3 and LNCAP prostate cancer cell line were purchased from Amirican Type Culture Collection (ATCC, USA). These cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (GIBCO BRL), 50 units/ml penicillin, and 50 μg/ml streptomycin. MCF-7 cells were maintained in a humidified 37 °C incubator with 5% CO_2, changed every 3 days with complete medium, and subculture when confluence was reached.

2. Transfection of cells

A total of 5 x 10^5 MCF-7 cells were seeded into each well of a six-well tissue culture plate (For Protein analysis) and 5 x 10^4 cells were seeded into each well of 24-well tissue culture plate (for proliferation assay). The next day when cells were 40-50 % confluent, cells was transfected with small interfering RNA (siRNA). The siRNA against WT1 ($siRNA_{WT1}$) was synthesized by Invitrogen. $siRNA_{WT1}$ actually consisted of a mixture of three siRNA duplexes targeting three different regions of WT1 mRNA, namely, $siRNA_{WT1}$HSS111388 ($siRNA_{WT1}$R88), $siRNA_{WT1}$HSS111389 ($siRNA_{WT1}$R89) and $siRNA_{WT1}$HSS111390 ($siRNA_{WT1}$R90). The first regions located in exon7 and the last two regions located in exon8. Cells were transfected with only transfection agent as a control. All procedures were performed in an RNase-free environment. Briefly, the transfection of cells with siRNA duplexes was performed using LipofectAMINE™ 2000 (invitrogen), at a final concentration of 0.2%. To minimize the cytotoxicity of the reagent itself, cells were washed once with PBS, and media were changed at 5 h after transfection. The cells were harvested at different time points (0, 12, 24, 48, 72, 96 and 120 h) and different concentrations of siRNA (0, 25, 50, 100, 200, 400 and 800 nM).

3. Protein Analysis

3.1 Protein extraction

. Cells were harvested at different time points and concentrations by trypsinization, washed twice with 2 ml of cold PBS for each of six-cm plate, and centrifuged at 1,500 rpm for 5 min. The pellet was resuspended in SDS lysis buffer (10% glycerol, 2% SDS and 10 μg/ml of proteinase inhibitor) and mixed by pipetting, subsequently boiled in water bath 100 °C for 5 min followed by incubation on ice for 1 min before stored at -20 °C. The protein concentration was determined by Lowry method (DC Kit, BioRAD)(Appendix A).

3.2 Immunoblotting (Graidist et al., 2004)

The protein samples were then subjected to 12% SDS-PAGE (Appendix A) and transferred to nitrocellulose membrane. The membrane was blocked by blocking buffer (5% skim milk in TBS-T (0.1% Tween 20, 154 mM NaCl, 48 mM Tris-base, pH 6.8) for 1 h and washed two times for 5 min each time with 1% skim milk in TBS-T buffer. The membrane was then incubated with primary antibody anti-WT1 (1:200) and anti-actin (1: 1000) (diluted with 1% skim milk in TBS-T) (Santa Cruz Biotechnology, Inc) for 1 h. The blot was washed three times for 5 min each time with 1% skim milk in TBS-T buffer. The membrane was then incubated with Polyclonal IgG-anti-rabbit antibody (1:5000) for WT1 and actin (diluted with 1% skim milk in TBS-T) for 1 h and, then washed three times for 10 min each time with TBS-T buffer. Bound antibodies were detected by ECL chemiluminescence detection method (Amersham). The excess detection reagent was drained off with 3 MM paper. Then the membrane was placed in the film cassette containing autoradiography film (Hyperfilm, Amersham), and exposed for 5 min. The X-ray film was developed using developing machine.

4. Cellular proliferation assay (Freshney, 1994)

After transfection, $siRNA_{WT1}$-transfected cell and control cell were harvested at specific time point and concentration by a brief trypsinization. Cells were then transferred into 1.5 ml microcentrifuge tube and centrifuged at 3,000 rpm for 5 min. Floated and attached cells were subjected to the assay. After centrifugation, the pellet was resuspended with 100 µl of new medium and 100 µl of 0.4% of trypan blue stained solution (GIBCO). The viable cells will be counted under inverted microscope to calculate the amount of viable cells compared with the total cells (survival cell and death cell). Each transfection was carried out at least three times.