รายงานวิจัยฉบับสมบูรณ์

โครงการ ศึกษาการทำงานร่วมกันของ WT1 กับ p53 ในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MDA-MB-468

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เมษายน 2557

สัญญาเลขที่ MRG5480124

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

รูปแบบ Abstract (บทคัดย่อ)

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Project Title : ศึกษาการทำงานร่วมกันของ WT1 กับ p53 ในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MDA-MB-468

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บทคัดย่อภาษาไทยและภาษาอังกฤษ

ยีน *WT1* มีส่วนของ zinc finger ทำหน้าที่กระตุ้นหรือยับยั้งยีนหลายชนิดที่เกี่ยวข้องกับการพัฒนา การเจริญเติบโต และการตายแบบอะพอพโตซิสของเซลล์ หน้าที่ที่แตกต่างกันขึ้นอยู่กับชนิดของเซลล์ที่แสดงออก ไอโซฟอร์มของ WT1 และโมเลกุลที่เกี่ยวข้อง การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่าง WT1 และโปรตีนต่างๆ ในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MCF-7 และ MDA-MB-468 โดยยับยั้งการแสดงออกของ WT1 ด้วย siRNA และใช้โปรติโอมิกส์ในการวิเคราะห์รูปแบบการแสดงออกของโปรตีนด้วยเทคนิคการแยกโปรตีนแบบสองมิติ (2-DE) และการแยกโปรตีนแบบหนึ่งมิติ (1-DE) พร้อมระบุชนิดของโปรตีนด้วยเครื่องแมสสเปกโตรเมทรีชนิด LC-MS/MS ผลการศึกษาด้วยเทคนิค 2D LC MS/MS พบโปรตีน ที่มีการแสดงออกแตกต่างกันระหว่างสภาวะที่มี WT1 เปรียบเทียบกับสภาวะที่ไม่มี WT1 ทั้งหมด 27 ชนิด และ 70 ชนิดในเซลล์ MCF-7 และ MDA-MB-468 ตามลำดับ ในขณะที่ผลการศึกษาด้วยเทคนิค 1D LC-MS/MS พบโปรตีนที่มีการแสดงออกที่แตกต่างกันระหว่างสภาวะที่มี WT1 เปรียบเทียบกับสภาวะที่ไม่มี WT1 ทั้งหมด 164 ชนิดในเซลล์ MCF-7 และ MDA-MB-468 โปรตีนเหล่านั้นทำหน้าที่เกี่ยวข้องกับการตายของเซลล์แบบอะพอพโทสิส การส่งสัญญาณภายในเซลล์ การขดตัวของโปรตีน กระบวนการเมตาบอลิสึม โปรตีนโครงสร้าง การเกาะติดของเซลล์ การแสดงออกของยีน การขนส่ง การสลายโปรตีน กระบวน redox ภายในเซลล์ และโปรตีนที่ยังไม่ทราบหน้าที่แน่ชัด จากการวิเคราะห์พบโปรตีนที่เกี่ยวข้องกับการตายของเซลล์แบบอะพอพโทสิส แสดงออกเฉพาะในสภาวะที่ไม่มี WT1 ในเซลล์ MCF-7 ได้แก่ Cathepsin D, apoptosis inducing factor และ apoptosis stimulating of p53 protein เท่านั้น และพบโปรตีนที่เกี่ยวข้องกับกระบวนการส่งสัญญาณภายในเซลล์ ในสภาวะที่มี WT1 ในเซลล์ MCF-7 ชนิด 14-3-3 epsilon, signal transducing adaptor protein 1, phospholipase C และ metabotropic glutamate

receptor ส่วนในเซลล์ MDA-MB-468 พบโปรตีนที่น่าสนใจ ได้แก่ ALG-2 interacting protein x, apoptosis- inducing factor 1 guanine nucleotide binding protein, neuropolypeptide h3, platelet derived growth factor receptor (PDGFRA) และ Rho guanine nucleotide exchange factor 1 แสดงออกเฉพาะในสภาวะที่มี WT1 ในขณะที่ IBTK protein, SH2 domain containing protein และ mitogaliain จะแสดงออกเฉพาะในสภาวะที่ไม่มี WT1 เท่านั้น จากการศึกษาครั้งนี้สามารถสรุปได้ว่าเซลล์ MCF-7 WT1 น่าจะมีความสัมพันธ์กับโปรตีนที่เกี่ยวข้องกับอะพอพโทสิสชนิด Cathepsin D, apoptosis-inducing factor 1 และ apoptosis stimulating factor of p53 protein 2 นอกจากนี้ WT1 น่าจะมีความเกี่ยวข้องกับโปรตีนในกระบวนการส่งสัญญาณภายในเซลล์ชนิด 14-3-3 epsilon, signal transducing adaptor protein 1, phospholipase C, and metabotropic glutamate receptor ส่งผลให้ WT1 นั้นน่าจะมีบทบาทเป็น oncogene และโปรตีนต้านการตายของเซลล์แบบอะพอพโทสิส ส่วนในเซลล์ MDA-MB-468 นั้นคาดว่า WT1 น่าจะมีความสัมพันธ์กับโปรตีนที่เกี่ยวข้องกับอะพอพโทสิสชนิด mitogaligin และโปรตีนที่เกี่ยวข้องกับการส่งสัญญาณภายในเซลล์ชนิด platelet derived growth factor receptor alpha และ rho guanine nucleotide exchange factor 1 ส่งสัญญาณผ่านทาง mTOR รวมทั้งอาจจะควบคุมการทำงาน Raf kinase inhibitor protein ซึ่งส่งผลให้การส่งสัญญาณผ่านทาง Raf/MAP kinase pathway เกิดได้และส่งเสริมกระบวนการ metastasis อย่างไรก็ตาม ควรทำการยืนยันผลการทดลองเพิ่มเติมเพื่อยืนยันสมมติฐานดังกล่าว

The Wilms' tumor 1 (*WT1*) gene encodes a zinc finger acting as a transcriptional activator or repressor for many genes involved in cell differentiation, growth, and apoptosis. These functions depend on the cell types, WT1 isoforms, and the status of targeted molecules. To determine the relationship between WT1 and related proteins, WT1 was silenced with siRNA in MCF-7 and MDA-MB-468 breast cancer cell lines. The protein expression patterns were analyzed by proteomics techniques: two-dimensional gel electrophoresis (2-DE) and one-dimensional gel electrophoresis (1-DE) combined with LC-MS/MS mass spectrometry. For 2-DE LC-MS/MS analysis, 27 protein spots (15 spots in $siRNA_{neg}$ (present WT1) and 12 spots in $siRNA_{WT1}$ (without WT1)) were found to have a significant change in expression level. However, in MDA-MB-468, 70 protein spots (61 spots in $siRNA_{neg}$ (present WT1) and 9 spots in $siRNA_{WT1}$ (without WT1)) had a significant change in expression level. While, 1-DE LC-MS/MS showed 164 proteins differentially expressed between $siRNA_{neg}$ and $siRNA_{WT1}$ in MCF-7 and MDA-MB-468. These proteins could be classified by their functions in apoptosis, cell signaling, protein folding, metabolism, structural, cell adhesion, gene expression, transport, redox-regulation, protein degradation and unknown functions. In MCF-7, the proteins involving apoptosis were cathepsin D, apoptosis inducing factor, and apoptosis stimulating of p53 protein and were found only in silenced WT1 condition. In the presence of WT1, the following proteins involving signal transduction pathway were found: 14-3-3 epsilon, signal transducing adaptor protein 1, phospholipase C, and metabotropic glutamate 5 receptor. In MDA-MB-468, proteins involving apoptosis

including ALG-2 interacting protein x and apoptosis- inducing factor 1 were up-regulated in the presence of WT1, while mitogaligin, an apoptosis related molecule, was identified when WT1 was silenced. On the other hand, proteins related in the signaling pathway were detected in both $siRNA_{neg}$ and $siRNA_{WT1}$ but the type of proteins were different. For example, IBTK protein, and SH2 domain containing protein were present in $siRNA_{WT1}$ condition, while the platelet derived growth factor receptor (PDGFRA**),** rho guanine nucleotide exchange factor 1, guanine nucleotide binding protein, and neuropolypeptide h3 were expressed in $siRNA_{neg}$. From these results it may be assumed that WT1 could be related with proteins involved in apoptosis: cathepsin D, apoptosis-inducing factor 1, and apoptosis stimulating factor of p53 protein 2 and may play a role as an anti-apoptosis in MCF-7. While in the signal transduction pathway, WT1 may crosstalk with 14-3-3 epsilon, signal transducing adaptor protein 1, phospholipase C, and metabotropic glutamate receptor resulting in cell growth or cell proliferation. Thus, WT1 acts as an oncogene in MCF-7. In MDA-MB-468, WT1 relates to mitogaligin and behaves as an anti-apoptotic molecule. Moreover, WT1 may be associated with the platelet derived growth factor receptor alpha, rho guanine nucleotide exchange factor 1 that activates proliferation via the mTOR pathway. Furthermore, WT1 may act as a negative regulator or block Raf kinase inhibitor resulting in activation of the MAPK pathway and promote metastasis. However, validation of the selected protein is necessary to confirm these hypotheses.

Executive Summary

วัตถุประสงค์

To study the relationship between WT1 and p53 in breast cancer cell lines: MCF-7 (p53 wild type) and MDA-MB-468 (p53 mutation).

วิธีการทดลอง

1. Cell culture

The human breast cancer cell lines MCF-7 (p53 wild-type, estrogen dependent) and MDA-MB-468 (p53 mutation, estrogen independent) (Figure 10) were kindly given from Assist. Prof. Dr. Potchanapond Graidist (Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University). MCF-7 and MDA-MB-468 cell lines were cultured in RPMI-1640 and DMEM (Dulbecco's Modified Eagle Medium) medium (GIBCO BRL), respectively. The medium was supplemented with 10% fetal bovine

serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM glutamine. Then the cells were incubated in a 37°C incubator with 5% CO_2 (Graidist *et al*., 2010).

2. Small interference RNA (siRNA) transfection

MCF-7 and MDA-MB 468 cell lines at $1x10^5$ cells were seeded in each well of 24-well culture plate and incubated in a 37°C incubator with 5% CO_2 for 24 hours. The cells were 40-50% confluent in next day and transfected with small interference RNA (siRNA). The siRNA against WT1 ($siRNA_{WT1}$) (Invitrogen) consisted of a mixture of two 25-nt duplexes, labeled as $siRNA_{WT1}$R88 (5'-AAATATCTCTTATTGCAGCCTGGGT3') and $siRNA_{WT1}$R90 (5'-TTTCACACCTGTATGTCTCCTTTGG-3'). The formal sequence was located on exon 7 while the later one was located on exon 8. The cells were transfected with 100 and 200 nM siRNA duplexes for MCF-7 and MDA-MB-468, respectively. The cells were transfected with Stealth Select RNAi (Invitrogen) as a negative control. All procedures were performed in RNase-free environment. The transfection cells with siRNA-duplexes were performed using Lipofectamine 2000 reagent (Invitrogen) at the final concentration of 1% in serum free medium (Opti-MEM® I, Gibco BRL). The mixure was incubated for 15 minutes at room temperature and then mixed with serum free medium (Opti-MEM® I, Gibco BRL). The old medium was removed from cells and washed once with PBS. The mixture was added into the plate. To avoid the cytotoxicity of the reagent, the cells were washed once with PBS, and the media was changed six hours after transfection (Navakanit *et al*., 2007). The cells were harvested at 72 hours after transfection. The total of extracted protein in transfected cells was investigated expression by Western blot analysis.

3. Western blot analysis

To investigate the WT1 protein level, the cells were harvested by trypsinization. Protein was extracted with radio immunoprecipitation assay (RIPA) buffer (Pierce, Rockford, USA). Then the concentration of protein was determined by Bradford assay (Bio-Rad, Hercules, USA) (APPENDIX A). The 50 µg of protein samples were loaded to 12% SDS-polyacrylamide gel electrophoresis (APPENDIX A) and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked by blocking solution (5% low fat dry milk in 1x TTBS (0.1% Tween®20, 154 mM NaCl, 48 mM Tris base)) for 1 hour and washed 5 minutes for three times with washing solution (1% low fat dry milk in 1x TTBS buffer). After blocking, the membrane was incubated with primary antibody anti-WT1 (1:200) and anti-actin (1:1,000) antibodies in 1% low fat dry milk in 1x TTBS for 2 hours and washed 5 minutes for three times with washing solution. The membrane was then incubated with secondary antibody polyglonal anti-IgG rabbit (1:10,000) antibody in 1% low fat dry milk in 1x TTBS for WT1 and actin for 1 hour. After blotting with secondary antibody, the membrane was detected by SuperSignal

West Femto chemiluminescence Substrate (Pierce, Rockford, USA) for WT1 and SuperSignal West Pico chemiluminescence Substrate (Pierce, Rockford, USA) for actin . Then the membrane was placed in the film cassette containing CL-XPosure X-ray film (Pierce, Rockford, USA) and exposed for 1-10 minutes. The X-ray film was developed by developing machine (Navakanit *et al*., 2007)

4. Proteomics analysis

4.1 One- dimension electrophoresis (1-DE)

4.1.1 Sample preparation

After transfection, $siRNA_{WT1}$ and $siRNA_{neg}$ transfected cells from both MCF-7 and MDA-MB 468 were extracted with 0.5% SDS and incubated at 37°C for 15 minutes. Then, the suspension was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was transferred to new steriled eppendorf tube. Protein quantification was calculated using Lowry method. The absorbance at 690 nm (OD_{690}) was measured and the protein concentration was calculated using the standard curve, plotted between OD_{690} on Y-axis and BSA concentration (μg/ml) on X-axis.

4.1.2 One dimension-polyacrylamide gel electrophoresis (1D-PAGE)

Total protein samples of 50 μg were loaded onto 12.5% SDS-polyacrylamide gel (APPENDIX A) and a marker lane (Low range marker, GE Healthcare) was added for calculation of the molecular weight of the protein bands. The gel was run at 20 mA/gel for 1.45 hours with ATTO AE 6540 electrophoresis apparatus (Figure 15). After electrophoresis, the gel was fixed in fixing solution (40% ethanol, 10% acetic acid) and stained with Colloidal Comassie blue G-250 (8% ammonium sulfate, 0.8% phosphoric acid, 0.08% Coomassie Brilliant blue G-250, and 20% methanol). Then the gel was scanned with EPS 601 scanner (Bio-rad) and the gel bands were fractionated to 15 slices (Figure 16) and excised from the bottom to the top of the gel lane and cut each slice into 1 mm cubes. The gel pieces were transferred into a well of low binding 96-well plate.

4.2 In gel digestion

Selected protein spots from 1D-gel were placed in low binding 96-well plate and destained by washing twice with 25 mM ammonium bicarbonate in 50% methanol. The gel plugs were dehydrated with 100% acetonitrile (ACN). Then, gel plugs were reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. To perform in-gel digestion of proteins, 10 μl of trypsin solution (10 ng/μl sequencing

grade modified porcine trypsin (Promega, USA) in 10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gels, then the gels were incubated at room temperature for 10 min in a shaker and dried in 40°C incubator overnight. Peptides extracted were collected and pooled together in the new tube. The pool extracted peptides were dried by vacuum centrifuge and kept at -80°C for further mass spectrometric analysis.

4.3 LC-MS/MS and protein identification

The dried extracted peptides were resuspended with 12 µl of 0.1% formic acid and transferred to low binding microcentrifuge tube. Then the solution was centrifuged at 10,000 rpm for 10 minutes and transferred to vial tube. The resuspended peptide was injected to LC-MS/MS (HPLC-ESI-Quad-TOF Mass spectrometry) (Figure 17). LC-MS/MS analysis of digested peptide mixtures was performed using a Waters SYNAPT™ HDMS™ system. The 1D-nanoLC was carried out with a Waters nanoACQUITY UPLC system. Four microlitre of tryptic digests was injected onto the RP analytical column (20 cm x 75 µm) packed with a 1.7 µm Bridged Ethyl Hybrid (BEH) C18 material (Waters). Peptides were eluted with a linear gradient from 2% to 40% acetonitrile developed over 60 minutes at a flow rate of 350 nl/min. This was followed by a 15 min period of 80% acetonitrile to clean the column before returning to 2% acetonitrile for the next sample. The effluent samples were electrosprayed into a mass spectrometer (Synapt HDMS) for MS/MS analysis of peptides and then generated the spectral data for further protein identification against database search. For 2D-PAGE, the peptide sequences from LC-MS/MS were analyzed by Mascot Search and identified by NCBInr data base (Figure 18). On the other hand, protein quantification and identification of 1D-PAGE was analyzed by The DeCyder™ MS 2.0 Differential Analysis Software (GE Healthcare) and Mascot, respectively (Figure 19). To normalization, the peptides of selected range (range 7 for this study) were spiked with known amount of bovine serum albumin peptides.

4.4 Bioinformatics

After proteins were identified from LC-MS/MS and Mascot search, all proteins were grouped into particular functions using the GO categorizer (GOCat), a tool to navigate the Gene Ontology. Moreover, the proteins were clustered, visualized, and classified by Multi Experiment Viewer (MeV) version 4.6.1. Finally, protein interactions

were predicted by Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) 9.05 database, a known- predicted protein-protein association.

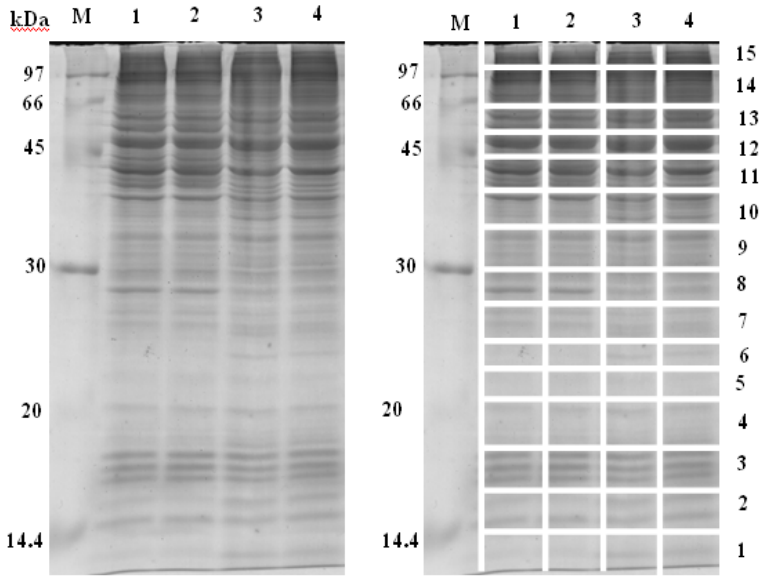
STRING is a database and web resource dedicated to protein–protein interactions, including both physical and functional interactions. It weights and integrates information from numerous sources, including experimental repositories, computational prediction methods and public text collections, thus acting as a meta-database that maps all interaction evidence onto a common set of genomes and proteins providing the most comprehensive view on protein–protein interactions currently available. STRING can be reached at http://string-db.org/ (Jensen *et al*., 2009). The lasted version 9.05 contains information on 5.2 millions proteins from 1133 species.

ผลการทดลอง

One dimensional gel electrophoresis (1D-PAGE)

The quantitative proteomic, one-dimensional gel electrophoresis (1D-PAGE) was also carried out to determine the protein expression patterns between $siRNA_{neg}$ compared to $siRNA_{WT1}$ in MCF-7 and MDA-MB-468. Figure 1 represents the protein patterns obtained from 1D-PAGE. Lane 1 and 2 shows the protein bands of $siRNA_{neg}$ and $siRNA_{WT1}$ in MCF-7. While lane 3 and 4 shows the protein bands of $siRNA_{neg}$ and $siRNA_{WT1}$ in MDA-MB-468. After 1D-PAGE, the gels were cut into 15 slices as shown in Figure 1B.

A **B**



M = Marker
1= MCF-7 control cell ($siRNA_{neg}$)
2 = MCF-7 transfected with $siRNA_{WT1}$
3 = MDA-MB-468 control cell ($siRNA_{neg}$)
4 = MDA-MB-468 transfected with $siRNA_{WT1}$

Figure 1 12.5% SDS-gel image of protein pattern between $siRNA_{neg}$ compared to $siRNA_{WT1}$ in MCF-7 and MDA-MB-468 (A) and gel after fractionation into 15 slices (B)

1 Identification of differential protein expression by 1-DE and LC-MS/MS

The quantification of protein from 1D-PAGE was analyzed by the DeCyder™ MS 2.0 Differential Analysis Software (GE Healthcare). The protein expressions of all four conditions were compared together including MCF-7 $siRNA_{WT1}$ (MS), MCF-7 $siRNA_{neg}$ (MN), MDA-MB-468 $siRNA_{WT1}$ (DS), and MDA-MB-468 $siRNA_{neg}$ (DN). The protein expressions with different intensity among these four conditions were shown in Venn's diagram which demonstrated all possible relations of protein expressions in all four conditions (Figure 2). There were 12, 11, 12, and 14 proteins expressed only in MS, MN, DS, and DN, respectively. Furthermore, there were 23 proteins expressed in both MS and MN, 14 proteins expressed in DS and DN, 9 proteins expressed in MS and DS, 11 proteins expressed in MS and DN, 4 proteins expressed in MN and DS, and 5 proteins expressed in MN and DN. Moreover there were 13 proteins expressed along in MS, MN, and DN, 13 proteins expressed in MN, DN, and DS, 15 proteins expressed in MS, DN, and DS as well as 11 proteins expressed in MS, MN, and DS. However, there were 219

proteins expressed together in MN, MS, DN, and DS. The protein names and their biological functions have been listed in Table 1-15.

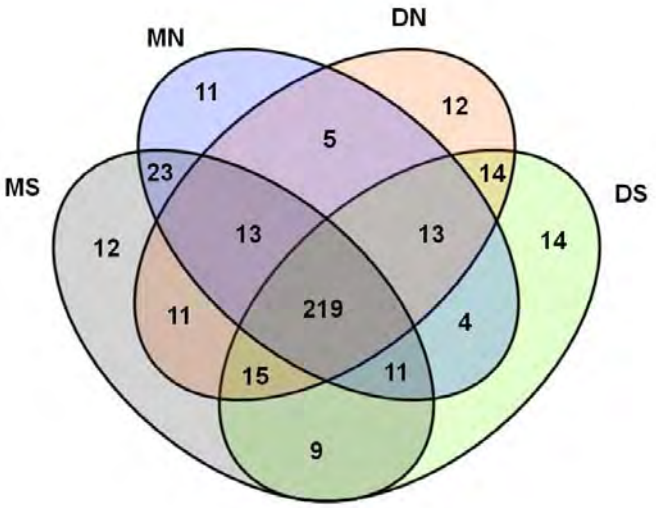


Figure 2 Venn's diagram of protein expression with different intensity between siRNA$_{WT1}$ transfection and siRNA$_{neg}$ control of MCF-7 and MDA-MB-468 (MN= MCF-7 siRNA$_{neg,}$ MS = MCF-7 siRNA$_{WT1}$, DS = MDA-MB-468 siRNA$_{WT1}$, and DN = MDA-MB-468 siRNA$_{neg}$)

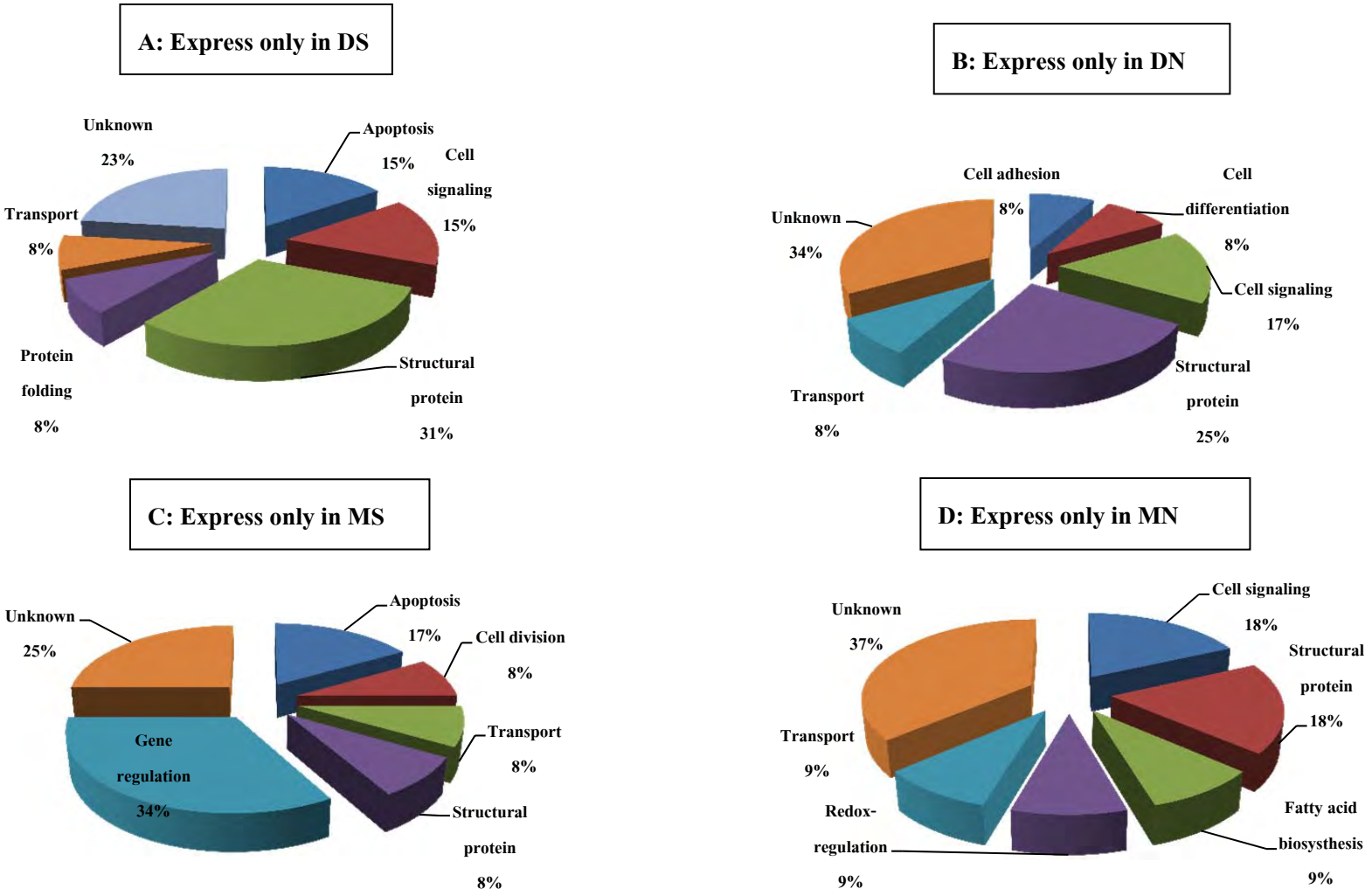
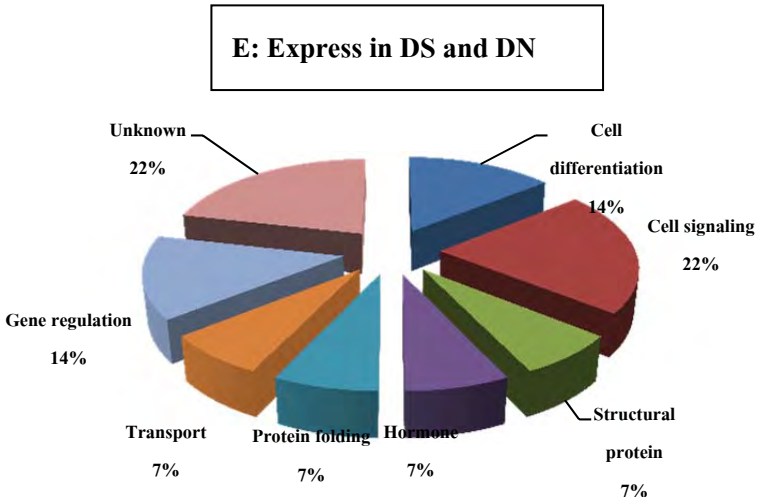
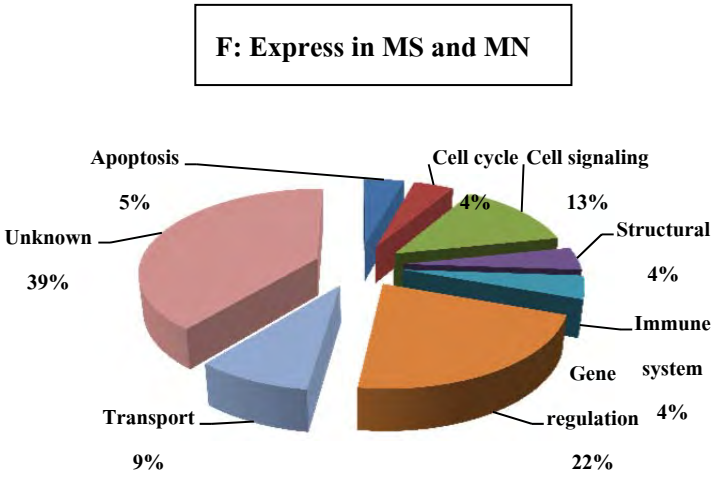
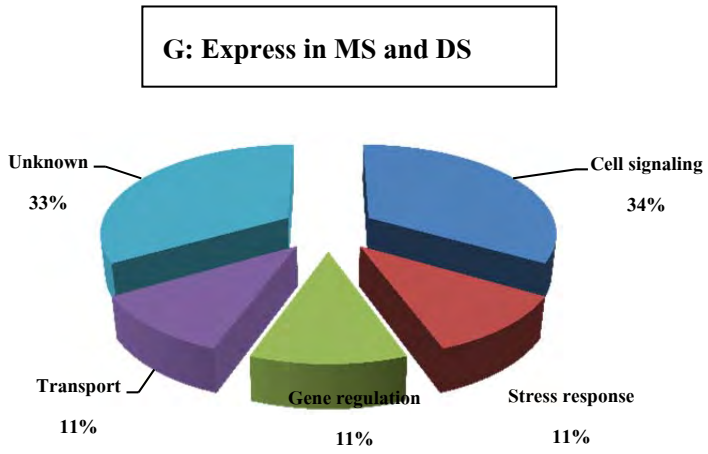


Figure 3 Functions of differentially expressed proteins between $siRNA_{WT1}$ and $siRNA_{neg}$ in MDA-MB-468 and MCF-7







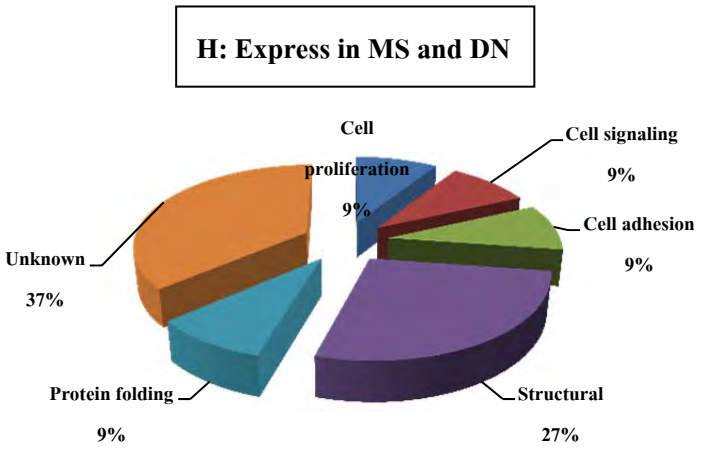
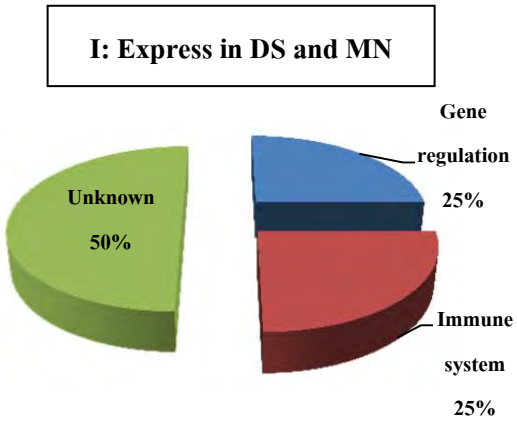
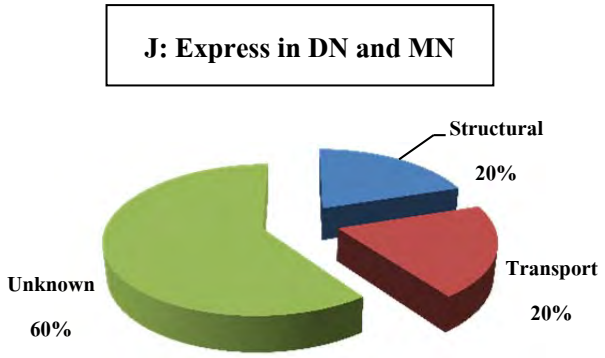
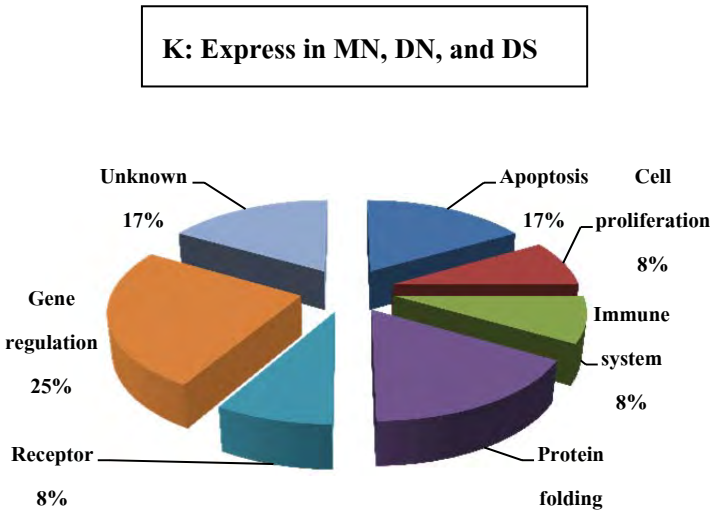


Figure 3 (Continued)







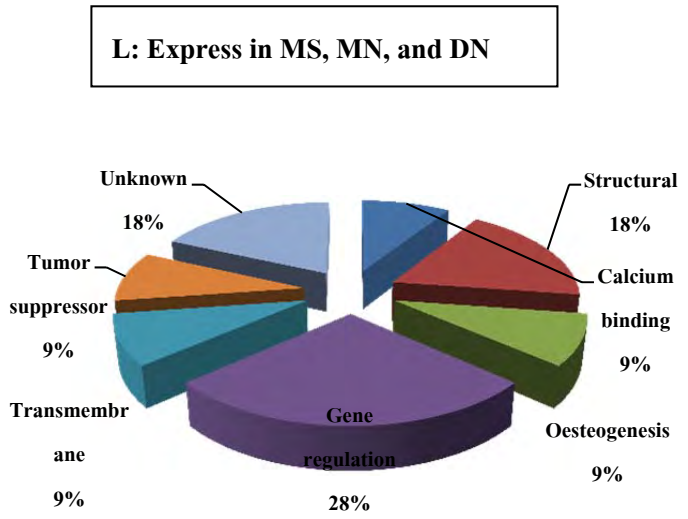
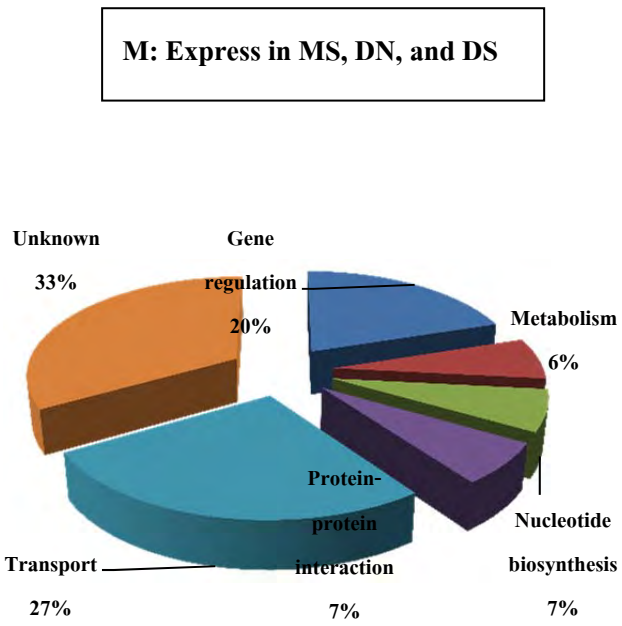


Figure 3 (Continued)



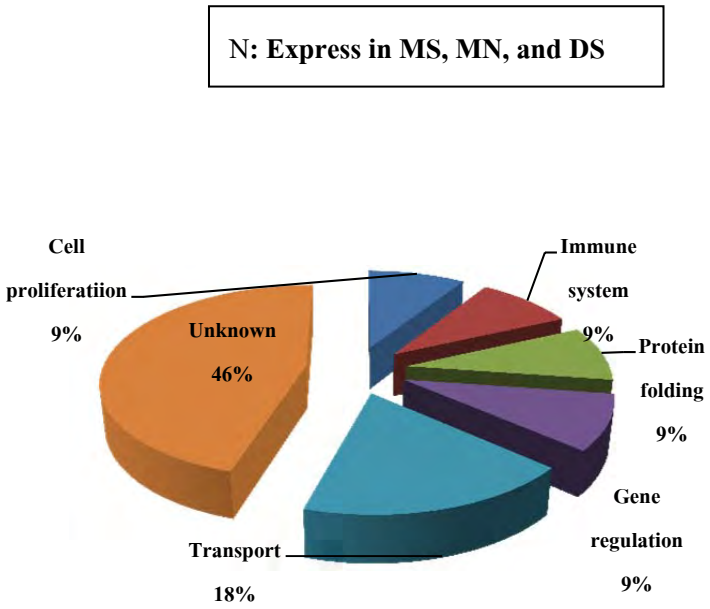


Figure 3 (Continued)

O: Express in MN, MS, DN, and DS

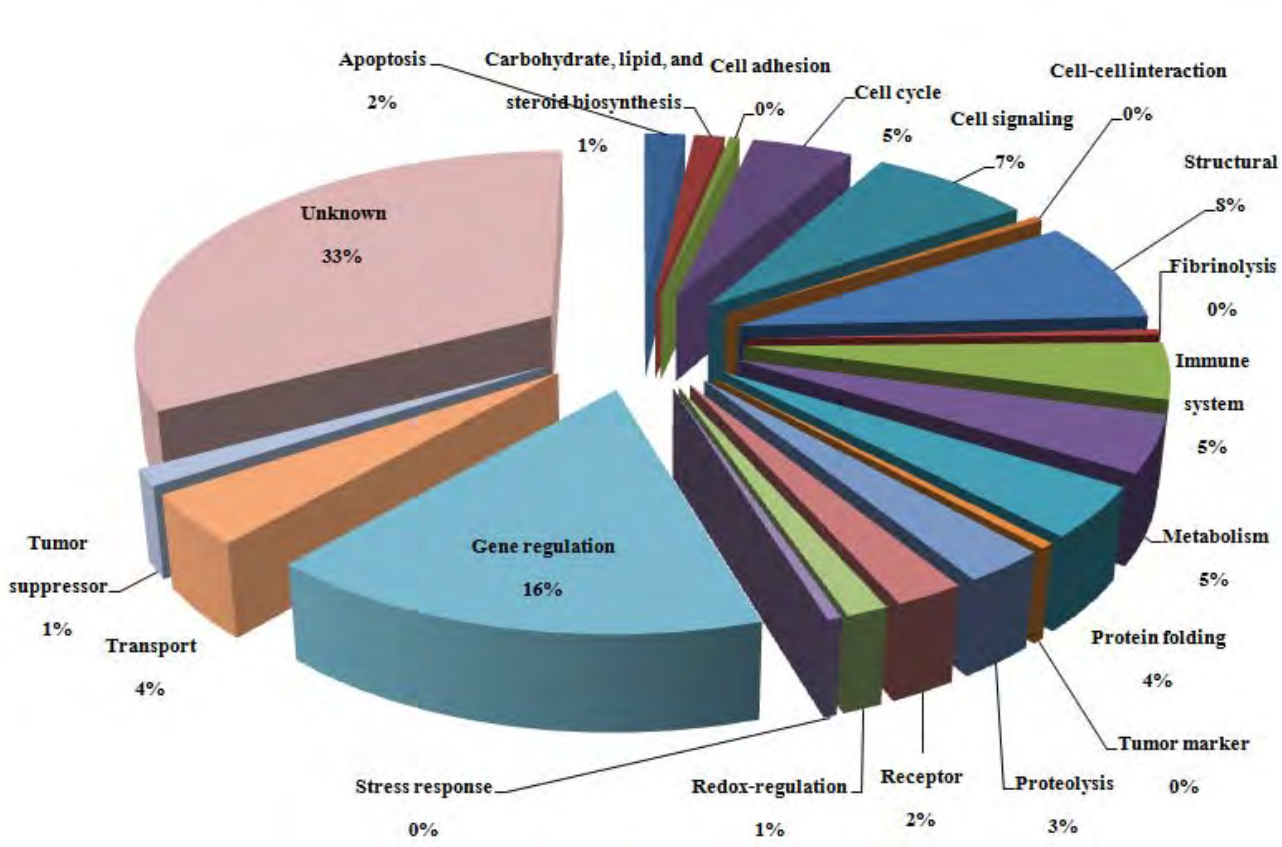


Figure 3 (Continued)

Table 1 Identification of expressed proteins only found in MDA-MB-468 $siRNA_{WT1}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Apoptosis			
mitogaligin	gi|12005991	AWRMGEPACWGR	9.50
Cell signaling			
IBTK protein, partial	gi|34192875	SLDVLSDGVLK	27.56
SH2 domain-containing protein 3C isoform a	gi|41281821	RSSASISR	11.47
Structural protein			
cytokeratin 9	gi|435476	GGSGGSYGGGGSGGGYGGGSGSR	91.06
Keratin 10	gi|21961605	SQYEQLAEQNRK	50.17
keratin, type II cytoskeletal 1	gi|119395750	SLNNQFASFIDK	98.71
type I keratin 16	gi|1195531	APSTYGGGLSVSSR	30.64
Protein folding			
Ankyrin repeat domain-containing protein 62	gi|302393830	LNDLNDRDK	13.03
Gene regulation			
SON DNA binding protein isoform E	gi|17046381	NRDKGEKEK	10.73
Redox-regulation			
selenoprotein I	gi|119621096	KMAASTRVEASR	5.30
Transport			
synaptosomal-associated protein 23 isoform SNAP23A	gi|18765729	KLIDS	4.17

Table 1 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Unknown			
hCG2042301	gi|119611404	TGGDRTKAQRHEIISLS	11.14
unknown protein IT12	gi|2792366	SGARAMAKAKK	7.15
unnamed protein product	gi|21757251	LINDSTNK	19.40

Table 2 Identification of expressed proteins only found in MDA-MB-468 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Cell adhesion			
vang-like protein 1 isoform 1	gi|20373171	HMAGLK	12.95
Cell differentiation			
METRNL protein, partial	gi|30047763	VFEPVPEGDGHWQGR	10.04
Cell signaling			
PDGFRA protein	gi|39645305	VPSIKLVYTLTVPEATVK	11.73
rho guanine nucleotide exchange factor 11 isoform 1	gi|7662086	SSNSK	6.04

Table 2 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Structural			
Keratin 5	gi|18999435	LAELEEALQK	23.61
peroxisome assembly protein 26 isoform a	gi|8923625	KSDSSTSAAPLR	6.59
hHa7 protein	gi|50949256	NTLNGHEK	12.35
Transport			
Na+/K+ -ATPase alpha 3 subunit variant	gi|62898870	LNIPVSQVNPR	14.46
Unknown function			
unnamed protein product	gi|194390014	MFHLAAFKLK	22.44
hCG2042050	gi|119579649	ASTVPDLK	7.42
chromosome 9 open reading frame 39	gi|119579068	LLEGQSLALSPR	11.96
hypothetical protein LOC286076	gi|119602615	DVGDALPR	29.47

Table 3 Identification of expressed proteins only found in MCF-7 siRNA$_{WT1}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Apoptosis			
Apoptosis-stimulating of p53 protein 2	gi|33860140	ENGVNSPR	37.14
apoptosis-inducing factor 1, mitochondrial isoform 5 precursor	gi|195927006	QMASSGASGGK	9.7

Table 3 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Cell division			
centrosomal protein of 63 kDa isoform b	gi|109255239	KQMREFRGNTK	5.37
Transport			
solute carrier family 12 member 9 isoform 1	gi|31881740	ILHALAR	28.14
Structural proteins			
keratin 19	gi|7594732	AALEDTLAETEAR	80.11
Gene regulation			
GATA zinc finger domain-containing protein 1	gi|88759346	SSPFPTVPTRPEK	19.38
M-phase phosphoprotein, mpp8, isoform CRA_a	gi|119628633	YQKRHDSDK	12.38
cap-specific mRNA (nucleoside-2'-O-)-methyltransferase 1	gi|24307983	NFVLDNTDR	7.37
eukaryotic translation initiation factor 4 gamma, 3, isoform CRA_a	gi|297283576	AGKIVVNLTGR	1.87
Unknown function			
SRSQ1913	gi|37181514	AVALDLPGFGNSAPSK	11.91
hCG1781582	gi|119603082	KTRMEDTFWNSRLDNISR	11.78
unnamed protein product	gi|14042413	KKSKPCLIK	14.36

Table 4 Identification of expressed proteins only found in MCF-7 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Cell signaling			
Phospholipase C, delta 1 variant	gi|62089310	GAVATQVSPAVPLPPR	10.01
metabotropic glutamate receptor 5 isoform B precursor	gi|4504143	RLMETPNAR	16.38
Structural protein			
tubulin alpha-1C chain	gi|14389309	DVNAAIATIK	40.29
keratin, type I cytoskeletal 18	gi|4557888	AQIFANTVDNAR	57.19
Fatty acid biosynthesis			
fatty acid synthase	gi|119610151	LQVVDQPLPVR	41.03
Redox-regulation			
Cu/Zn-superoxide dismutase	gi|1237406	KHGGPK	4.35
Transport			
anion exchange transporter isoform a	gi|16306483	KFYTDLMNMIQK	13.97
Unknown function			
hCG1739111, isoform CRA_a	gi|119613684	MMSGPVPQCLR	1.26
Hypothetical protein DKFZp434O1826 variant	gi|62089384	QGTEERQPRSR	3.11

Table 4 (Continued)

Protein name	Accession number	Peptide	Mowse Score
unnamed protein product	gi|193788364	MEGKKPRVMAGTLK	10.55
Chromosome 9 open reading frame 139	gi|124376896	LAGSLATDLSR	15.44

Table 5 Identification of expressed proteins found in MDA-MB-468 $siRNA_{WT1}$ and MDA-MB-468 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Structural protein			
LMNA protein	gi|21619981	SGAQASSTPLSPTR	44.82
Cell differentiation and survival			
Nance-Horan syndrome protein isoform 2	gi|42384238	KTISGIPR	26.98
sestrin-2	gi|13899299	KLSEINK	21.68
Cell signaling			
S100 calcium binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11)), isoform CRA_b	gi|119573783	NALSGAGEASAR	11.49
Chain A, Catalytic Domain Of Human Phosphodiesterase 4b In Complex With Piclamilast	gi|58177395	GMEISPMXDK	8.66
protein S100-A6	gi|7657532	LQDAEIAR	43.91

Table 5 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Hormone			
C-type natriuretic peptide precursor	gi|13249346	YKGANKKGLSK	10.08
Protein folding			
heat shock protein	gi|4204880	IINEPTAAAIAYGLDKK	27.1
Transport			
ras association domain-containing protein 9	gi|114155158	ADAFLPVPLWR	6.35
Gene regulation			
TTLL5 protein	gi|33877151	MGNTMDKR	10.31
39S ribosomal protein L15, mitochondrial	gi|7661806	CGRGHK	16.37
Unknown function			
hCG16415, isoform CRA_f	gi|119611935	GAECCPGGPVK	10.83
FLJ00258 protein	gi|18676718	GSMSR	8.83
pyruvate dehydrogenase E1 alpha subunit	gi|861534	EEIPPHSYR	6.28

Table 6 Identification of expressed proteins found in MCF-7 $siRNA_{WT1}$ and MCF-7 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Cell signaling			
orphan G protein-coupled receptor HG20	gi|4836218	FQGSEPPK	21.41
MAPKBP1 protein	gi|71297458	WACLGEGTTPKPR	12.72
calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1B isoform 1	gi|4505677	SDAVPSEVR	17
Structural protein			
Chain A, Crystal Structure Of Human Full-Length Vinculin	gi|83753119	KLEAXTNSKQSIAK	9.63
Immune system			
PREDICTED: HLA class II histocompatibility antigen, DRB1-7 beta chain-like isoform 3	gi|310124860	LRKSPGMLEK	11.26
Gene regulation			
non-histone chromosomal protein HMG-14	gi|48255933	RKVSSAEGAAK	5.73
serine/threonine-protein kinase SMG1	gi|62243658	LSSGGGGGGTKYPR	6.52
zinc finger protein 38, isoform CRA_b	gi|119597017	ASVSMRASAPTR	6.45
ORF	gi|434765	AAGIR	4.95
U2AF1-RS1	gi|1125020	IKKEKEEAAK	11.82

Table 6 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Transport			
Golgin subfamily A member 7B	gi|58219040	GLLLTDPVERGMR	16.12
stomatin-like protein 3 isoform 2	gi|221316744	VLAAEGEMNASK	14.3
Apoptosis			
NACHT, LRR and PYD domains-containing protein 7 isoform 2	gi|75709196	MGPCSFAELISK	7.37
Cell cycle			
NIMA-related kinase 6	gi|10121890	MLHRSPSGTRARPR	8.17
Unknown function			
FLJ00020 protein	gi|10440371	QGRPLPR	18.8
hCG1783738, isoform CRA_b	gi|119606482	KRHEASMFR	2.96
SLC44A1 protein, partial	gi|17390479	EAGKGGVADSR	13.48
unnamed protein product	gi|10433066	QGAKEKQLLK	39.4
protein FAM181B	gi|54873602	LALDKPGKSK	11.59
unnamed protein product	gi|194378218	DASQVSAPGTRR	8.81
similar to RIKEN cDNA 1700011J18	gi|119607333	LILVSKSLEFLDGK	14.65
KIAA0411 protein	gi|25535933	LSXISEDVIR	17.36
hypothetical protein LOC286076	gi|119602615	DVGDALPR	21.23

Table 7 Identification of expressed proteins found in MCF-7 siRNA$_{WT1}$ and MDA-MB-468 siRNA$_{WT1}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Cell signaling			
2-5A-dependent ribonuclease	gi|10863929	GGATALMDAAEK	10.33
centaurin beta2	gi|4688902	EAYIRAKYVER	4.90
PDE4D protein	gi|14249999	LSPVISPR	28.91
Stress response			
SGK-like protein SGKL	gi|17402861	KKRFTVYK	8.89
Gene regulation			
DNA-directed RNA polymerases I and III subunit RPAC2 isoform 1	gi|7705740	TSMAEGERK	14.28
Transport			
stathmin-like 3, isoform CRA_b	gi|119595656	AAAPSAAR	11.10
Unknown function			
KIAA0338	gi|2224617	GTPEKANERAGLR	6.93
unnamed protein product	gi|21751864	IKKANECASR	11.11
CYorf15A protein	gi|83405816	QGLSLSPR	29.27

Table 8 Identification of expressed proteins found in MCF-7 $siRNA_{WT1}$ and MDA-MB-468 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Cell proliferation			
Calcium homeostasis endoplasmic reticulum protein	gi|18204653	NSGPSRSRSR	14.73
Cell signaling			
Mitogen-Activated Protein Kinase Kinase Kinase 3	gi|83754682	SSSXK	9.13
Cell adhesion			
Neural cell adhesion molecule 1	gi|28703938	SHARVSSLTLK	4.4
Structural protein			
Keratin 8	gi|49256423	ISSSSFSR	55.86
sperm-associated antigen 17	gi|46240864	TRKEIETTQNYLMDIKNR	15.76
myosin regulatory light chain 10	gi|34147532	ESLALSPR	29.6
Protein folding			
90kDa heat shock protein	gi|306891	ADLINNLGTIAK	64.95
Unknown function			
hCG1813960	gi|119615973	MESLQCASGTLK	5.59
KIAA0483 protein	gi|3413926	IVPILKR	8.96

Table 8 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Unknown function			
KIAA1712 protein	gi|12697969	VMATGDLKRSLR	9.33
unnamed protein product	gi|16551723	MHNAAR	15.37

Table 9 Identification of expressed proteins found in MDA-MB-468 $siRNA_{WT1}$ and MCF-7 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Gene regulation			
RNA-binding protein 5	gi|5032031	MGSDK	1.11
Immune system			
MHC class II regulatory factor RFX1	gi|238859557	FEPVLQWTK	5.57
Unknown function			
unnamed protein product	gi|21755689	QDILDEMRK	14.97
N-acetylserotonin O-methyltransferase-like protein isoform 1	gi|117553627	MVLCPVIGK	8.56

Table 10 Identification of expressed proteins found in MDA-MB-468 $siRNA_{neg}$ and MCF-7 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Structural protein			
talin-1	gi|223029410	MATNAAAQNAIKKK	4.8
Transport			
mitochondrial dicarboxylate carrier isoform	gi|20149598	LFSGATMASSR	7.2
Unknown function			
hypothetical protein	gi|8246847	MGPTK	1.97
KIAA1311 protein	gi|7242977	QEVLEKQIECQK	14.75
unnamed protein product	gi|194375249	AEAGT	9.34

Table 11 Identification of expressed proteins found in MCF-7 $siRNA_{WT1}$, MCF-7 $siRNA_{neg}$, and MDA-MB-468 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Calcium binding protein			
calretinin	gi|825634	GSGMMSK	18.67

Table 11 (Continued)

Protein name	Accession number	Peptide	Mowse Score

Structural protein			
keratin type II	gi|914833	NKYEDEINKR	26.10
myosin-IIIa	gi|145275208	KEIVDMK	10.30
Oesteogenesis			
leucine-rich repeat-containing protein 17 isoform 2 precursor	gi|239582714	KASPGSVRSR	18.37
Gene regulation			
enhancer of mRNA-decapping protein 4	gi|45827771	VPAPR	5.32
heterogeneous nuclear ribonucleoproteins C1/C2 isoform	gi|117190174	VPPPPPIAR	9.87
chorion-specific transcription factor GCMb	gi|4758420	SETEAR	23.01
Transmembrane protein			
leucine-rich repeat neuronal 6A	gi|37675422	HLVSAK	8.59
Tumor suppressor			
adenomatous polyposis coli homolog APC2	gi|6018189	EDYRQVLR	9.70
Unknown function			
unnamed protein product	gi|194386918	ALLVG	5.56
unnamed protein product	gi|194376292	FGSIPK	5.59

Table 12 Identification of expressed proteins found in MCF-7 $siRNA_{neg}$, MDA-MB-468 $siRNA_{WT1}$, and MDA-MB-468 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Apoptosis			

lymphotoxin alpha transcript variant 6	gi|78370182	SPELSKKEF	4.96
inactive caspase-12	gi|300360580	AGADTHGRLLQGNICNDAVTK	20.82
Cell proliferation			
oncoprotein-induced transcript 3 protein precursor	gi|22749297	NSPLEIMSR	21.97
Immune system			
Ig heavy chain DJ region (clone C100-94) - human (fragment)	gi|345998	AMVXLLGPGT	31.6
Protein folding			
heat shock 60kDa protein 1 (chaperonin), isoform CRA_c	gi|119590557	VTDALNATR	37.44
heat shock protein 105 kDa	gi|42544159	ANEKK	3.89
Receptor			
sphingosine 1-phosphate receptor 3	gi|38788193	MATALPPR	24.17
Gene regulation			
LIM homeobox transcription factor 1-alpha	gi|28893581	GTAEEGKDHK	9.47
Zinc finger protein 181	gi|71297022	HQRIHTMEK	7.25
signal recognition particle 19 kDa protein isoform 1	gi|4507213	TIAEGR	2.27

Table 12 (Continued)

Protein name	Accession number	Peptide	Mowse Score

Unknown function			
hCG1649526, isoform CRA_a	gi|119583049	RSPAAGIR	15.13

Table 13 Identification of expressed proteins found in MCF-7 $siRNA_{WT1}$, MDA-MB-468 $siRNA_{WT1}$, and MDA-MB-468 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Gene regulation			
adenosine deaminase domain-containing protein 2 isoform 2	gi|223972690	QLLLATQGGPK	7.01
eukaryotic translation initiation factor 2B, subunit 4 delta, 67kDa, isoform CRA_f	gi|119620997	TPGKANAK	10.22
coiled-coil and C2 domain-containing protein 2A isoform	gi|257900481	QNKNSKVR	10.76
Metabolism			
glyceraldehyde-3-phosphate dehydrogenase	gi|31645	IISNASCTTNCLAPLAK	78.7
Nucleotide biosysthesis			
phosphoribosyl pyrophosphate synthase-associated protein 2 isoform 1	gi|4506133	NAVIVAK	15.58
Protein-protein interaction			
tetratricopeptide repeat protein 22 isoform 1	gi|166235180	AKMGLGGMPDR	10.29

Table 13 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Transport			
ATP synthase subunit alpha, mitochondrial isoform c	gi|50345982	VLSIGDGIAR	16.53
Tpr	gi|633226	AIVAAK	1.15
vacuolar proton ATPase	gi|313014	AEEEFNIEK	11.8
N-methyl D-aspartate subunit 3A]	gi|20372905	AEALWPR	20.96
Unknown function			
unnamed protein product	gi|10438636	KSRPLTNSVKL	11.17
unnamed protein product	gi|7021931	NRDNQSMLIT	12.75
hCG2040455	gi|119601467	QEEDCRKVSR	8.46
unnamed protein product	gi|194378218	DASQVSAPGTRR	8.81
ALS2CR11	gi|15823651	GNSSLIKEQK	4.2

Table 14 Identification of expressed proteins found in MCF-7 siRNA$_{WT1}$, MCF-7 siRNA$_{neg}$, and MDA-MB-468 7 siRNA$_{WT1}$ using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Cell proliferation			
Angiopoietin-like 5	gi|29351676	LLATGIQWGTWTK	8.06
Immune system			
immunoglobulin heavy chain variable region	gi|145939619	ATTGA	6.98
Protein folding			
heat shock protein beta-1	gi|4504517	QLSSGVSEIR	41.36
Gene regulation			
zinc finger MYM-type protein 3 isoform 1	gi|4827067	SPRMSLR	22.91
Transport			
mucolipin-3 isoform 1	gi|24496763	KLKFFFMNPCEK	9.89
vesicular acetylcholine transporter	gi|507744	NVGLLTR	17.9
Unknown function			
hCG1749575, isoform CRA_a	gi|119625824	DGRGIIIFPR	15.28
unnamed protein product	gi|194381006	AARAWEGDAR	7.73
hypothetical protein	gi|52545574	ITDYALIAIGR	12.5

Table 14 (Continued)

Protein name	Accession number	Peptide	Mowse Score

Unknown function			
hCG2014677, isoform CRA_d	gi|119617550	ATSKAPQGSNSK	7.84
unnamed protein product	gi|34527456	MDLKCKKMK	13.1

Table 15 Identification of expressed proteins found in MCF-7 $siRNA_{neg}$, MCF-7 $siRNA_{WT1}$, MDA-MB-468 $siRNA_{WT1}$, and MDA-MB-468 $siRNA_{WT1}$using DeCyder™ MS 2.0 Differential Analysis Software

Protein name	Accession number	Peptide	Mowse Score
Apoptosis			
caspase recruitment domain protein 10	gi|13488607	EEDPAPPK	40.78
dead end protein homolog 1	gi|34740339	AAAMAK	7.54
niban-like protein 2 isoform b	gi|148664236	KEVPLSR	13.88
protocadherin gamma-A4 isoform 2 precursor	gi|14196468	VAENENPGAR	4.54
Cell adhesion			
trophinin, isoform CRA_b	gi|119613608	MHTLLAATK	5.98

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Carbohydrate, lipid, and steroid biosysnthesis			

alpha-1,2-mannosyltransferase ALG9 isoform b	gi|118026933	LKGSGASSGDTAPAADK	21.34
ethanolamine kinase 2	gi|55960794	LGGGTAEGKTGR	15.3
steroid 21-hydroxylase	gi|253757549	LKQAIXKR	13.42
Cell cycle, cell division, cell growth, cell proliferation			
STON1 protein	gi|111309209	IDRLPDK	27.95
thrombopoietin	gi|3986139	QSLLGTQTR	10.79
Titin	gi|17066105	NAVGVSLPR	30.51
cell division cycle 2-like 5 isoform 2 variant	gi|62897667	QMGMTDDVSTIK	8.63
G1 to S phase transition 2	gi|23271293	LPIVDKYK	19.77
separase	gi|38349532	AVRADTGQER	13.31
synaptonemal complex protein 3	gi|24233580	ILNMFR	16.8
placental lactogen	gi|229348	VQTVPLSR	22.76
Chain B, Crystal Structure Of Human Gins Complex	gi|150261226	QVLEEXK	18.55
fibroblast growth factor 6 precursor	gi|15147343	GVVSLFGVRSALFVAMNSKGR	11.06

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Cell signaling			

beige-like protein	gi|21434741	LLASKSEGIR	13.76
dedicator of cytokinesis 4, isoform CRA_d	gi|119603875	GGKTN	5.82
adapter molecule crk isoform b	gi|41327710	GMIPVPYVEK	4.61
A-kinase anchor protein 9 isoform 2	gi|22538387	LEVTKREK	11.98
Chain A, Crystal Structure Of Vegfr2 In Complex With A 3,4,5-Trimethoxy Aniline Containing Pyrimidine	gi|209156455	IXDFGLAR	25.98
inositol 1,4,5-trisphosphate receptor type 1	gi|46107962	ACNNTXDRK	5.56
IQ motif containing GTPase activating protein 3, isoform CRA_	gi|119573332	NLLAMTDK	21.36
MOB kinase activator 3A	gi|18677731	ILSRLFR	28.28
protein kinase, cAMP-dependent, catalytic, gamma	gi|119582876	EFSEF	21.01
protein phosphatase 2A B'alpha1 regulatory subunit	gi| gi|31083236	IMEPLFR	25.71
RADIL protein	gi|33870359	NGPSGLR	16.11
Regulator of G-protein signaling 22	gi|92095662	HLEKMK	12.58
serine/threonine-protein kinase WNK2	gi|32455273	EQQDVGSPDK	12.63
Similar to protein tyrosine phosphatase, non-receptor type 18 (brain-derived), partial	gi|18999432	GAMSR	1.74

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Cell signaling			

SIRP-beta1	gi|2052058	VTTVSELTKR	23.63
V-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	gi|111305899	LENLMR	29.69
Cell-cell interaction			
AIDA-1b	gi|31746739	IMSSIDVGINNELK	18.8
Structural proteins			
nestin, isoform CRA_c	gi|119573310	VQGLEGPR	19.74
tektin 3 variant	gi|62898800	SQRVSENTMLPFVSNR	16.14
tektin-1	gi|16753231	LLQPPPK	20.61
tektin-1	gi|16753231	LLQPPPK	20.61
Abelson tyrosine-protein kinase 2 isoform d	gi|209862772	RNAPTPPK	17.63
beta-actin-like protein 2	gi|63055057	VAPDEHPILLTEAPLNPK	28.69
beta-tubulin	gi|2119276	LAVNMVPFPR	45.12
caldesmon 1, isoform CRA_d	gi|119604235	MRSQKGMIFLTK	10.55
cytokeratin	gi|1419564	LSELEAALQR	77.02
cytokeratin type II	gi|3901030	AQYEDIANR	43.55

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Structural proteins			

cytoplasmic dynein 1 light intermediate chain 2	gi|5453634	QPATPTR	15.32
DNAH1 variant protein	gi|34532301	NPGMR	1.97
integrin, alpha E	gi|119610886	LRGLQVVAVK	12.8
keratin	gi|1200072	QEELEAALQR	21.19
keratin 19	gi|7594734	IVLQIDNAR	63.6
keratin, type I cytoskeletal 17	gi|4557701	TKFETEQALR	23.83
mutant beta-actin (beta'-actin)	gi|28336	AGFAGDDAPR	62.28
myotubularin-related protein 6	gi|134142348	NMYHQFDR	5.65
protein 4.1 isoform 1	gi|260436831	LAPNQTK	9.85
Fibrinolysis			
Plasminogen	gi|38051823	LSSPADITDK	36.06
Immune system			
complement C4-B-like preproprotein	gi|338858017	FGLLDEDGKK	0.4
C-type lectin domain family 3 member A isoform 2	gi|348041279	GGILVIPR	22.39
human complement C1r	gi|179644	NEQKGEKIPR	9.91

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Immune system			

Ig lambda-chain (V-D-J) precursor, partial	gi|186110	SPQYLLRHR	13.06
IL25	gi|18034676	ASEDGPLNSR	27.98
immunoglobulin heavy chain variable region	gi|16075862	KAGSSVRVSCK	10.36
immunoglobulin lambda chain variable region	gi|16075980	RPXGLSPR	17.66
immunoglobulin light chain variable region	gi|109693140	GLTFGGGTK	12.82
T cell receptor beta chain CDR3	gi|3859246	TGGQFFG	12.71
TLR10	gi|67626189	AAVNVNVLATR	4.25
Metabolism			
ADPRHL2	gi|48146591	MAAAAMAAAAGGGAGAARSLSRFR	7.98
aldolase A protein	gi|28595	ELSDIAHR	10.11
DDX27 protein, partial	gi|32425487	ADTLKVKDR	13.32
enolase	gi|31179	IGAEVYHNLK	19.22
enoyl-CoA hydratase	gi|1922287	SLAMEMVLTGDR	10.6
glyceraldehyde-3-phosphate dehydrogenase	gi|31645	GALQNIIPASTGAAK	43.62
L-lactate dehydrogenase A chain isoform 2	gi|207028494	VTLTSEEEAR	27.21

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Metabolism			

lysosomal acid lipase/cholesteryl ester hydrolase precursor	gi|51317399	IINLMR	12.8
short-chain dehydrogenase/reductase family 9C member 7	gi|22507405	LKNIMQVAEPR	14.26
UDP-glucose 6-dehydrogenase isoform 3	gi|296040438	LAANAFLAQR	57.95
Protein folding			
78 kDa glucose-regulated protein precursor	gi|16507237	ITITNDQNR	39.47
chaperonin (HSP60)	gi|306890	GYISPYFINTSK	31.34
HSP70-HOM	gi|4529894	TTPSYVAFTDTER	58.04
mitochondrial heat shock 60kD protein 1 variant 1	gi|189502784	TVIIEQSWGSPK	16.48
prefoldin subunit 4	gi|12408677	AATMK	1.54
proteasome 26S ATPase subunit 1 variant	gi|62896895	AVANQTSATFLR	8.33
proteasome subunit	gi|565651	NISRIMR	24.31
protein unc-45 homolog A isoform 3	gi|89179321	QFAEGSTLK	16.8
TRAP1	gi|3273383	GVVDSEDIPLNLSR	87.94
Tumor marker			
breast cancer resistance marker 1	gi|30231005	IAGTK	13.7

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Proteolysis			
Cocaine esterase	gi|46576349	RLHRLRAR	6.9

DDB1- and CUL4-associated factor 15	gi|78486540	ISGQLSPR	26
DIP2B protein	gi|38014007	YHPIDIETSVSR	10.55
epidermal type II transmembrane serine protease	gi|45861650	TVGFGTRSR	20.42
F-box protein 16, isoform CRA_a	gi|119583916	AQSMMSLSASSPLK	20.73
procollagen galactosyltransferase 1 precursor	gi|31377697	LMNLMR	30.11
Receptor			
dopamine receptor D2longer	gi|7381416	AHLRAPLK	23.71
glutamate receptor, ionotropic, delta 2 variant	gi|62088216	LENNMR	20.78
nicotinic acetylcholine receptor beta-3 subunit	gi|34988	LPKLLCMK	22.62
olfactory receptor 51B6	gi|52353945	TVMGIGSGGER	9.15
oxytocin receptor	gi|32307152	FLCCSASYLK	19.4
Redox-regulation			
cytochrome c oxidase subunit	gi|119622335	KLTERRK	15.99
Chain A, Monomeric Human Cu,Zn Superoxide Dismutase Without Zn Ligands	gi|240104588	SGGPK	10.37
peroxiredoxin-1	gi|4505591	QITVNDLPVGR	41.73

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Stress response			
oxidation resistance 1, isoform CRA_c	gi|119612311	GTANR	7.44
Gene regulation			
cullin-1	gi|32307161	MSSTR	0.49
DNA repair protein RAD50	gi|19924129	INEDMR	21.41
ubiquitin specific peptidase 25, isoform CRA_c	gi|119630448	NLPFMR	28.65
ubiquitin-like modifier-activating enzyme ATG7 isoform a	gi|5453668	TADKK	14.03
cullin-4B isoform 1	gi|121114298	IMIIFR	32.82
thymidylate synthase	gi|18150851	MPVAGSELPR	10.78
BTB/POZ domain-containing protein 1 isoform 2	gi|59814146	IRSLNMR	31.03
histone H2A.Z	gi|4504255	AGLQFPVGR	43.13
histone H2B	gi|1568557	LLLPGELAK	27.53
histone H4	gi|4504301	ISGLIYEETR	47.64
histone RNA hairpin-binding protein	gi|5729862	SRCSDWASAVEEDEMR	11.11
N6-adenosine-methyltransferase 70 kDa subunit	gi|21361827	EPAKK	0.47
ARP1 actin-related protein 1 homolog B	gi|119622328	RRCSTRCQTAAR	7.98

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
histone RNA hairpin-binding protein	gi|5729862	SRCSDWASAVEEDEMR	11.11
N6-adenosine-methyltransferase 70 kDa subunit	gi|21361827	EPAKK	0.47
ARP1 actin-related protein 1 homolog B	gi|119622328	RRCSTRCQTAAR	7.98
BCL-6 corepressor isoform b	gi|183396785	KMAPTVLVHSR	10.49
DNMT1 protein	gi|62204780	LAGVTLGQR	15.29
lysine-specific demethylase 4A	gi|109157941	MTLISPLMLK	27.02
myocardin isoform 2	gi|23957692	EPNEQMVR	17.16
orphan nuclear receptor	gi|1163077	VPSASQVQAIK	4.95
probable ATP-dependent RNA helicase DDX5	gi|4758138	APILIATDVASR	33.49
protein Jumonji isoform 2	gi|388490158	LNDEMR	13.55
ribosomal protein L4	gi|40889023	KPVVGKK	12.35
ribosome-binding protein 1	gi|110611218	QQLSEMK	11.4
heterogeneous nuclear ribonucleoprotein A2/B1, isoform CRA_d	gi|119614244	GGGGNFGPGPGSNFR	8.83
nuclear receptor co-repressor 2, isoform CRA_c	gi|119618857	EPTPR	1.02
putative zinc finger protein H140, partial	gi|4098632	ENSKDNSXLTK	14.75
serine/arginine-rich splicing factor 11 isoform 1	gi|4759100	TPSSSRHR	5.98

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
splicing factor 3A subunit 1 isoform 2	gi|53831995	IHEATGMPAGK	7.01
transcription factor; zinc-finger DNA-binding protein	gi|1587214	ERSGGPVTR	12.16
transcriptional coactivator p75	gi|4050036	KKGQEGKQPK	17.45
zinc finger protein 276, isoform CRA_b	gi|119587092	VNASPAGRR	8.12
tuftelin-interacting protein 11	gi|8393259	KDPSGSKK	10.87
40S ribosomal protein S14	gi|5032051	TPGPGAQSALR	16.9
40S ribosomal protein SA	gi|9845502	FAAATGATPIAGR	39.08
double stranded RNA activated protein kinase	gi|6467479	DGIISDIXDKK	5.3
EEF2 protein, partial	gi|33869643	VFSGLVSTGLK	52.26
elongation factor	gi|4503471	IGGIGTVPVGR	62.05
Transport			
alpha1A-voltage-dependent calcium channel	gi|9711929	GPGSRK	2.23
ATPase Na+/K+ transporting alpha 4	gi|33324437	LTLEELSTK	18.53
Golgin subfamily A member 6-like protein 2	gi|182662391	ATDTK	5.18
importin subunit beta-1	gi|19923142	NSAKDCYPAVQK	2.74
mitochondrial ATP synthase, H+ transporting F1 complex beta subunit	gi|89574029	IGLFGGAGVGK	29.16

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Transport			
NOP14 protein	gi|19684184	GGPAK	1.41
sorcin isoform B	gi|38679884	LSPQAVNSIAK	2.92
voltage-gated potassium channel	gi|186798	REAETLRER	13.18
ras-related protein Rab-23	gi|34485714	NEEAEALAK	6.87
Tumor suppressor			
ADAMTS18 protein	gi|19171150	KIQCVQKKPFQK	16.87
breast cancer-associated antigen BRCA1	gi|20800447	ATVVNNTK	20.58
mitochondrial tumor suppressor 1, isoform CRA_c	gi|119584210	KAEILINK	10.27
Unknown function			
ANKHZN	gi|6759376	RGSGAAEQVDNK	8.33
C5orf47 protein	gi|6716764	DAAKK	2.02
Chain A, Solution Structure Of Rsgi Ruh-022, A Myb Dna-Binding Domain In Human Cdna	gi|159163338	GSSGSSGDKEWNEK	4.61
Chain B, Crystal Structure Of The Beta-CateninICAT COMPLEX	gi|24987641	MNREGAPAK	10.89
Chain B, Pwwp Domain Of Human Bromodomain And Phd Finger-Containing Protein 1 In Complex With Trimethylated H3k36 Peptide	gi|297343131	PATGGVXKPHRY	8.58

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Unknown function			
Chromosome 1 open reading frame 105	gi|23468238	QRSSLPR	27.86
FLJ46481 protein	gi|223461603	KQHEAKLAVTPLK	18.82
hCG16178, isoform CRA_a	gi|119600436	EAGCPAGRLYR	8.94
hCG1748746	gi|119592021	NRWESAGAR	8.19
hCG1785581, isoform CRA_b	gi|119602089	VAALGR	3.02
hCG1821234	gi|119612665	GLALGTASGTGLGP	10.92
hCG1990378, isoform CRA_c	gi|119588266	INCSGK	18.23
hCG19906, isoform CRA_a	gi|119610045	CVQASTAPGGR	16.1
hCG2000808	gi|119624486	LGPAIPPK	20.95
hCG2011944	gi|119629431	MEMEPAGTKCEK	13.14
hCG2021576	gi|119608597	KTEEYGTR	12.71
hCG2038600	gi|119602014	QVSGAAQGRPTGQVHK	14.18
hCG2039044	gi|119604361	QGLTLSPR	36.77
hCG2040112	gi|119620669	MSAGALGAGRGR	2.58
hCG2040199	gi|119625937	QPPLLLPK	25.36

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Unknown function			
hCG2040385	gi|119577445	NMHSPL	14.77
hCG2040772	gi|119605533	CELGNSSL	7.5
hCG2041280, isoform CRA_a	gi|119603553	RAPSAAGGAGGCR	12.96
hCG2041407	gi|119585548	ATSSSKTLAAK	6.5
hCG2041770	gi|119620833	KMSTSNTLK	4.32
hCG2042040	gi|119588660	VDRGCEK	14.38
hCG2042887, isoform CRA_c	gi|119584883	STAPGHTSQLK	3.73
hCG2045077	gi|119577941	GAGLSSIPR	21.89
hCG2045247	gi|119588481	MSLACDRQR	5.7
hCG2045268	gi|119590405	VSPGA	12.32
hypothetical protein	gi|57161863	VPSLNGK	14.54
hypothetical protein BC006130	gi|119602791	KAAEAARMGRR	10.25
hypothetical protein FLJ37440, isoform CRA_b	gi|119572486	AAPPATASAR	10.58
kelch-like protein 35	gi|259013520	AALSAGSAYFR	8.81
KIAA1123 protein	gi|20521770	QLVVLMK	10.85

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Unknown function			
KIAA1692 protein	gi|12697929	RDRSLPR	38.5
LOC100135777 protein	gi|19263727	MGTLGQCSEK	4.9
LRRC37A2 protein	gi|219521268	INISLSIF	20.81
paraneoplastic antigen-like protein 6C	gi|283806576	ASADR	3.54
PRO2277	gi|11493445	NLENMR	23.83
protein FAM115A isoform	gi|7662276	GPNVK	12.91
protein FAM133A	gi|27734775	KKSGSSHKSR	6.82
putative	gi|553734	GITLSVRP	32.71
similar to Piccolo protein (Aczonin)	gi|51094943	VDAKVEIIK	19.3
uncharacterized protein	gi|197333715	QASDSGTGDQV	11.59
unknown	gi|37704379	NGLQTASSGAK	9.28
unknown	gi|14336678	DGTFR	1.69
unnamed protein product	gi|22761077	MVSDSLR	12.11
unnamed protein	gi|21755985	AADIIDGLRK	12.55
unnamed protein product	gi|194377686	KAKTGAAGKFK	11.1

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Unknown function			
unnamed protein product	gi|47076901	IVDRYRVGKQIGK	5.12
unnamed protein product	gi|7022134	NLIEVMRK	22.12
unnamed protein product	gi|34536392	RASVASPGEK	13.81
unnamed protein product	gi|194387024	WGHGGGRLFPR	14.38
unnamed protein product	gi|32488	DQVANSAFVER	32.68
unnamed protein product	gi|158261127	LGTMPLLPIR	13.17
unnamed protein product	gi|21757631	VQEGGFR	23.8
unnamed protein product	gi|221043958	ILMCQK	27.89
unnamed protein product	gi|194378218	DASQVSAPGTRR	8.81
unnamed protein product	gi|32486	TLTIVDTGIGMTK	23.56
unnamed protein product	gi|16553461	IGGKEVFR	25.26
unnamed protein product	gi|34531434	LLLETGMK	22.66
unnamed protein product	gi|21758470	RSLNLFR	24.53
unnamed protein product	gi|34535739	KSLALSPR	25.31
unnamed protein product	gi|34535739	KSLALSPR	28.22

Table 15 (Continued)

Protein name	Accession number	Peptide	Mowse Score
Unknown function			
unnamed protein product	gi|194391204	SAPPSLPR	25.11
unnamed protein product	gi|10432847	ALAKLTR	8.8
unnamed protein product	gi|28590	KVPEVSTPTLVEVSR	40.6
unnamed protein product	gi|28590	KVPEVSTPTLVEVSR	40.6
UPF0705 protein C11orf49 isoform 2	gi|51558748	LLLPFFR	24.92
uterus-ovary specific putative transmembrane protein UO	gi|10799170	DISSYK	17.58
WD repeat domain 46, isoform CRA_c	gi|119624108	IGSSVLRDQK	26.93

สรุปและวิจารณ์ผลการทดลอง

The differentially expressed proteins in various conditions have been summarized based on their biological functions including apoptosis, cell signaling, transport, structural, gene regulation, protein folding, redox-regulation, cell adhesion, cell cycle and differentiation, biomolecules biosynthesis, fibrinolysis, tumor marker, stress response, calcium binding protein, oesteogenesis, transmembrane protein, tumor suppressor, receptor, metabolism, hormone, immune system, protein-protein interaction, cell-cell interaction, proteolysis, and unknown function (Table 1). These functions were different and vary among conditions. Only the protein expressions in MS, MN, DN, and DS were further criticized in this study.

In DS, the protein functions were classified as follows: apoptosis (15%), cell signaling (15%), structural proteins (31%), protein folding (8%), transport (8%), and unknown (23%) (Figure 3A). The protein names are listed in Table 1, such as mitogaligin in apoptosis, IBtK protein and SH2 domain-containing protein in cell signaling, cytokeratin and keratin in structural proteins etc. In DN, the proteins were clustered in cell signaling (17%), structural proteins (25%), transport (8%), cell adhesion (8%), and unknown (34%) (Figure 3B). The protein names are shown in Table 2. For example, METRNL protein in cell differentiation, platelet derived growth factor receptor alpha (PDGFRA) and rho guanine nucleotide exchange factor in cell signaling, keratin, peroxisome assembly protein, and hHa protein in structural proteins etc. In MS, the protein functions were grouped as follws: apoptosis (17%), cell division (8%), transport (8%), structural proteins (8%), gene regulation (34%), and unknown (25%) (Figure 3C). The protein names are shown in Table 3, for instance, apoptosis-stimulating of p53 protein 2 (ASPP2) and apoptosis inducing factor 1 (AIF-1) in apoptosis, centrosomal protein of 63 kDa, keratin 19 in structural proteins etc. Moreover, in MN, the protein functions were divided in cell signaling (18%), structural protein (18%), fatty acid biosynthesis (9%), transport (9%), redox-regulation (9%) and unknown (37%) (Figure 3D). The proteins name are shown in Table 4, such as phospholipase C and metabotropic glutamate receptor 5 in cell signaling, tubulin-alpha and keratin in structural proteins etc.

WT1 plays a major role in the transcriptional regulatory function. The WT1 protein seems to perform two main functions, oncogene and anti-apoptosis. It regulates the transcription of a variety of target genes and is involved in post-transcriptional processing of RNA. As shown in this study, MCF-7 and MDA-MB-468 that were transfected with $siRNA_{neg}$ and $siRNA_{WT1}$ could alter hundreds of proteins. Therefore, the proteins implicated in apoptosis and cell signaling were emphasized in detail. Cathepsin D, apoptosis inducing factor, and apoptosis stimulating of p53 protein were up-regulated only when WT1 was silenced in MCF-7. Moreover, the proteins

associated with the signal transduction pathway: 14-3-3 epsilon, signal transducing adaptor protein 1, phospholipase C, and metabotropic glutamate 5 receptor (GRM5) were found only in $siRNA_{neg}$ (WT1 present) (Table 16). These selected proteins are described below.

Table 16 Unique Protein expression in $siRNA_{neg}$ and $siRNA_{WT1}$ in MCF-7

MCF-7 $siRNA_{neg}$		MCF-7 $siRNA_{WT1}$	
2-DE	**1-DE**	**2-DE**	**1-DE**
Cell signaling:		**Apoptosis:**	
14-3-3 epsilon	Phospholipase C	Cathepsin D	Apoptosis inducing factor
Signal transducing adaptor protein 1	Metabotropic 5 glutamate receptor		Apoptosis stimulating of p53 protein

Cathepsin D is an intracellular aspartic protease present in the endosomes and lysosomes of all mammalian cells. Cathepsin D is also a key mediator of apoptosis induced by many apoptotic agents such as IFN-γ, Fas/APO, and TNF-α (Deiss *et al*., 1996). The role of cathepsin D in apoptosis showed in Figure 4. After the induction of apoptosis, selective permeabilization of lysosomal membrane results in the release of mature cathepsin D into cytosol. The release of cathepsin D may cleave Bid, following formation of active Bax conformation and insertion in the outer mitochondrial membrane, or may interact with unknown partners (Beaujouin *et al*., 2006) leading to release of the cytochrome c from the mitochondria. The release of cytochrome c activates caspase-9 and caspase-3 (Heinrich *et al*., 2004) resulting in apoptosis. Alternatively, presence of cathepsin D in cytosol may trigger Bax activation via Bid-independent pathway, resulting in release of apoptosis inducing-factor (AIF) resulting to apoptosis (Bidere *et al*., 2003). Many studies reported that cathepsin D synthesis is regulated by estrogen in estrogen receptor positive breast cancer (Duffy *et al*., 1991). Cathepsin D can act as a prognostic marker in breast cancer (Tandon *et al*., 1990). Furthermore Liaudet *et al*, (2006) showed that cathepsin D over-expressed and acted as a poor prognosis marker in breast cancer.

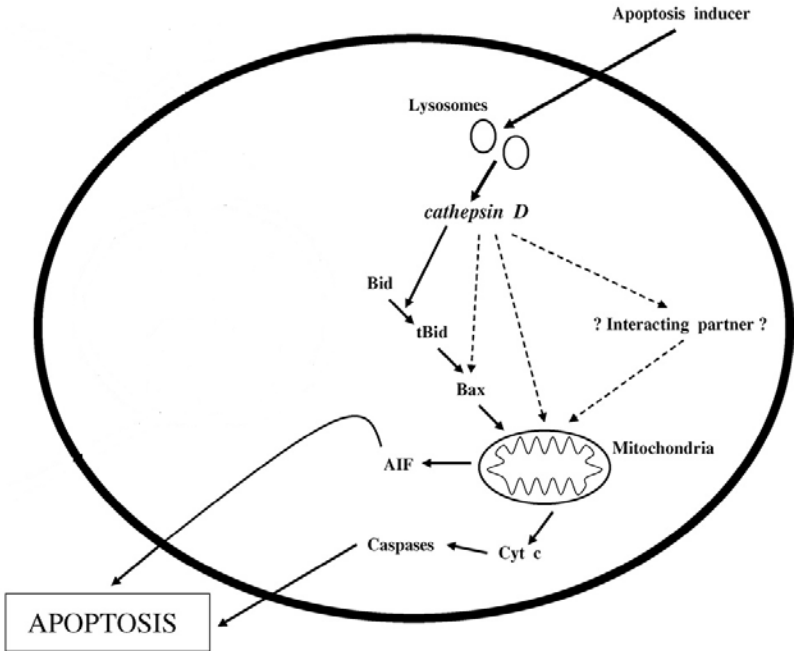


Figure 4 The role of cathepsin D in apoptosis (Adapted from: Benes, vetvicka and Fusek, 2008)

Apoptosis-inducing factor 1 (AIF-1) mitochondrion is a protein triggering chromatin condensation and DNA degradation in a cell in order to induce a caspase-independent pathway of apoptosis (Joza *et al*., 2001). It normally presents in the mitochondrial inter-membrane space and is released in response to death stimuli (Susin *et al*., 1999). The process of stimulating apoptosis starts when the mitochondrion releases AIF-1, which exits through the mitochondrial membrane, enters the cytosol, and finally ends up in the cell nucleus where it signals the cell to condense its chromosomes and fragment its DNA molecules in order to prepare for cell death (Hangen *et al*., 2010).

Apoptosis stimulating factor of p53 protein 2 (ASPP2), also referred to as 53BP2L, enhances p53-induced apoptosis (Samuels *et al*., 2001) and is downstream of E2F suggesting that it functions as a common link between the p53/p73 and Rb/E2F apoptotic pathways (Chen *et al*., 2005).

14-3-3 epsilon, a protein of the 14-3-3 family mediates signal transduction by binding to phosphoserine-containing proteins. It interacts with cell division cycle 25 phosphatases (CDC25A, CDC25B and CDC25C) (Forrest and Gabrielli, 2001). Different CDC25s participate in different phases of cell cycle. CDC25A takes part in regulation of G1/S transition, whereas CDC25B and CDC25C regulate G2/M transition via CDK1 (Sancar *et al*., 2004) (Figure 5). Furthermore, Zuo *et al*, (2009) reported that

TNF-α stimulation enhances the interaction between 14-3-3 epsilon and some key components in MAPK pathway locating at the upstream of NF-kB, including transforming growth factor-beta activated kinase-1 (TAK1) and its interacting protein and protein phosphatase 2Cβ. These studies revealed that 14-3-3 epsilon coordinates the crosstalked between the MAPK signal module and other molecular pathways or biological processes including protein metabolism and synthesis, DNA repair, and cell cycle regulation.

Signal transducing adaptor protein 1 is a protein involved in a signal transduction pathway. Signal transducing adaptor protein 1 contains a variety of protein-binding modules that link protein-binding partners together and facilitate the creation of larger signaling complexes. It contains Src homology 2 (SH2) and SH3 domains which allow specific interactions with several other specific proteins. SH2 domains recognize specific amino acid sequences within proteins containing phosphotyrosine residues and SH3 domains recognize proline-rich sequences within specific peptide sequence contexts of proteins (National Library of Medicine-Medical Subject Headings, 2011).

Phospholipase C (PLC) plays a key role in the signal transduction process for many receptors. PLC cleaves a phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). DAG remains bound to the membrane, and IP_3 is released into the cytosol. IP_3 then diffuses through the cytosol to bind to IP_3 receptors, particular calcium channels in the smooth endoplasmic reticulum (ER). This causes calcium level increasing in cytosol, causing a cascade of intracellular changes and activity. In addition, calcium and DAG together work to activate protein kinase C, which goes on to phosphorylate other molecules, leading to alter cellular activity (Figure 6) (Alberts *et al*., 2008).

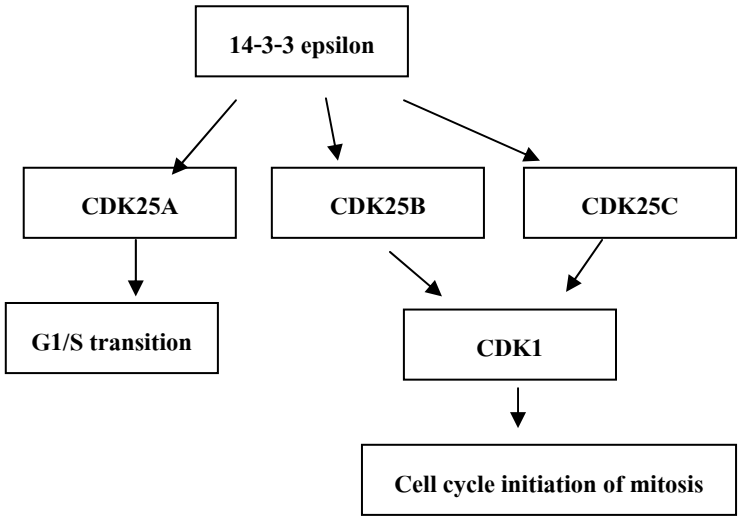


Figure 5 Role of 14-3-3 epsilon in cell cycle regulation

Adapted from: http://pathwaymaps.com/maps/647/#647_11466620

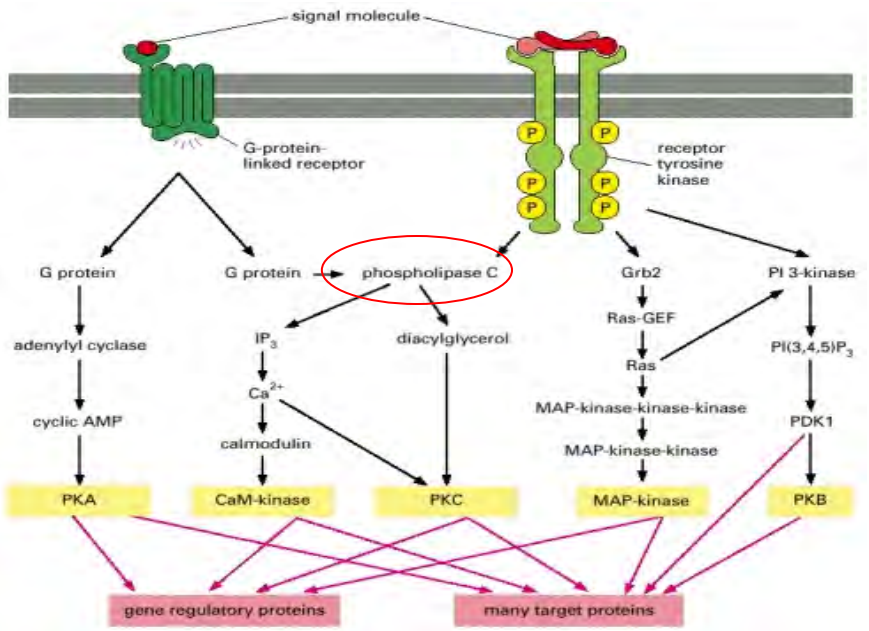


Figure 6 Role of Phospholipase C and other molecules in signal transduction pathway (Alberts *et al*., 2008)

Metabotropic glutamate 5 receptor (GRM5); The metabotropic glutamate receptors belong to a family of G protein-coupled receptors, that have been divided into 3 groups. Group I includes GRM1 and GRM5 and these receptors have been shown to activate phospholipase C. Group II includes GRM2 and GRM3 while Group III includes GRM4, GRM6, GRM7 and GRM8. Group II and III receptors are linked to the inhibition of the cyclic AMP cascade (Nicoletti *et al*., 2011).

Additionally, in MDA-MB-468, the proteins involving apoptosis, including ALG-2 interacting protein x, apoptosis- inducing factor 1 were found when WT1 was present ($siRNA_{neg}$) while mitogaligin was detected in the $siRNA_{WT1}$ condition. Furthermore, the proteins related in the signaling pathway found in $siRNA_{neg}$ included guanine nucleotide binding protein, neuropolypeptide h3, PDGFRA and Rho guanine nucleotide exchange factor 1 while IBTK protein, and SH2 domain containing protein were expressed in $siRNA_{WT1}$ (Table 17).

Table 17 Unique Protein expression in $siRNA_{neg}$ and $siRNA_{WT1}$ in MDA-MB-468

MDA-MB-468 $siRNA_{neg}$		MDA-MB-468g $siRNA_{WT1}$	
2-DE	1-DE	2-DE	1-DE
Cell signaling:		Cell signaling:	

Guanine nucleotide binding protein	PDGFRA	-	IBtK protein
Neuropolypeptide h3	Rho guanine nucleotide exchange factor 1	-	SH2 domain containing protein
Apoptosis:		-	
ALG-2 interacting protein x	-	-	Mitogaligin
Apoptosis-inducing factor 1	-	-	-

ALG-2-interacting protein X (Alix) or Hp95, also known as AIP1 was reported to interact with the calcium-binding protein ALG-2 (apoptosis-linked gene 2), which was necessary for cell death (Missotten *et al*., 1999).

Mitogaligin, a cell death protein, contains a mitochondrial targeting sequence and promotes the release of cytochrome c into the cytosol. Additionally, mitogaligin localizes in nucleus and induces cell death through a pathway exhibiting typical properties of apoptosis causing cell shrinkage, cytoplasm vacuolization, nuclei condensation, and eventually cell death (Robinet *et al*., 2010).

Platelet derived growth factor receptor alpha (PDGFRA) is a cell surface tyrosine kinase receptor important factors which is an important regulating cell proliferation, cellular differentiation, cell growth, and development (Heldin *et al*., 1989). After ligand binding, the platelet-derived growth factor receptor (PDGFR), dimerize via autophosphorylation (P) and recruit adaptor proteins (such as GRB2 and SHC) that activate various downstream effectors via MAPK or PI3K pathway resulting in cell proliferation (Alberts *et al*., 2008).

Rho guanine nucleotide exchange factor is an intracellular signaling molecule that regulates cytoskeleton organization, gene expression, cell cycle progression, cell motility, and other cellular processes. It represents the activating enzymes of Rho GTPases by serving to relay a variety of signals to catalyze GDP/GTP exchange of specific Rho GTPases (Shang *et al*., 2013). Rho-GEF is related in Rho GTPases activity which controlled by three types of proteins: Rho-guanine nucleotide exchange factors (Rho-GEF), which catalyses the exchange of GDP for GTP, resulting the protein active, GTPase activating proteins (GAPs), which stimulate the intrinsic

GTPase activity, turning off the GTPase, and guanine nucleotide dissociation inhibitors (GDIs), whose role appears to block spontaneous activation (Figure 7)

Inhibitor of Bruton's tyrosine kinase (IBtK) is a negative regulator of the Bruton tyrosine kinase (BtK), which play a major role in B-cell differentiation and down-regulated BtK kinase activity (Liu *et al*., 2001). However Janda *et al*, (2011) reported that IBtK is phosphorelated with at serine 87 and 90 by PKC. This phosphorylation causes the dissociation of the interaction between BtK and IBtK and allows BtK to translocate to the plasma membrane.

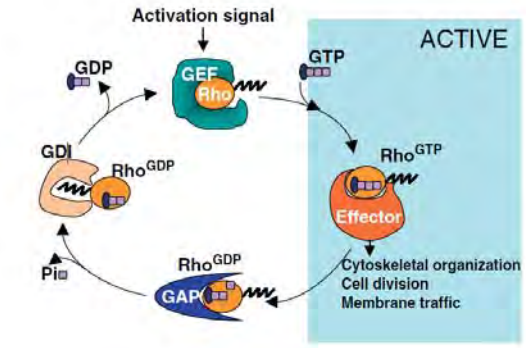


Figure 7 The role of Rho-GEF in Rho-GTPase activity of signaling pathway (Garcia *et al*., 2006)

SH2 domain containing prorein is a sequence specific phosphotyrosine-binding module present in many signaling molecules including tyrosine kinase. In cytoplasmic tyrosine kinases, the SH2 domain is located N-terminally to the catalytic kinase domain (SH1) where it mediates cellular localization, substrate recruitment, and regulation of kinase activity (Filippakopoulos, Mu ller and Knapp, 2009).

Guanine nucleotide binding protein or G-protein is important for relaying signal from G-protein linked receptor to intracellular enzymes or ion channel (Alberts *et al*., 2008). G-protein activates phospholipase C which cleaves a phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) leading to altered cellular activity or it may activate adenylyl cyclase relating in signal transduction pathway via cyclic AMP-dependent pathway (Figure 6) (Alberts *et al*., 2008).

Neuropolypeptide h3, also known as Raf kinase inhibitor protein as been shown to inhibit Raf and thereby negatively regulate growth factor signaling by the Raf/MAP kinase pathway. RKIP has also been shown to suppress metastasis (Shemon *et al*., 2010)

Protein identification from 1-DE and 2-DE combined with LC-MS/MS was classified into various groups according to biological function, such as cell signaling,

apoptosis, structural proteins, protein folding, metabolism, unknown etc. However, the proteins involved in apoptosis and cell signaling were only discussed in each cell line in this study.

1. WT1 and apoptosis pathway in MCF-7

The evidence of the relationship between WT1 and other proteins in the apoptosis pathway was explored using STRING 9.05 database. Figure 8A shows the association of WT1 and other proteins relevant to the apoptosis pathway. The action modes of these proteins have been shown in different colors, such as green, red, blue, and light blue. They refer to activation, inhibition, binding, and phenotypes, respectively. Grey lines refer to the relationship of proteins but the mode of actions has not been reported (Figure 8B). The result revealed the binding of WT1 and p53 which was agreeable from previous studies that WT1 closely interacts with the p53, tumor suppressor gene. Interaction between p53 and WT1 leads to stabilize the expression of p53 resulting in p53 over-expression and long half-life. WT1 appears to inhibit the apoptotic effect of p53 but not its ability to induce cell cycle arrest (Maheswaran *et al*., 1995). However, the direct association between WT1 and the other proteins related in the apoptosis pathway has not been found the STRING 9.05 database. In this study, cathepsin D, AIF-1, and ASPP2 were indirectly related to WT1 via p53 protein (Figure 8).

MCF-7 contains estrogen and progesterone receptor, no HER2 expression, p53 wild type, and expresses IGFBP. Under $siRNA_{WT1}$ transfection, proteins involved in the apoptosis pathway were up-regulated. This communication was elicited through p53. Without WT1 in the cell, p53 was released and allowed to trigger an intracellular signal transduction cascade leading to gene activation of cathepsin D and ASPP2. Alternatively, presence of cathepsin D triggers Bax activation via the Bid-independent pathway, resulting in release of AIF-1 leading to apoptosis. Previous studies reported that cathepsin D synthesis was regulated by estrogen in estrogen positive breast cancer (Duffy *et al*., 1991). In this study, the possible relationship between WT1 and related molecules has been proposed in Figure 9.

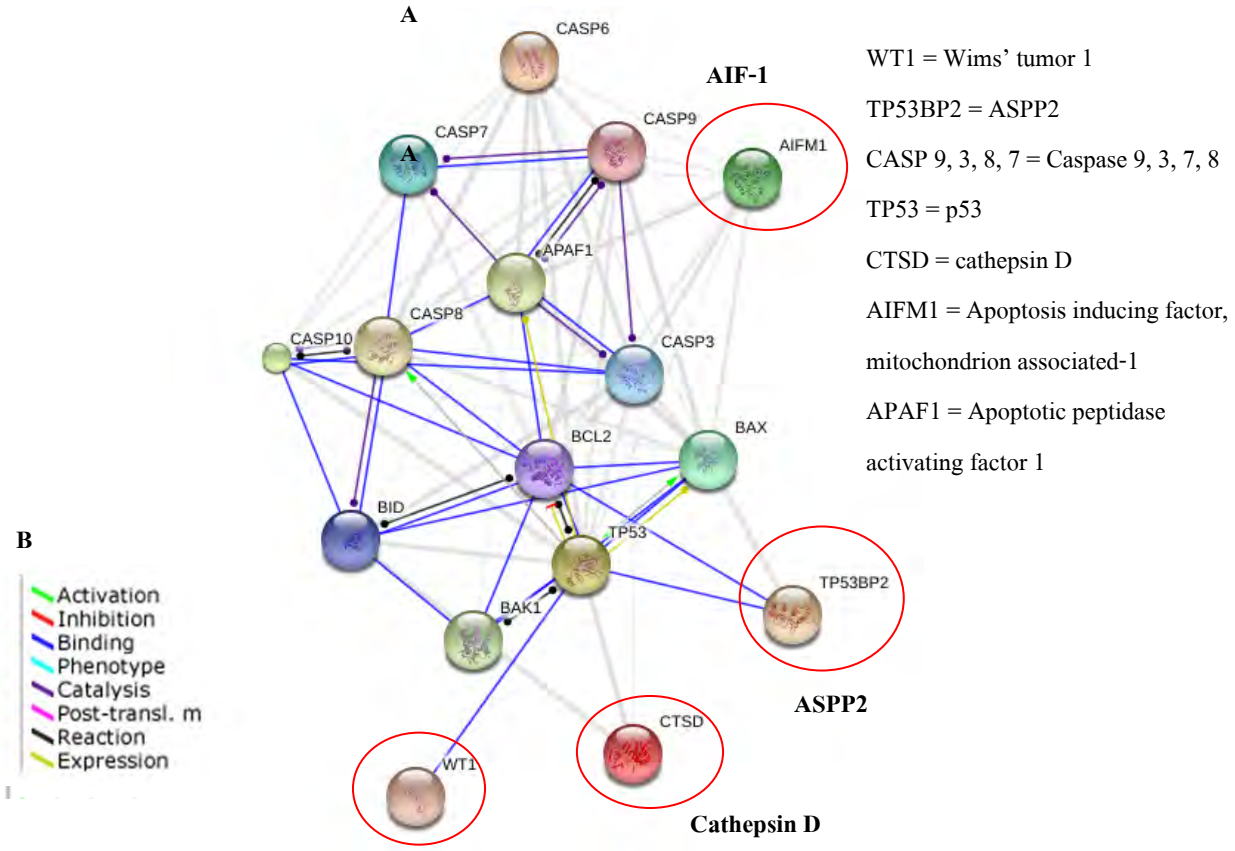


Figure 8 (A) The involvement of WT1 and proteins in apoptosis pathway in MCF-7 (STRING 9.05). (B) Modes of action are shown in different colors. The red circle shows the proteins found in this study.

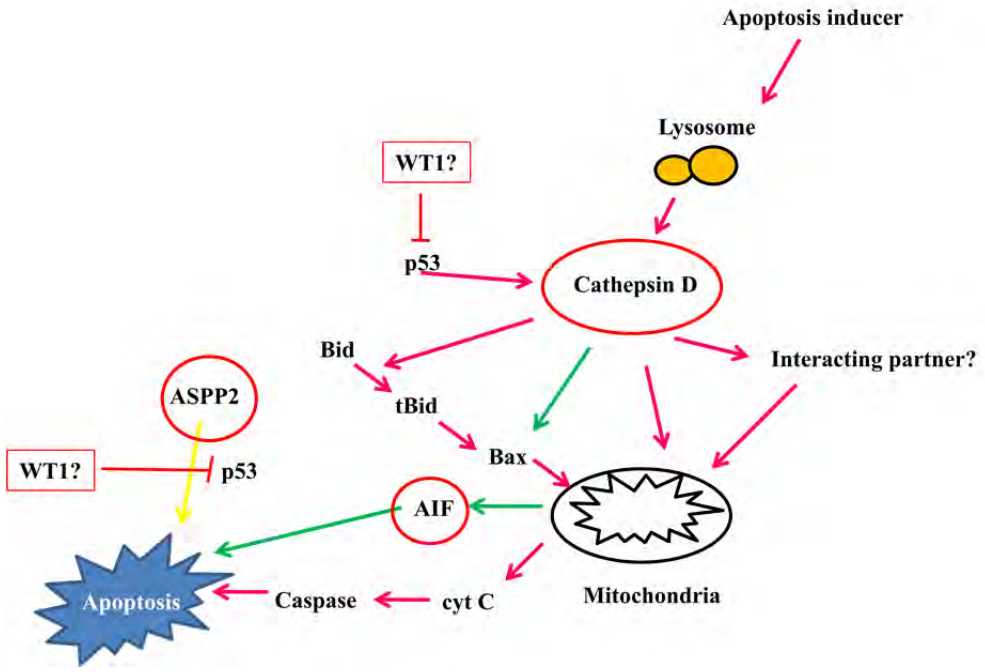


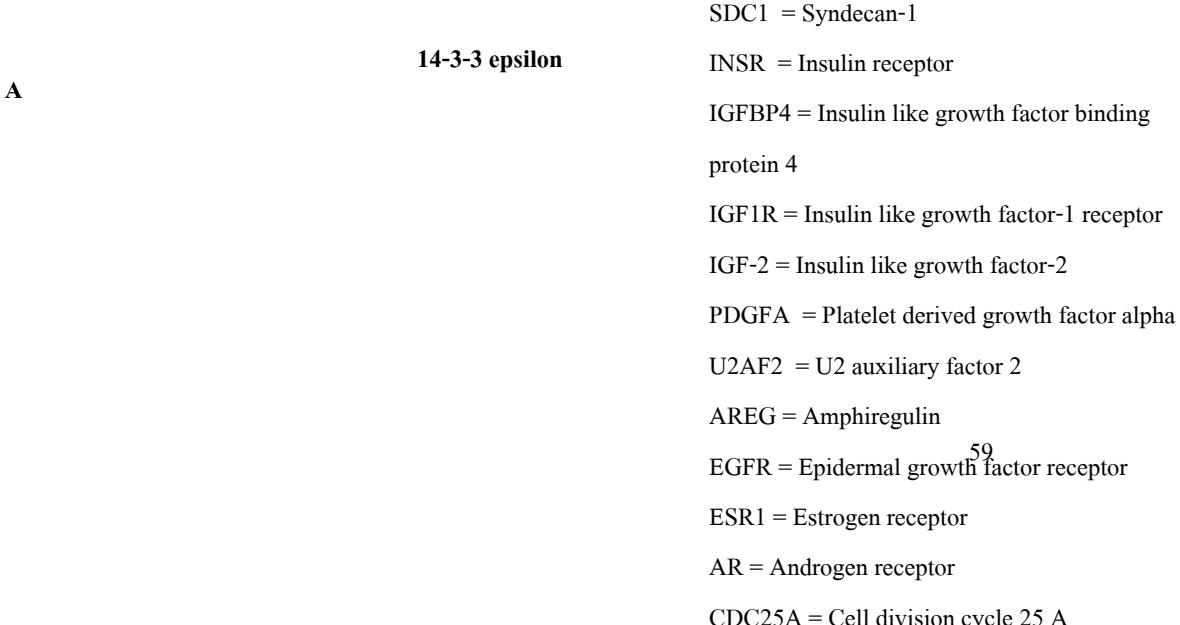
Figure 9 The possible relationship between WT1 and cathepsin D, AIF-1, and ASPP2 in MCF-7 when knockdown WT1 with $siRNA_{WT1}$. (Adapted from: Benes, vetvicka and Fusek, 2008)

2. WT1 and signaling pathway in MCF-7

When WT1 is present, 14-3-3 epsilon, signal transducing adaptor protein 1, phospholipase C, and metabotropic glutamate receptor 5 protein were up-regulated. The STRING 9.05 showed that WT1 interacted with many genes involved in cell survival and cell growth, such as IGFR-1, EGFR, C-Myc, Syndecan-1, etc. (Figure 10). However, the direct association between WT1 and 14-3-3 epsilon, signal transducing adaptor protein 1 (STAP1), phospholipase C (PLC), and metabotropic glutamate receptor 5 protein (GRM5) has never been reported according to STRING 9.05 database.

The possible correlation between WT1 and 14-3-3 epsilon, STAP1, PLC, and GRM5 is shown in Figure 11. WT1 may crosstalk with STAP1, PLC, and GRM5. PLC cleaves a PIP_2 to IP_3 and DAG. IP_3 activates the release of Ca^{2+} into cytosol to bind with calmodulin. The binding of Ca^{2+}/calmodulin triggers Ca^{2+}/calmodulin-dependent kinase (CAM-kinase) to phosphorylate many targeted proteins involved in cell growth. In addition, Ca^{2+} and DAG together activated protein kinase C (PKC) leading to phosphorylate other molecules, resulting in cell proliferation. Moreover, WT1 may crosstalk with 14-3-3 epsilon to trigger cyclin-dependent kinases (CDKs) or activate the MAPK pathway. These relationships may occur at transcription or translation level resulting to cell proliferation or cell growth.

Consequently, WT1 plays an oncogenic role in MCF-7. When WT1 is present, the proliferative signaling pathway has been amplified through 14-3-3 epsilon, PLC, and GRM5. Unlike in the $siRNA_{WT1}$ condition, WT1 behaves as an anti-apoptotic molecule by activating cathepsin D and ASPP2 through p53.



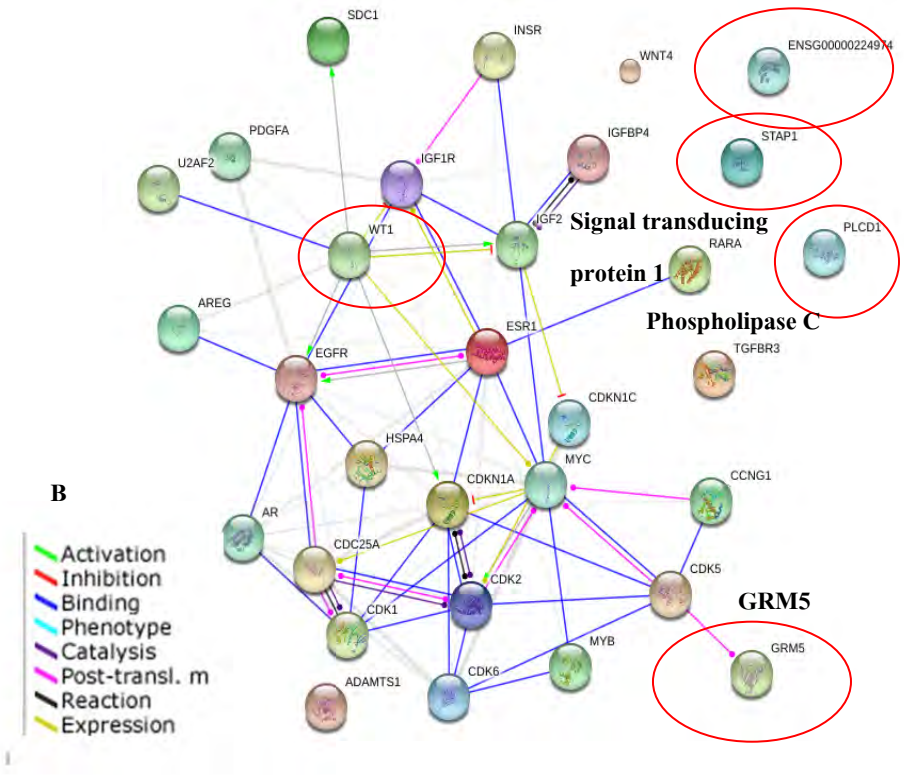
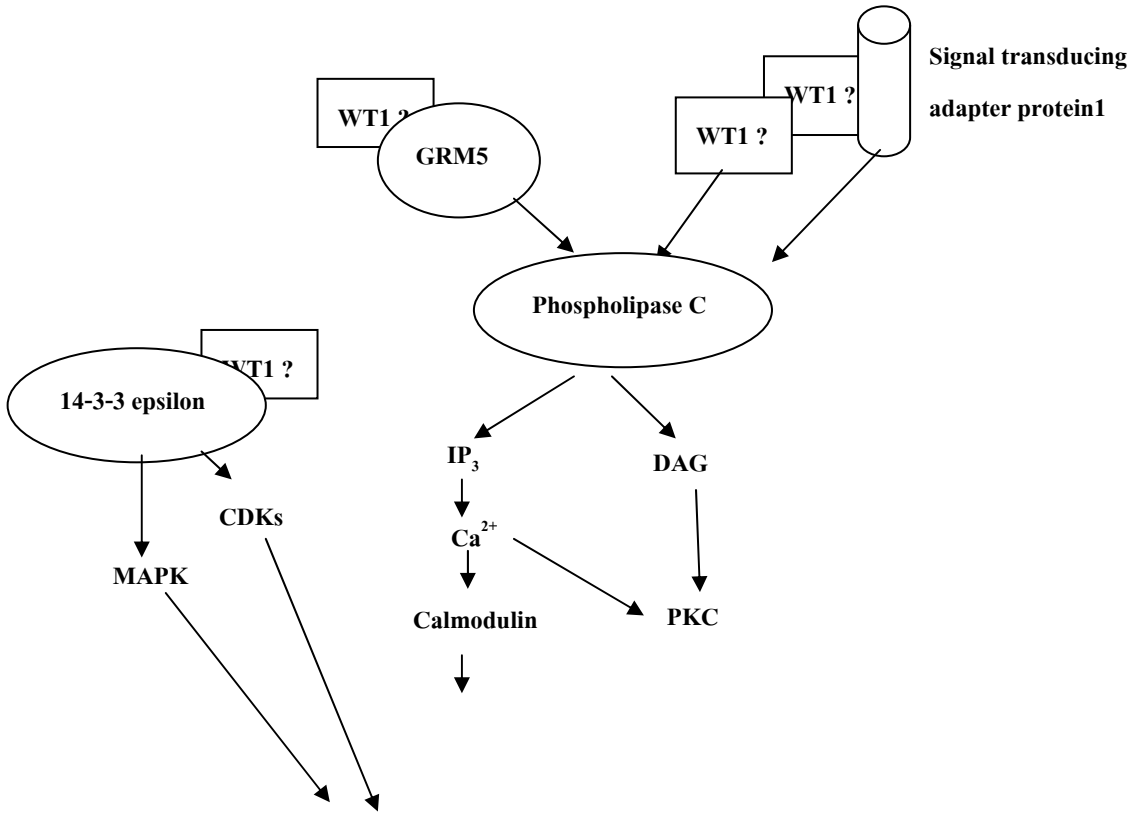


Figure 10 (A) The involvement of WT1 and proteins in signal transduction pathway in MCF-7 (STRING 9.05). (B) Modes of action are shown in different colors. The red circle shows the proteins found in this study.



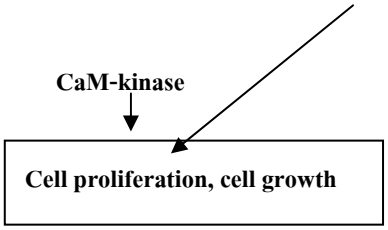
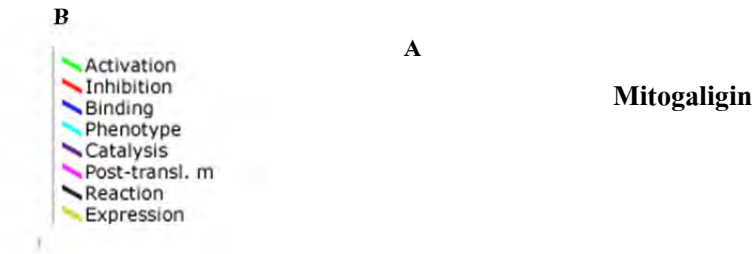


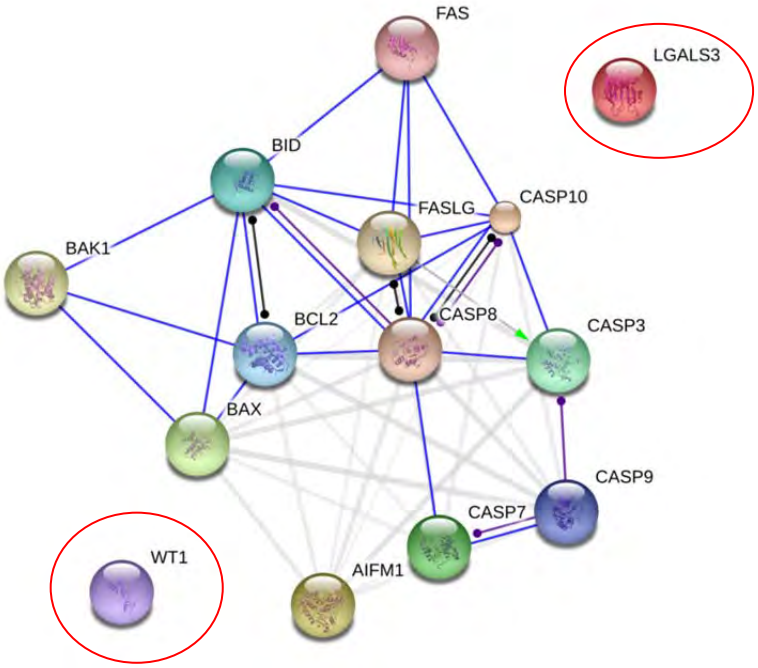
Figure 11 The possible relationship between WT1 and phospholipase C, GRM5, Signal transducing adapter protein1, and 14-3-3 epsilon in signal transduction pathway.
Adapted from: Alberts *et al*., 2008

3. WT1 and apoptosis in MDA-MB-468

Due to p53 mutation in MDA-MB-468, the apoptosis pathway may occur via p53 independently. Surprisingly, a novel target protein of WT1, mitogaligin, was found in MDA-MB-468 when WT1 was silenced. The STRING shows no correlation between WT1 and mitogaligin from previous studies (Figure 12).

Mitogaligin contains a mitochondrial targeting sequence and promotes the release of cytochrome C. It induces cell death through the apoptosis pathway (Robinet *et al.,* 2010). The relationship between WT1 and motogaligin assumed that WT1 may act as negative regulator of mitogaligin at transcription or translation level. However, the relationship between WT1 and ALG-2 interacting protein x, apoptosis-inducing factor 1 has not been elucidated. There was not enough evidence to clarify the correlation of WT1 and selected molecules. Therfore, further works will be required to investigate this hypothesis.





BAK1 = Bcl-2 homologous antagonist killer

BID = BH3 interacting domain death agonist

FASLG = Fas ligand

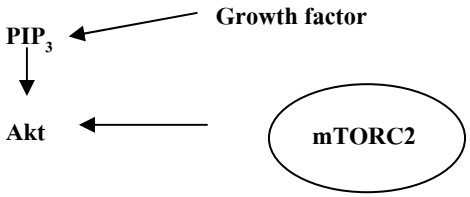
BCL-2 = B-cell lymphoma-2

BAX = Bcl-2–associated X protein

Figure 12 (A) The involvement of WT1 and p53-independent apoptosis pathway in MDA-MB-468 (STRING 9.05). (B) Modes of action are shown in different colors. The red circle shows the proteins found in this study.

4. WT1 and signaling pathway in MDA-MB-468

The signal transduction pathway in MDA-MB-468 breast cancer cell line was related with the mTOR signaling pathway that regulates cell growth, proliferation, differentiation, and survival (Yu *et al*., 2001). mTOR protein exists in two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 contains the protein *raptor* while mTORC2 contains the protein *rictor*. In the presence of growth factors, activated Akt phosphorylates and inhibits tuberous sclerosis protein 2 (Tsc2), thereby promoting the activation of Rheb. Activated Rheb (Rheb-GTP) helps activate mTORC1, which in turn stimulates cell growth. Forthermore, mTORC2 phosphorylates Akt at Ser473 and regulates the actin cytoskeleton and cell motility (Zhou *et al*., 2010) (Figure 13). Recently, Razmara *et al*, (2013) demonstrated that PDGFRs are essential for multiple growth factor signaling pathways that lead to PI3K/Akt activation. The pathway from PDGFR leads to phosphorylation of Akt which involves both the mTORC2 and PLCγ/PKC pathways.



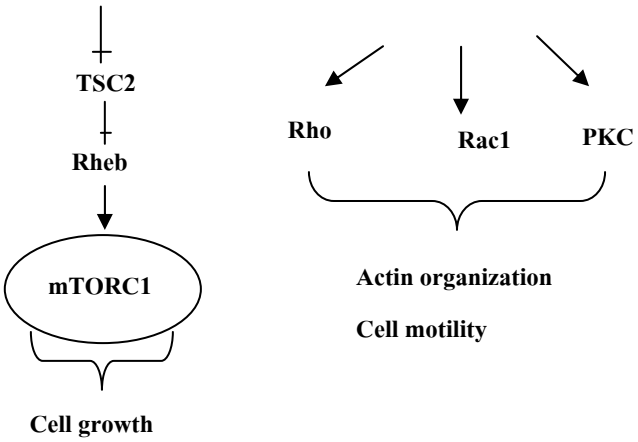
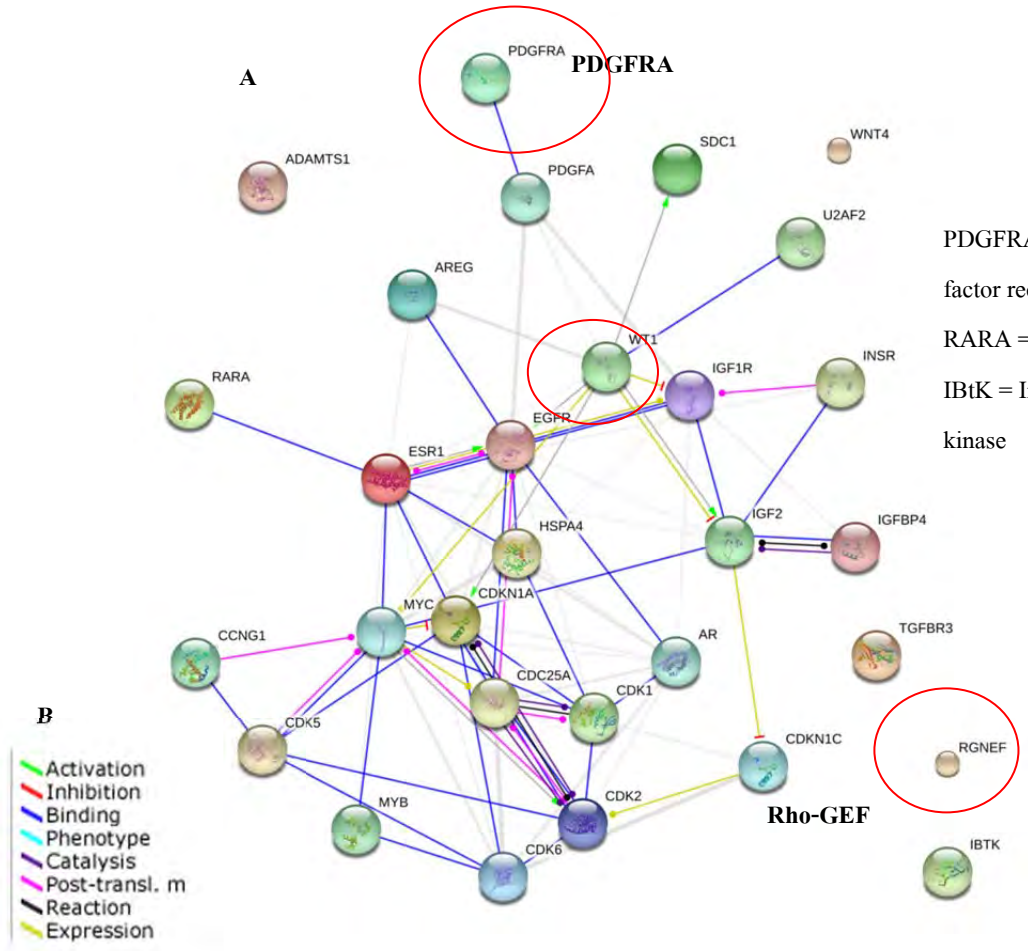


Figure 13 Activation of mTOR by the PI-3-kinase-Akt signaling pathway
Adapted from: Zhou and Huang, 2010; Albert *et al.,* 2008

WT1 interacted with many genes involved in the cell signaling pathway (Figure 14). In this study, the proteins involved in the cell signaling pathway, PDGFRA and rho-GEF were found when WT1 was present in MDA-MB-468, while G-protein, SH2 domain containing protein, and neuropolypeptide h3 were found when the cell was without WT1. However, the STRING 9.05 showed that these molecules were not associated.



PDGFRA = Platelet derived growth factor receptor alpha
RARA = Retinoic acid receptor alpha
IBtK = Inhibitor of Bruton's tyrosine kinase

Figure 14 (A) The involvement of WT1 and proteins in signal transduction pathway in MDA-MB-468 (STRING 9.05). (B) Modes of action are shown in different colors. The red circle shows the proteins found in this study.

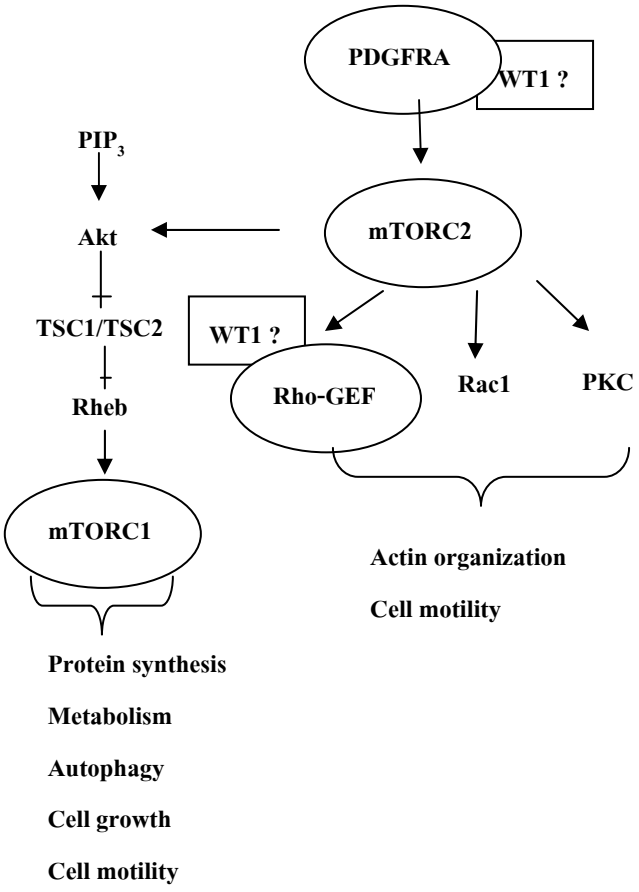


Figure 15 The possible relationship between WT1 and PDGFRA, Rho-GEF in signal transduction pathway in MDA-MB-468 (Adapted from: Zhou and Huang 2010)

In this study, WT1 may relate with PDGFRA leading to activation of Akt/ TSC1, TSC2/mTOR2 pathway resulting in cell growth. Moreover, WT1 may also associate with mTOR2/Rho-GEF resulting in cell motility (Figure 15). Thus, WT1 plays an oncogenic role in MDA-MB-468. Moreover, when WT1 was silenced with $siRNA_{WT1}$, IBtK, SH2 domain containing protein, G-protein, and neuropolypeptide h3 were up-regulated. The relationship between WT1 and these proteins in signaling pathway in MDA-MB-468 has not prior been elucidated. WT1 may behave as a negative regulator of IBtK that bind to SH2 domain of BtK tyrosine kinase receptor resulting in IBtK inactivate leading to B-cell differentiation. Furthermore, WT1 possibly be a negative regulator of Raf kinase inhibitor resulting to activation MAPK pathway and promote metastasis. The overview of the relationship between WT1 and proteins in MCF- and MDA-MB-468 were shown in Figure 14-15. The red alphabet refers to the proteins found in this study.

ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

This work should be further functional studies such as immunuprecipitation, functional proteomics (affinity chromatograpy, protein-protein interaction, protein-promoter interaction etc.) should be performed to determine these hypotheses.

กิตติกรรมประกาศ

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Keywords : WT1, p53, breast cancer, MCF-7, MDA-MB-468, proteomics
(คำหลัก)

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ
   - **Kanokwiroon K,** Graidist, P. The association of oncogenic role between WT1 and p53 in breast cancer. FEBS Journal 2012; 279 (SI): 59.
   - Chesor M, Roytrakul S, Graidist P and **Kanokwiroon K**. 2014. Proteomics analysis of siRNA-mediated silencing of Wilms' tumor 1 in the MDA-MB-468 breast cancer cell line. *Oncology Reports* 31: 1754-1760. IF 2.297

2. การนำผลงานวิจัยไปใช้ประโยชน์
   ไม่มี

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)
   - **Kanokwiroon K**, Nasomyon T, Graidist P. A study of the oncogenic functions of WT1 in breast cancer using siRNA. *The 3rd Biochemistry and Molecular Biology (BMB) Conference* 6-8 April 2011, the Empress Convention Centre, Chiang Mai. p49.
   - **Kanokwiroon K**, Graidist P. The association of oncogenic role between WT1 and p53 in breast cancer**.** *The 22nd IUBMB & 37th FEBS Congress*, 4-9 September 2012; the Seville Conference and Exhibition Centre, Seville, Spain. p59.

การวิเคราะห์โปรติโอมิกส์ของเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MCF-7 เมื่อยับยั้ง WT1 ด้วย siRNA

Proteomics Analysis of siRNA Mediated WT1 in MCF-7 Breast Cancer Cell Line

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* Correspondence: kkanyana@medicine.psu.ac.th

Abstract

The Wilms' tumor 1 (*WT1*) gene encodes a zinc finger acting as a transcriptional activator or repressor for many genes involved in cell differentiation, growth, and apoptosis. These functions depended on the cell types, WT1 isoforms, and the status of targeted molecules. To determine the relationship between WT1 and related proteins, WT1 was silenced with siRNA in MCF-7 breast cancer cell line and the protein expression pattern was analyzed by proteomics including two-dimensional gel electrophoresis (2-DE) and LC-MS/MS mass spectrometry. The results showed that 27 spots were significantly changed in silencing WT1 compared to control. These proteins could be classified their functions in apoptosis, cell signaling, protein folding, metabolism, structural, cell adhesion, gene expression, transport, redox-regulation, protein degradation and unknown function. In this study, our proteomics analysis revealed that apoptotic protein, cathepsin D may be targeted protein of WT1. This relationship acts as anti-apoptosis and oncogene in MCF-7 breast cancer cell lines. However, validation of proteomic results and further study are necessary to confirm this result.

Keywords: Proteomics, WT1, siRNA, MCF-7, 2-DE

บทคัดย่อ:

ยีน *WT1* มีส่วนของ zinc finger ทำหน้าที่กระตุ้นหรือยับยั้งยีนหลายชนิดที่เกี่ยวข้องกับการพัฒนา การเจริญเติบโต และการตายแบบอะพอพโตซิสของเซลล์ หน้าที่ที่แตกต่างกันขึ้นอยู่กับชนิดของเซลล์ที่แสดงออก ไอโซฟอร์มของ WT1 และโมเลกุลที่เกี่ยวข้อง การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่าง WT1 และโปรตีนต่างๆ ในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MCF-7 การศึกษาโดยยับยั้งการแสดงออกของ WT1 ด้วย siRNAและวิเคราะห์รูปแบบการแสดงออกของโปรตีนด้วยเทคนิคการแยกโปรตีนแบบสองมิติ (2D-E) และระบุชนิดของโปรตีนด้วยเครื่องแมสสเปกโตรเมทรีชนิด LC-MS/MS ผลการศึกษาพบโปรตีน 27 ชนิดที่มีการแสดงออกแตกต่างกันระหว่างสภาวะที่มี WT1 เปรียบเทียบกับสภาวะที่ไม่มี WT1 ซึ่งโปรตีนเหล่านั้นทำหน้าที่เกี่ยวข้องกับการตายของเซลล์แบบอะพอพโทสิส การส่งสัญญาณภายในเซลล์ การขดตัวของโปรตีน กระบวนการเมตาบอลิสซึม โปรตีนโครงสร้าง การเกาะติดของเซลล์ การแสดงออกของยีน การขนส่ง การสลายโปรตีน กระบวน redox ภายในเซลล์ และโปรตีนที่ยังไม่ทราบหน้าที่ การศึกษานี้พบโปรตีนในกลุ่มของอะพอพโทสิสคือ cathepsin D ซึ่งน่าจะเป็นโปรตีนเป้าหมายของ WT1 ดังนั้น WT1 จึงน่าจะมีบทบาทในการเป็นโปรตีนต้านการตายของเซลล์แบบอะพอพโทสิส และโปรตีน oncogene ในเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MCF-7 อย่างไรก็ตาม ควรทำการทดลองเพิ่มเติม และศึกษาถึงความสัมพันธ์อย่างละเอียด เพื่อยืนยันผลการทดลองดังกล่าว

คำสำคัญ: โปรติโอมิกส์, WT1, siRNA, MCF-7, 2-DE

Introduction:

Breast cancer is the most common cancer and the leading cause of death in women worldwide, accounting for 23% (1.38 million cases) of the total new cancer cases and 14% (458,400 cases) of the total cancer deaths in 2008 [1]. The development of breast cancer might result from interaction between the change in genetic elements, environmental factors, and also the difference in ethnicity [2]. There are several genes are reported to be associated with breast cancer, such as *ERBB2, c-Myc, CCND1, TP53, PTEN*, and *WT1* [3].

The human Wilms' tumor 1 (*WT1*) gene is located at chromosome locus 11p13 comprising 10 exons. Recently, Loeb *et al*. (2001) have demonstrated that WT1 mRNA and protein was detected in nearly 90% of breast cancers but not in most normal breast samples [4]. Moreover, Navakanit *et al.* (2007) reported that the siRNA against WT1 inhibited both WT1 protein expression level and growth of breast cancer cell line MCF-7, in a dose and time-dependent manner. These results suggested that WT1 could act as oncogenic in breast cancer cell line MCF-7. These results suggest that WT1 plays a role in the pathogenesis of breast cancer as an oncogene rather than a tumor suppressor gene as in leukemia [5]. Additionally, the high level expression of WT1 mRNA detected by real time RT-PCR can predict a poor prognosis in breast cancer patients [6] and the absence of mutations through the whole 10 exons of the *WT1* gene in the 36 cases of primary breast cancer [7].

WT1 encodes a zinc finger acting as a transcriptional activator or repressor for many genes involved in cell differentiation, growth, and apoptosis. These functions depend on the type of cells, WT1 isoforms and the status of targeted molecules. There are several targeted molecules for WT1. However, the relationship between WT1 and the targeted molecules involved in breast cancer remains unclear and the overview study of the relationship between WT1 and the related molecules has not been reported.

In this study, siRNA against WT1 mRNA has been used to silence WT1 expression and then compared the protein pattern to the control. The relationship between WT1 and related proteins in breast cancer cell line MCF-7 was investigated by proteomics analysis. The proteins were further identified by LC-MS/MS and database searching. These studies may provide more evidences to understand the mechanism of WT1 and the related molecules in breast cancer.

Materials and Methods:

Cell culture:

The human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (ATCC, USA). MCF-7 was cultured in RPMI 1640 medium (GIBCO BRL) supplementing with 10% fetal bovine serum, 100 units/ml of penicillin and 100 µg/ml of streptomycin, and 10% glutamine. Then the cells was incubated in a 37°C incubator with 5% CO_2 [5].

Small interference RNA (siRNA) transfection:

MCF-7 at $1x10^5$ cells were seeded in each well of 24-well culture plate and incubated in CO_2 incubator at 37°C for 24 hours. The cells were transfected using Lipofectamine 2000 reagent (Invitrogen) in 24-well plates with 100 nM siRNA duplexes (Optimal siRNA condition performed in preliminary study). The siRNA against WT1 ($siRNA_{WT1}$) (Invitrogen) consisted of a mixture of two 25-nt duplexes, namely, $siRNA_{WT1}$R88 (5'-AAATATCTCTTATTGCAGCCTGGGT3') and $siRNA_{WT1}$R90 (5'-TTTCACACCTGTATG TCTCCTTTGG-3'). To minimize the cytotoxicity of the reagent itself, the cells were washed once with PBS, and the media was changed six hours after transfection [5]. After 72 hours, the cells were harvested and investigated the protein level by Western blot analysis.

Western blot analysis

Cell pellets were harvested by trypsinization and extracted with radio immunoprecipitation assay (RIPA) buffer (Pierce, USA). Then the concentration of protein was determined by Bradford assay (Bio-Rad, Hercules, USA). The 50 µg of protein samples were loaded to 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitocellulose membrane (Bio-Rad). The membrane was blocked by blocking solution [5% low fat dry milk in 1xTTBS (0.1% Tween 20, 154 mM NaCl, 48 mM Tris base)] for 1 hour and washed 5 minutes for three times with washing solution (1% low fat dry milk in 1xTTBS buffer). After blocking, the blot was incubated with primary antibody anti-WT1 (1:200) and anti-actin (1:1,000) antibodies (diluted with 1% skim milk in 1xTTBS) for 2 hours and washed 5 minutes for three times with washing solution. The membrane was then incubated with secondary antibody polyglonal anti-IgG rabbit (1:10,000) antibody in 1% low fat dry milk in 1xTTBS for 1 hour and washed three times (10 mins/wash). The proteins were visualized using an chemiluminescent detection kit (Pierce, USA) and exposed to X-ray film [5].

Proteomics analysis:

Sample preparation for 2D-PAGE

After transfection**,** cell pellets were harvested and extracted with 2D lysis buffer [7 M urea, 2 M Thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), and 10 µl/ml Protease inhibitor cocktail] and removed the contaminants by 2D Clean-Up kits (GE Healthcare Life Sciences, Sweden). Total content of samples were measured using Bradford assay (Biorad, USA) and diluted to desired concentration of 2 µg/ml with rehydration buffer [7 M Urea, 2 M Thiourea, 2% CHAPS, 60 mM DTT, 0.5% Immobilline pH gradients (IPG) buffer, and 0.002% Bromophenol blue] [8].

2D-PAGE (Two dimension polyacrylamide gel electrophoresis)

For first dimension, proteins were separated using 7 cm immobilized pH gradients strip (IPG strip) pH 3-10. IPG Strips were rehydrated overnight in 125 µl of rehydration solution (7 M Urea, 2 M Thiourea, 2% CHAPS, 60 mM DTT, 0.5% IPG buffer, and 0.002% Bromophenol blue) containing 250 µg of protein samples and overlaid with ImmobilineDrystrip Cover Fluid. The rehydrated IPG strips were transferred to an EttanIPGphor II Manifold, covered with ImmobilineDrystrip Cover Fluid, and run at 500 volts, 1000 volts, and 8000 volts for 1 hour, 1 hour, and 2 hours, respectively [9]. After IEF, the strips were equilibrated in first equilibration buffer (6 M urea, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 375 mM Tris-HCl pH 8.8, and 2% DTT) for 10 min and then with second equilibration buffer (2.5% iodoacetamide (IAA)) for another 10 min. For the second dimension, IPG strip was placed onto 12% SDS polyacrylamide gel and sealed using 1% agarose. The gel was run at 150 volts for 2 hours. Three independent protein samples were run for each cell lines. After electrophoresis, the gel was fixed in fixing solution (40% ethanol, 10% acetic acid) and stained with Colloidal Coomassie blue G-250 (8% ammonium sulfate, 0.8% phosphoric acid, 0.08% Coomassie Brilliant blue G-250, and 20% methanol) to visualize protein spots following the manufacturer's protocol (Amercham Biosciences Inc, Sweden). The stained gels were examined using Image Scanner™. The search for differentially expressed proteins was carried out using the Image Master™ 2D Platinum 7.0 program (Amersham Biosciences Inc, Sweden). Molecular masses were determined by running Pink Plus Prestained Protein Ladder markers, in a range of 10–175 kDa (GeneDirex).

In gel digestion

Selected protein spots from 2D-gel were picked from the gels and destained by washing twice with 25 mM ammonium bicarbonate in 50% methanol. Gel pieces were further washed with 100% acetonitrile. Dried gel pieces were added with 20 µl of 10 mM DTT in 10 mM ammonium bicarbonate and incubated at 56°C for 1

hour, followed by adding 100 mM iodoacetamide in 10 mM ammonium bicarbonate. The gel pieces were then digested with 10 µl of 10 ng/µl sequencing grade modified porcine trypsin (Promega, USA) in 10 mM ammonium bicarbonate solution and incubated at 37°C overnight. The peptides were extracted by addition of 30 µl of 50% acetonitrile in 0.1% formic acid and dried at 40°C overnight.

LC-MS/MS and protein identification

The dried extracted peptides were resuspended with 12 µl of 0.1% formic acid and transferred to low binding microcentrifuge tube. Solution was centrifuged at 10,000 rpm for 10 minutes and transferred to vial tube. The resuspended peptide was injected to LC-MS/MS (ESI-Quad-TOF Mass spectrometry). The peptide sequences from LC-MS/MS were analyzed by Mascot Search and identified by NCBInr data base.

Results:

siRNA against WT1 transfection in MCF-7 cell line

MCF-7 breast cancer cell line was transfected with 100 nM of siRNA against WT1 ($siRNA_{WT1}$) compared to control ($siRNA_{neg}$) for 72 hours. After transfection the cells were detected WT1 level by Western blot analysis. The results showed that knockdown of WT1 led to decrease in WT1 protein expression in MCF-7 (Figure 1).

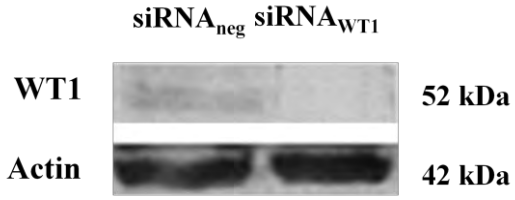

Figure 1 $siRNA_{WT1}$ transfection in MCF-7 cell line. MCF-7 was transfected with 100 nM of $siRNA_{WT1}$ for 72 hours. 50 µg of total protein was detected by Western blot analysis.

Proteomics analysis of $siRNA_{WT1}$ transfection in MCF-7

Two-dimensional gel electrophoresis (2D-PAGE)

Image analysis revealed that there were 27 spots significantly change (fold change ≥ 1.5, *p*-value < 0.05) between $siRNA_{WT1}$ and $siRNA_{neg}$. 15 spots were up-regulated in MCF-7 $siRNA_{neg}$ and 12 spots were up-regulated in $siRNA_{WT1}$ (Figure 2).

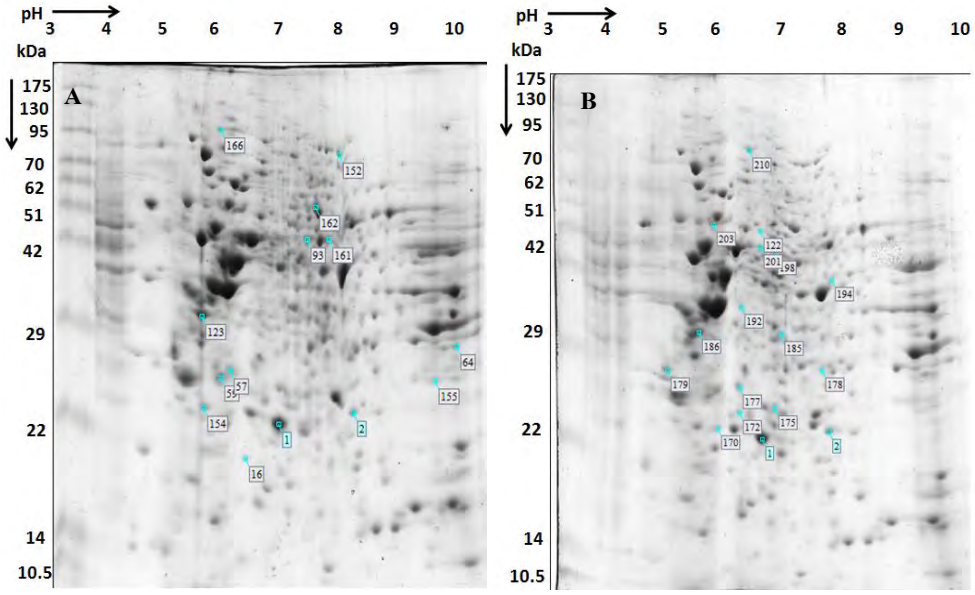

Figure 2 Protein patterns of 2D-PAGE containing of 250 µg of total protein extracted from $siRNA_{WT1}$ transfection in MCF-7 (A) and $siRNA_{neg}$ (negative control) in MCF-7 (B).

Identification of differential protein expression by LC-MS/MS

The excised spots from 2D-gel images of MCF-7 were digested with trypsin and injected into LC-MS/MS. The identification of the selected spots was searched in NCBInr database by Mascot Search. The proteins were grouped in various groups according to biological functions: apoptosis, protein folding, metabolism, structural, cell adhesion, gene expression, transport, redox-regulation, protein degradation, and unknown function (Table 1-2). Interestingly, we found the proteins involving in apoptosis and transport up-regulated only in MCF-7 $siRNA_{WT1}$ but not in MCF-7 $siRNA_{neg}$

Table 1 Identification of expressed proteins of $siRNA_{neg}$ in MCF-7

Spot ID	Protein name	NCBInr Acc. No.	Calculated MW/pI	Exp. Mw/PI	score	match peptide	% Cov.
up-regulated in MCF-7 $siRNA_{neg}$							
Protein folding							
122	26S protease (S4) regulatory subunit	AAA35484	49.3/5.68	50/5.7	33	1	2
170	Heat shock protein beta-1	NP_001531	22.8/5.9	22.8/5.7	167	5	21
Metabolism							
185	GDP-L-fucose synthase	NP_003304	36.0/6.12	32/6.25	102	2	7
175	Triosephosphateisomerase 1	AAH17917	26.9/6.45	25.5/6.6	139	5	16
Cell adhesion							
201	embigin precursor	NP_940851	37.3/6.14	45/6.0	31	1	2
Gene regulation							
203	Transformation upregulated nuclear protein	CAA51267	51.3/5.13	52/5.0	224	3	3
186	Human elongation factor 1 - delta	CAA79716	31.3/4.95	31.5/4.8	79	1	4
Transport							
198	Annexin VII isoform 1	BAD96272	50/6.5	50.48/6.47	218	4	10
177	Chondroitin sufate proteoglycan 4 precursor	NP_001888	25.03/5.27	25.5/5.5	25	2	10
Redox-regulation							
172	Thioredoxin peroxidase	2PN8_A	24.1/5.68	24/5.8	128	3	13

Table 2 Identification of expressed proteins of $siRNA_{WT1}$ in MCF-7

Spot ID	Protein name	NCBInr Acc. No.	Calculated MW/pI	Exp. Mw/PI	score	match peptide	% Cov.
Apoptosis							
59	**Chain B, crystal structure of native and inhibited forms of human cathepsin D**	**1LYA_B**	**26.4/5.31**	**26.5/6.1**	**108**	**44**	**12**
57	**Cathepsin D prepoprotein**	**NP_001900**	**25.0/6.1**	**26/5.2**	**196**	**43**	**12**
Structural proteins							
162	WDR1 protein	AAA05045	58.5/6.41	58/6.8	67	44	4
123	TPMSK3	AAL84570	28.9/4.72	31/4.8	146	44	16
Gene expression							
161	KUP protein	CAA34595	49.4/6.06	49/6.1	19	44	1
152	Elongation factor 2	NP_001952	96.2/6.41	95/6.6	81	43	3
transport							
64	Porin 31HM	AAB30246	30.7/8.63	28/9.6	218	44	12
Redox-regulation							
16	Glutathione transferase M3	AAA60964	27.1/5.37	25/5.0	139	44	22
Protein degradation							
155	calcyclin-binding protein isoform 1	NP_055227	26.3/8.28	25.5/8.8	96	44	12
Protein folding							
166	Heat shock protein 70 kDa protein 4	NP_002145	95.1/5.11	95/5.2	80	44	2

Conclusion and discussion

The *WT1* gene encodes a zinc finger acting as a transcriptional activator or repressor for many genes involved in cell differentiation, growth, and apoptosis. These functions depended on the cell types, WT1 isoforms and the status of targeted molecules. In order to determine the relationship between WT1 and the targeted molecules, WT1 was silenced with siRNA and the protein expression pattern was analyzed by two-dimensional gel electrophoresis (2D-PAGE) and LC-MS/MS mass spectrometry. We have found 28 proteins having significant changes in their expression level (Table 1) between $siRNA_{WT1}$ and $siRNA_{neg}$. These proteins can be classified their function in apoptosis, protein folding, metabolism, structural, cell adhesion, gene expression, transport, and protein degradation (Table 1).

Our results found the protein involving in apoptosis, cathepsin D, when WT1 was transfected with siRNA in MCF-7 breast cancer cell line. Cathepsin D is an intracellular aspartic protease found in the endosomes and lysosomes of

all mammalian cells. It is also key mediator of apoptosis induced by many apoptotic agents such as IFN-γ, Fas/APO, and TNF-α [10]. Furthermore Liaudet *et al*, (2006) shown that cathepsin D over-expressed and acted as poor prognosis marker in breast cancer [11]. However, validation of proteomic results and the further study are necessary to confirm this result.

Acknowledgments

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Proteomics analysis of siRNA-mediated silencing of Wilms' tumor 1 in the MDA-MB-468 breast cancer cell line

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Abstract. The Wilms' tumor 1 (*WT1*) gene encodes a zinc finger which appears to be a transcriptional activator or repressor for many genes involved in cell differentiation, growth and apoptosis. In order to determine the relationship between WT1 and related proteins, WT1 was silenced with small interfering RNA (siRNA) and the protein expression pattern was analyzed by proteomics analysis including one-dimensional gel electrophoresis (1-DE) and LC-MS/MS mass spectrometry. The results revealed that 14 proteins were expressed in WT1-silenced cells ($siRNA_{WT1}$) and 12 proteins were expressed in the WT1-expressing cells ($siRNA_{neg}$), respectively. These proteins may be classified by their functions in apoptosis, cell signaling, protein folding, gene expression, redox-regulation, transport, structural and unknown functions. Mitogaligin, an apoptosis-related molecule, was identified when WT1 was silenced while the proteins related to the signaling pathway were detected in both $siRNA_{neg}$ and $siRNA_{WT1}$ but the type of proteins were different. For example, the IBtK protein and the SH2 domain-containing protein were present in $siRNA_{WT1}$ conditions, while the platelet-derived growth factor receptor α (PDGFRA) and Rho guanine nucleotide exchange factor 1 (Rho-GEF 1) were expressed in $siRNA_{neg}$. Of these, Rho-GEF was selected for validation by western blot analysis and demonstrated to be present only in the presence of WT1. In conclusion, WT1 is related to mitogaligin via EGFR and behaves as an anti-apoptotic molecule. Moreover, WT1 may be associated with PDGFRA and Rho-GEF 1 that activates proliferation in MDA-MB-468 cells.

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Key words: proteomics, Wilms' tumor 1, siRNA, mitogaligin, platelet-derived growth factor receptor α, Rho guanine nucleotide exchange factor 1

Introduction

Breast cancer is the most common cancer and the leading cause of mortality in women worldwide, accounting for 23% (1.38 million cases) of the total new cancer cases and 14% (458,400 cases) of all cancer deaths in 2008 (1). The development of breast cancer may result from interaction between the change in genetic elements, environmental factors and also the difference in ethnicity (2). There are several genes reported to be associated with breast cancer, such as *ERBB2*, *c-Myc*, *CCND1*, *TP53*, *PTEN* and *Wilms' tumor 1* (*WT1)* (3).

The human *WT1* gene is located at chromosome locus 11p13 comprising 10 exons. Alternative splicing occurs at exon 5 (plus or minus 17AA) and exon 9 (plus or minus KTS) in mRNA of WT1. These two alternative splicing sites yield four different isoforms: WT1+/+, WT1+/-, WT1-/+ and WT1-/- (4,5). Loeb *et al* demonstrated that WT1 mRNA and protein was detected in nearly 90% of breast cancers but not in most normal breast samples (6). Moreover, Navakanit *et al* reported that the siRNA against WT1 inhibited both WT1 protein expression level and growth of breast cancer cell line MCF-7, in a dose- and time-dependent manner. These results suggested that WT1 may act as an oncogene in the breast cancer cell line MCF-7. Furthermore, WT1 may play a role in the pathogenesis of breast cancer as an oncogene rather than a tumor suppressor gene as in leukemia (7). Additionally, the high level expression of WT1 mRNA detected by real-time RT-PCR can predict a poor prognosis in breast cancer patients (8) and the absence of mutations through the whole 10 exons of the *WT1* gene in the 36 cases of primary breast cancer (9). *WT1* encodes a zinc finger acting as a transcriptional activator or repressor for many genes involved in cell differentiation, growth and apoptosis. These functions depend on the type of cells, WT1 isoforms and the status of targeted molecules. There are several targeted molecules for WT1 including growth factor genes: IGF-II, PDGF-A, CSF-1 and TGF-β1, growth factor receptor genes: insulin receptor, IGF-1R and EGFR, and transcription factor and other genes, including: Egr1, PAX4, p53, c-myc, Bcl-2, cyclin E, Bak, Bax (10,11). However, the relationship between WT1 and the targeted molecules involved in breast cancer remains unclear

and the overview study of the relationship between WT1 and the related molecules has not been reported.

MDA-MB-468 breast cancer cells are estrogen receptor (ER), progesterone receptor (PR) and HER2-negative (12). The cells have a very high number of the epidermal growth factor receptors (EGFRs) which is growth inhibited by EGF and mediated apoptosis (13,14). Moreover, the cells have a p53 mutation, G -> A mutation in codon 273 of the p53 gene resulting in an Arg -> His substitution (15).

In the present study, we used siRNA against WT1 mRNA to silence WT1 expression and the relationship between WT1 and related proteins in the breast cancer cell line MDA-MB-468 was investigated by proteomics analysis. The proteins were further identified by LC-MS/MS and database searching. These studies may provide further evidence to understand the relationship between WT1 and the related molecules in breast cancer.

Materials and methods

Cell culture. The human breast cancer cell line MDA-MB-468 was purchased from American Type Culture Collection. MDA-MB-468 was cultured in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin and 10% glutamine. Then, the cells were incubated in a 37˚C incubator with 5% CO_2 (7).

Small interfering RNA (siRNA) transfection. MDA-MB-468 at $1x10^5$ cells were seeded in each well of 24-well culture plates and incubated in a CO_2 incubator at 37˚C for 24 h. The cells were transfected using Lipofectamine® 2000 reagent (Invitrogen) in 24-well plates with 200 nM siRNA duplexes (optimal siRNA conditions performed in preliminary study and data not shown). The siRNA against WT1 ($siRNA_{WT1}$) (Invitrogen) consisted of a mixture of two 25-nt duplexes, i.e., $siRNA_{WT1}$R88 (5'-AAATATCTCTTATTGCAGCCT GGGT-3') and $siRNA_{WT1}$R90 (5'-TTTCACACCTGTATGTCT CCTTTGG-3'). To minimize the cytotoxicity of the reagent itself, the cells were washed once with PBS and the media was changed 6 h after transfection (7). After 72 h, the cells were harvested and the protein level was investigated by western blot analysis.

Western blot analysis. Cell pellets were harvested by trypsinization and extracted with radioimmunoprecipitation assay (RIPA) buffer (Pierce, USA). Then, the concentration of protein was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). The 50 µg of protein samples were loaded to 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked by blocking solution [5% low fat dry milk in 1X TTBS (0.1% Tween-20, 154 mM NaCl, 48 mM Tris-base)] for 1 h and washed 5 min for three times with washing solution (1% low fat dry milk in 1X TTBS buffer). After blocking, the blot was incubated with primary antibody anti-WT1 (1:200), anti-rho-GEF (1:1,000) and anti-actin (1:1,000) antibodies (diluted with 1% low fat dry milk in 1X TTBS) for 2 h and washed 5 min for three times with washing solution. The membrane was then incubated with secondary antibody polyclonal anti-IgG rabbit (1:10,000) antibody in 1% low fat dry milk in 1X TTBS for 1 h and washed three times (10 min/wash). The proteins were visualized using a chemiluminescent detection kit (Pierce) and exposed to X-ray film (7).

Proteomics analysis

One-dimensional electrophoresis (1-DE). After transfection, $siRNA_{neg}$- and $siRNA_{WT1}$-transfected cell pellets from MDA-MB-468 were extracted with 0.5% SDS. Protein quantification was calculated using the Lowry method. Total protein samples of 50 µg were loaded onto 12.5% SDS-polyacrylamide gel and a marker lane (low range marker; GE Healthcare) was added for calculation of the molecular weight of the protein bands. The gel was run at 20 mA/gel for 1.45 h. After electrophoresis, the gel was fixed in fixing solution (40% ethanol, 10% acetic acid) and stained with Colloidal Coomassie Blue G-250 (8% ammonium sulfate, 0.8% phosphoric acid, 0.08% Coomassie Brilliant Blue G-250 and 20% methanol). Then, the gel was scanned with EPS 601 scanner (Bio-Rad) and the gel bands were fractionated to 15 slices and excised from the bottom to the top of the gel lane and each slice was cut into 1 mm cubes. The gel pieces were transferred into a well of low binding 96-well plates.

In-gel digestion. The gel pieces were destained by washing twice with 25 mM ammonium bicarbonate in 50% methanol and further washed with 100% acetonitrile. Dried gel pieces were added with 20 µl of 10 mM DTT in 10 mM ammonium bicarbonate and incubated at 56˚C for 1 h, followed by the addition of 100 mM iodoacetamide in 10 mM ammonium bicarbonate. The gel pieces were then digested with 10 µl of 10 ng/µl sequencing grade modified porcine trypsin (Promega, USA) in 10 mM ammonium bicarbonate solution and incubated at 37˚C overnight. The peptides were extracted by addition of 30 µl of 50% acetonitrile in 0.1% formic acid and dried at 40˚C overnight.

LC-MS/MS and protein identification. The dried extracted peptides were resuspended with 12 µl of 0.1% formic acid and transferred to low binding microcentrifuge tube. Solution was centrifuged at 10,000 rpm for 10 min and transferred to vial tube. The resuspended peptide was injected to LC-MS/MS (ESI-QUAD-TOF mass spectrometry). The peptide sequences from LC-MS/MS were analyzed by Mascot Search and identified by NCBInr database.

Results and Discussion

siRNA against WT1 transfection in MDA-MB-468 cell line. The MDA-MB-468 breast cancer cell line was transfected with 200 nM of siRNA against WT1 ($siRNA_{WT1}$) compared to control ($siRNA_{neg}$) for 72 h. After transfection of the cells we detected WT1 level by western blot analysis. The results showed that knockdown of WT1 led to decrease in WT1 protein expression in MDA-MB-468 (Fig. 1A).

1-DE. The quantitative proteomic, one-dimensional gel electrophoresis (1D-PAGE) was carried out to determine the protein expression patterns between $siRNA_{neg}$ compared to $siRNA_{WT1}$ in MDA-MB-468. Fig. 1B1 represents the protein patterns obtained from 1D-PAGE. Lane 1 and 2 show the

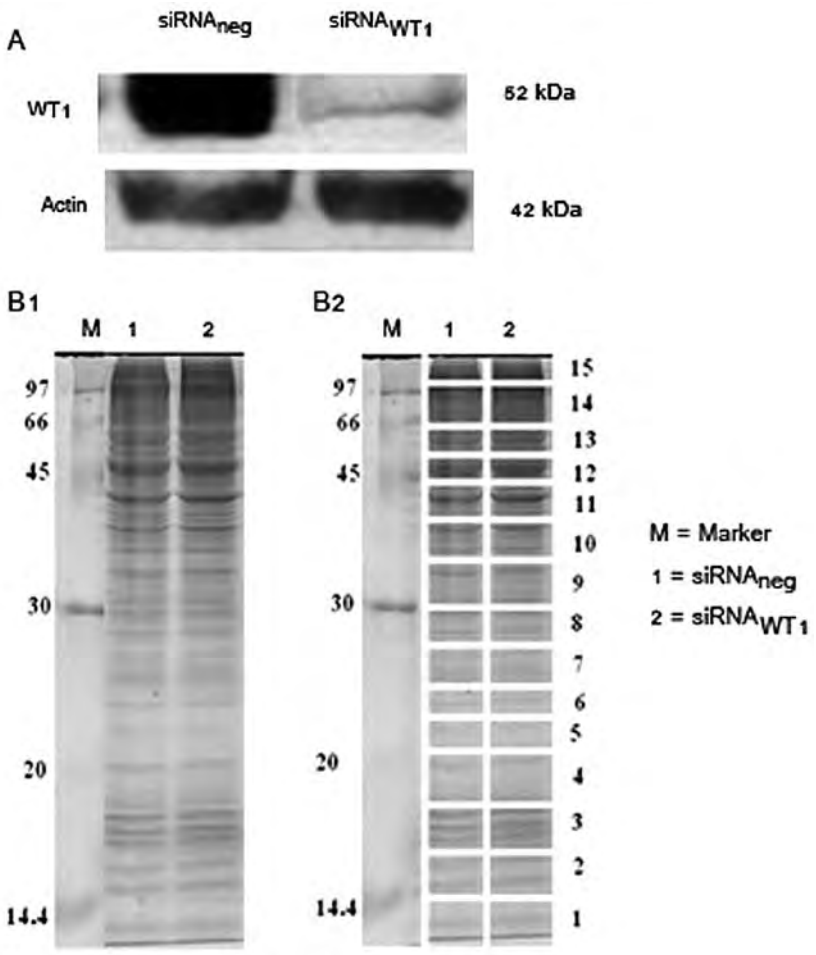

Figure 1. (A) $siRNA_{WT1}$ transfection in the MDA-MB-468 cell line. MDA-MB-468 was transfected with 200 nM of $siRNA_{WT1}$ for 72 h. Total protein (50 µg) was detected by western blot analysis; (B) SDS-gel image (12.5%) of protein pattern between $siRNA_{neg}$ compared to $siRNA_{WT1}$ in MCF-7 and MDA-MB-468 (B_1) and gel after fractionation into 15 slices (B_2). siRNA, small interfering RNA; WT1, Wilms' tumor 1.

protein bands of $siRNA_{neg}$ and $siRNA_{WT1}$, respectively. After 1D-PAGE, the gels were cut into 15 slices as shown in Fig. 1B2.

The quantification of protein from 1D-PAGE was analyzed by the DeCyder™ MS 2.0 Differential Analysis Software (GE Healthcare). The protein expressions of $siRNA_{WT1}$ and $siRNA_{neg}$ were compared. The different intensity of protein expression in both conditions is shown in Venn's diagram (Fig. 2A). These demonstrated all possible relations of protein expressions in two conditions. The protein names and their biological functions of expressed proteins found only in $siRNA_{WT1}$ are listed in Table I and the expressed proteins found only in $siRNA_{neg}$ are listed in Table II. Table III shows the protein names and the biological functions of expressed proteins found in $siRNA_{WT1}$ and $siRNA_{neg}$. Rho guanine nucleotide exchange factor 1 (Rho-GEF) was selected to validate by western blot analysis. The result showed the presence of Rho-GEF only in WT1 presence in the cell (Fig. 2B).

Due to p53 mutation in MDA-MB-468 (15), the apoptosis pathway may occur via p53 independently. Notably, a novel target protein of WT1, mitogaligin, was found when WT1 was silenced. Mitogaligin is a 96 amino acid protein highly cationic and rich in tryptophan (16). This protein contains two localization signals, mitochondria and nucleus. Mitogaligin is mainly localized in mitochondria and promotes the release

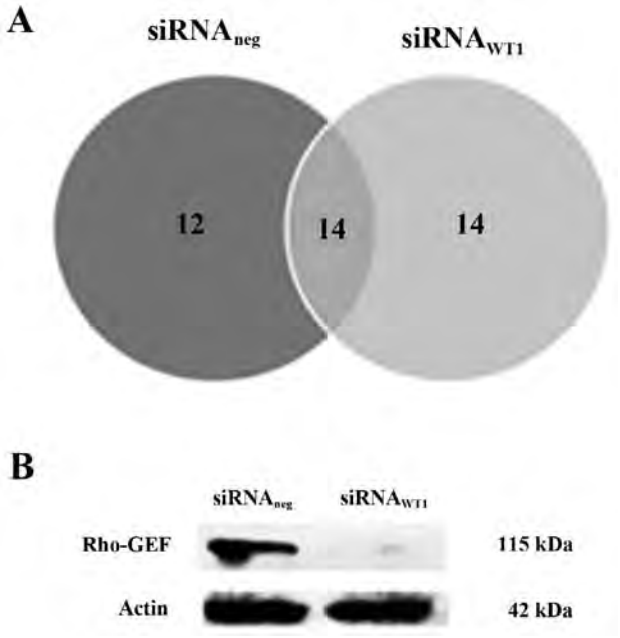

Figure 2. (A) Venn's diagram of protein expression with different intensity between $siRNA_{WT1}$ transfection and $siRNA_{neg}$ control of MDA-MB-468; (B) Rho-GEF was validated by western blot analysis. siRNA, small interfering RNA; WT1, Wilms' tumor 1; Rho-GEF, Rho guanine nucleotide exchange factor 1.

Table I. Identification of expressed proteins found only in MDA-MB-468 $siRNA_{WT1}$ using DeCyder™ MS 2.0 Differential Analysis Software.

Protein name	Accession no.	Peptide	Mowse score
Apoptosis			
Mitogaligin	gi|12005991	AWRMGEPACWGR	9.50
Cell signaling			
IBTK protein, partial	gi|34192875	SLDVLSDGVLK	27.56
SH2 domain-containing protein 3C isoform a	gi|41281821	RSSASISR	11.47
Structural protein			
Cytokeratin 9	gi|435476	GGSGGSYGGGGSGGGYGGGSGSR	91.06
Keratin 10	gi|21961605	SQYEQLAEQNRK	50.17
Keratin, type II cytoskeletal 1	gi|119395750	SLNNQFASFIDK	98.71
Type I keratin 16	gi|1195531	APSTYGGGLSVSSR	30.64
Protein folding			
Ankyrin repeat domain-containing protein 62	gi|302393830	LNDLNDRDK	13.03
Gene regulation			
SON DNA binding protein isoform E	gi|17046381	NRDKGEKEK	10.73
Redox-regulation			
Selenoprotein I	gi|119621096	KMAASTRVEASR	5.30
Transport			
Synaptosomal-associated protein 23 isoform SNAP23A	gi|18765729	KLIDS	4.17
Unknown			
hCG2042301	gi|119611404	TGGDRTKAQRHEIISLS	11.14
Unknown protein IT12	gi|2792366	SGARAMAKAKK	7.15
Unnamed protein product	gi|21757251	LINDSTNK	19.40

siRNA, small interfering RNA; WT1, Wilms' tumor 1.

of cytochrome *c* resulting in the induction of cell death (17). Moreover, it can also be directed to the nucleus and can play a role in apoptotic properties leading to cell death (18). The STRING shows the correlation between WT1 and mitogaligin via EGFR (Fig. 3A). WT1 may act as negative regulator of mitogaligin through the EGFR leading cell death.

WT1 interacts with many genes involved in the cell signaling pathway. In the present study, the proteins involved in the cell signaling pathway, platelet-derived growth factor receptor α (PDGFRA) and Rho-GEF were found when WT1 was present in MDA-MB-468, while SH2 domain-containing protein and IBtK were found when the cell was without WT1. However, the STRING 9.05 showed that these molecules were not associated with WT1 (Fig. 3B).

The signal transduction pathway in the MDA-MB-468 breast cancer cell line was related to the mTOR signaling pathway that regulates cell growth, proliferation, differentiation and survival (19). The mTOR protein exists in two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 contains the protein *raptor* while mTORC2 contains the protein *rictor*. In the presence of growth factors, activated Akt phosphorylates and inhibits tuberous sclerosis protein 2 (Tsc2), thereby promoting the activation of Rheb. Activated Rheb (Rheb-GTP) helps activate mTORC1, which in turn stimulates cell growth. Furthermore, mTORC2 phosphorylates Akt at Ser473 and regulates the actin cytoskeleton and cell motility (20). Recently, Razmara *et al* demonstrated that PDGFRs are essential for multiple growth factor signaling pathways that lead to PI3K/Akt activation. The pathway from PDGFR leads to phosphorylation of Akt which is involved in both the mTORC2 and PLCγ/PKC pathways (21).

The WT1 protein has two nuclear localization domains: within zinc fingers I and within zinc fingers II and III. It is responsible for transcription and RNA processing (22). However, WT1 can be detected in the cytoplasm of various cell lines including breast cancer and shuttles the nucleus and the cytoplasm (23,24). PDGFRA is a tyrosine-protein kinase that acts as a cell surface receptor for PDGFA and plays a role in the regulation of cell proliferation and survival (25). Rho-GEF is an intracellular signaling molecule that regulates cytoskeleton organization, gene expression, cell cycle progression, cell motility and other cellular processes. It represents the activating enzymes of Rho GTPases by serving to relay a variety of signals to catalyze GDP/GTP exchange of specific Rho GTPases (26).

Table II. Identification of expressed proteins found only in MDA-MB-468 siRNA$_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software.

Protein name	Accession no.	Peptide	Mowse score
Cell adhesion			
Vang-like protein 1 isoform 1	gi|20373171	HMAGLK	12.95
Cell differentiation			
METRNL protein, partial	gi|30047763	VFEPVPEGDGHWQGR	10.04
Cell signaling			
PDGFRA protein	gi|39645305	VPSIKLVYTLTVPEATVK	11.73
Rho guanine nucleotide exchange factor 11 isoform 1	gi|7662086	SSNSK	6.04
Structural			
Keratin 5	gi|18999435	LAELEEALQK	23.61
Peroxisome assembly protein 26 isoform a	gi|8923625	KSDSSTSAAPLR	6.59
hHa7 protein	gi|50949256	NTLNGHEK	12.35
Transport			
Na+/K+-ATPase α 3 subunit variant	gi|62898870	LNIPVSQVNPR	14.46
Unknown function			
Unnamed protein product	gi|194390014	MFHLAAFKLK	22.44
hCG2042050	gi|119579649	ASTVPDLK	7.42
Chromosome 9 open reading frame 39	gi|119579068	LLEGQSLALSPR	11.96
Hypothetical protein LOC286076	gi|119602615	DVGDALPR	29.47

siRNA, small interfering RNA; WT1, Wilms' tumor 1. PDGFRA, platelet-derived growth factor receptor α.

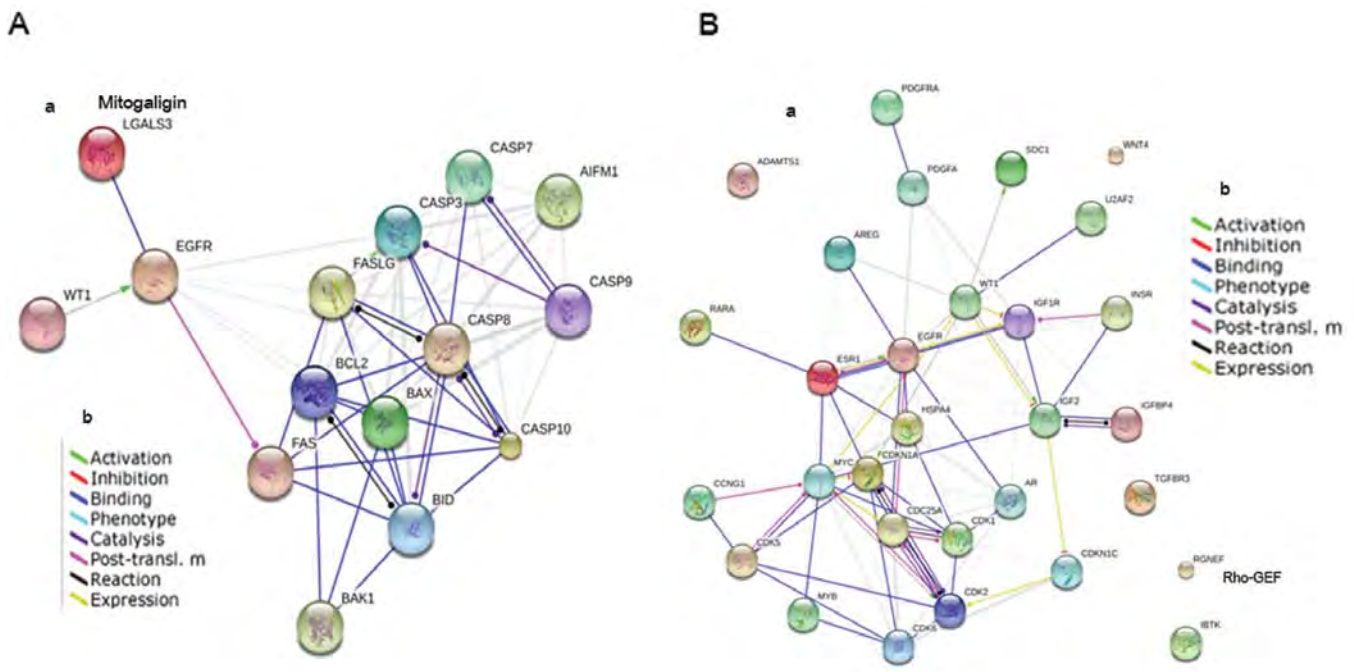

Figure 3. (A-a) The involvement of WT1 and p53-independent apoptosis pathway in MDA-MB-468 (STRING 9.05). (b) Modes of action are shown in different colors. The red circle shows the proteins found in this study. (B-a) The involvement of WT1 and proteins in signal transduction pathway in MDA-MB-468 (STRING 9.05). (b) Modes of action are shown in different colors. The red circle shows the proteins found in this study. WT1, Wilms' tumor 1; BAK1, Bcl-2 homologous antagonist killer; BID, BH3 interacting domain death agonist; FASLG, Fas ligand; BCL-2, B-cell lymphoma-2; BAX, Bcl-2-associated X protein; LGALS3, mitogaligin; EGFR, epidermal growth factor receptor; PDGFRA, platelet-derived growth factor receptor α; RARA, retinoic acid receptor α; IBtK, inhibitor of Bruton's tyrosine kinase.

Table III. Identification of expressed proteins found in MDA-MB-468 $siRNA_{WT1}$ and MDA-MB-468 $siRNA_{neg}$ using DeCyder™ MS 2.0 Differential Analysis Software.

Protein name	Accession no.	Peptide	Mowse score
Structural protein			
LMNA protein	gi|21619981	SGAQASSTPLSPTR	44.82
Cell differentiation and survival			
Nance-Horan syndrome protein isoform 2	gi|42384238	KTISGIPR	26.98
Sestrin-2	gi|13899299	KLSEINK	21.68
Cell signaling			
S100 calcium binding protein A10 [Annexin II ligand, calpactin I, light polypeptide (p11)], isoform CRA_b	gi|119573783	NALSGAGEASAR	11.49
Chain A, catalytic domain of human phosphodiesterase 4b in complex with piclamilast	gi|58177395	GMEISPMXDK	8.66
Protein S100-A6	gi|7657532	LQDAEIAR	43.91
Hormone			
C-type natriuretic peptide precursor	gi|13249346	YKGANKKGLSK	10.08
Protein folding			
Heat shock protein	gi|4204880	IINEPTAAAIAYGLDKK	27.1
Transport			
Ras association domain-containing protein 9	gi|114155158	ADAFLPVPLWR	6.35
Gene regulation			
TTLL5 protein	gi|33877151	MGNTMDKR	10.31
39S ribosomal protein L15, mitochondrial	gi|7661806	CGRGHK	16.37
Unknown function			
hCG16415, isoform CRA_f	gi|119611935	GAECCPGGPVK	10.83
FLJ00258 protein	gi|18676718	GSMSR	8.83
Pyruvate dehydrogenase E1 α subunit	gi|861534	EEIPPHSYR	6.28

siRNA, small interfering RNA; WT1, Wilms' tumor 1.

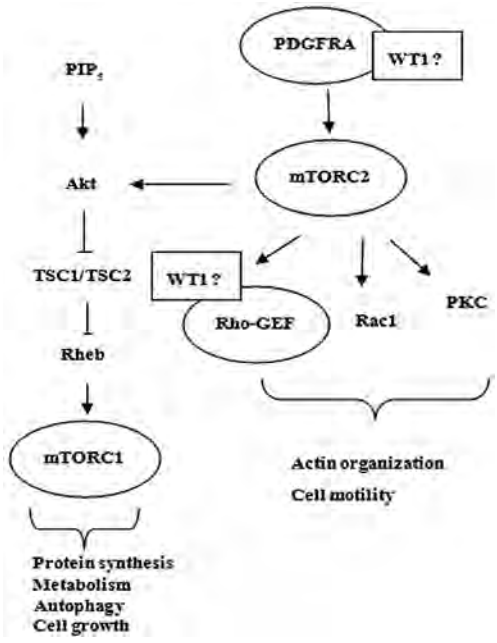

Figure 4. The possible relationship between WT1 and PDGFRA, Rho-GEF in signal transduction pathway in MDA-MB-468 [adapted from Zhou and Huang (20)]. WT1, Wilms' tumor 1; PDGFRA, platelet-derived growth factor receptor α; Rho-GEF, Rho guanine nucleotide exchange factor 1.

WT1 may be related to PDGFRA leading to activation of Akt/TSC1, TSC2/mTOR2 pathway resulting in cell growth. Moreover, WT1 may also be associated with mTOR2/Rho-GEF resulting in cell motility (Fig. 4).

Thus, WT1 plays an oncogenic role in MDA-MB-468. Moreover, when WT1 was silenced with $siRNA_{WT1}$, IBtK, SH2 domain-containing protein were upregulated. The relationship between WT1 and these proteins in the signaling pathway in MDA-MB-468 has not previously been elucidated. WT1 may behave as a negative-regulator of IBtK that binds to SH2 domain of BtK tyrosine kinase receptor resulting in IBtK inactivate leading to B-cell differentiation (27).

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