CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND MATERIALS

The name of chemicals and reagents, and instruments are shown in Appendix A and B, respectively. The detail of reagents and/or buffers used in this study is shown in Appendix C.

2.2 CELL CULTURES AND PLASMID TRANSFECTION

2.2.1 Cell Lines and Culture Conditions

Breast adenocarcinoma cells (MDA-MB-231) (ATCC, USA) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS HyClone, Thermo Scientific, USA), 1xAntibiotic-Antimycotic (Invitrogen, USA.) at 37°C in a humidified incubator of 5% CO_2. All experiments were initiated with cells in log phase of growth, and designed to be completed before 80% confluence.

2.2.2 Expression Constructs

The full length cDNAs of 6xHis-FLAG tagged wild-type maspin, ovalbumin and two maspin/ovalbumin chimeric mutants are kindly provided by Professor S.S. Twining, Medical College of Wisconsin, USA. The chimeric mutants are maspin mutant containing the ovalbumin RCL (MOM) and ovalbumin mutant containing the

maspin RCL (OMO). All of these constructs were subcloned into a mammalian expression vector pcDNA3.1 (Invitrogen, USA) at *Not l* (5') and *Sal l* (3') restriction sites by PCR-based method.

All constructs were transformed into competent *E. Coli* strain DH5α (RBC Bioscience, Taiwan) by a standard heat-shock transformation method. Briefly, 20uL of competent cells (10^8) were mixed with 2 uL plasmid DNA (500 ng) and incubated on ice for 20 min. Next, the cells were heat-shocked at 42°C for 45 sec, and then 500 uL of LB broths were added to the cells. After 1 h incubation at 37°C, the transformants were plated on a LB agar containing 100 ug/mL of ampicillin for colony selection, and incubated at 37 °C. Single colonies of each mutant were picked and grown in LB broth for plasmid isolation. The vector constructs were purified using Genopure Plasmid Midi Kit according to the manufacturer's protocol (Roche diagnostics, Germany). The amount and purity of the purified plasmids were calculated by UV-spectrophotometry using the absorbance value at 260 nm and the ratio of OD_{260}/OD_{280}, respectively.

2.2.3 Cell Transfection

MDA-MB-231 cells were transfected with pcDNA3.1-6xHis-FLAG-maspin wild type or pcDNA3.1-6xHis-FLAG-chimeric mutant expression vectors using TurboFect™ *in vitro* Transfection Reagent according to the manufacturer's protocol (Fermentas, Thermo Scientific, USA). In brief, MDA-MB231 cells with 90% cell confluency ($4x10^5$ cells/ 35-mm dish) were incubated with the TurboFect™/DNA mixture at 37°C in a humidified incubator of 5% CO_2. After 5 hours, the MDA-MB231 transfectants were replaced the transfection mixture with fresh growth

medium. Transfections of empty vector and pcDNA3.1-6xHis-FLAG-ovalbumin were used as the controls. Forty eight hours later, the expression of protein was analyzed by Western blotting. The experiments were repeated 3 times with at least triplicate per experiment.

2.2.4 Western Blot Analysis

The Western blot analysis is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or cell extract. It uses SDS-polyacrylamide gel electrophoresis (PAGE) to separate denatured proteins by the size of the polypeptide and electrotransferred onto nitrocellulose membrane. The transferred protein is detected using specific primary antibody and secondary antibody labeled with peroxidase enzyme followed by chemiluminescent substrate.

2.2.4.1 Total Protein Extraction

Total cell lysate of the MDA-MB231transfectants was collected using mammalian protein extraction buffer (GE Healthcare, USA) that contains protease inhibitor cocktail tablet (Roche diagnostics, Germany). Cell debris was eliminated by centrifugation at 12,000x g for 15 minutes at 4°C.

2.2.4.2 Determination of Protein Content

The protein concentration of cell pellets was quantified by Bio-Rad Protein Assay (Bio-Rad, USA). The Bio-Rad Protein Assay, based on the method of Bradford, is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. The dye exists in three forms: cationic (red), neutral (green), and anionic (blue). Under acidic conditions, the dye is predominantly

in the doubly protonated red cationic form (A_{max} = 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (A_{max} = 595 nm). The blue colored protein-dye complex is detectable at 595 nm using a spectrophotometer or microplate reader. The concentrations of protein are estimated by calculation using a standard curve of absorbance obtained from a series of standard protein dilutions, which are assayed alongside the unknown samples.

The concentrations of protein determination by the Bio-Rad Protein assay reagent was as follows: 250 µL of the assay reagent was added to each well of a 96-well microplate which contains bovine serum albumin (BSA) standard protein solution in various concentrations (25-750 µg/mL) or unknown samples. Then, the absorbance was measured at 595 nm using Synergy™ HT Multi-Detection Microplate Reader (Bio-TEK, USA). The absorbance values of all individual standard and unknown samples replicates were subtracted by that of the blank. The standard curve of protein was plotted as shown in **Figure 8** and used to determine the protein concentration of each unknown samples.

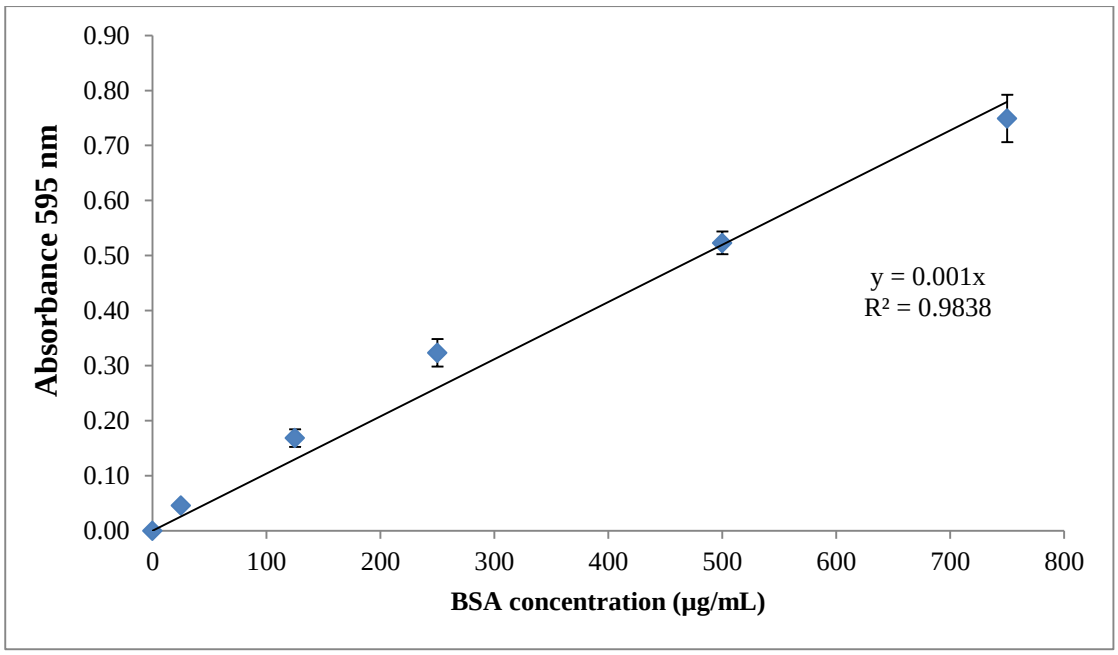

Figure 8 The Standard Curve of Bovine Serum Albumin (BSA)

2.2.4.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

SDS-PAGE maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH], and thus allows separation of proteins by their molecular weight. Protein samples become covered in the negatively charged SDS and move to the positively charged electrode through the polyacrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to their size.

The polyacrylamide gel was prepared according to the standard method described by Laemmli (51). The separating gel used for the fractionation of soluble proteins from mammalian cells contained 10% acrylamide. The equal amount of proteins in each sample were mixed with 5X sample buffer (0.125M Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 0.2M DTT, 0.02% bromophenol blue), boiled at 95^{o}C for 10 min before loading onto the gel. To estimate size of polypeptides, low molecular weight protein standard marker (Bio-Rad, USA) was used. Electrophoresis was performed in SDS electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3) at 90 Volts for 2 h. or until the tracking dye reached the bottom of the gel.

Next, proteins in the gel were transferred to a nitrocellulose membrane by Mini Trans-Blot® (Bio-Rad, USA) at 25 Volts for 16 h. The blot was then incubated sequentially with 5% skim milk in Tris-buffered saline solution (TBS) for 2 h at room temperature for blocking non-specific binding. Subsequently, the blot was incubated

with 0.5 µg/mL primary antibodies (anti-FLAG (M2) antibody (Sigma, USA), or polyclonal anti-ubiquitin antibody (kindly provided by Professor Arthur Haas, Louisiana State University, USA) in TBS containing 25 mg/mL BSA for 2 h at room temperature. Next, the blot was washed 5 times by TBS containing 0.15% Tween 20 (TBST) to remove excess antibodies. Later, the blot was incubated with secondary antibodies (goat anti-mouse/ or anti-rabbit IgG linked to peroxidase (Bio-Rad, USA)) at a 1:7500 dilution in 1% skim milk in TBST for 1 h at room temperature and washed again with the buffer 5 times. Finally, the specific band(s) were visualized by Western lightening Chemiluminescent HRP Substrate (PerkinElmer, USA), and captured on Kodak X-ray film.

2.3 BIOLOGICAL ASSAYS

2.3.1 *In vitro* Cell Migration Assay

The migration assay was performed as illustrated in **Figure 9** using polycarbonate 8 µm pore-sized Multiscreen MIC 96-well. MDA-MB231 transfectant cells ($2x10^4$ cells) in serum free DMEM medium were added to the upper wells of the filter plate. The culture medium containing 10% FBS were added to lower chambers. In addition, cells in duplicate wells without filter insert served as controls for cell proliferation and/or death during the incubation period. After 24 h of incubation at 37°C in 5% CO_2 incubator, the medium in the bottom of each well was carefully removed by aspirating. The adherent cells in both microplates were kept at -70˚C. Later, the cells in the bottom well were all labeled with fluorescent dye CyQuant (Molecular Probes, Invitrogen, USA) according to the manufacturer's protocol. Fluorescence was measured using Synergy™ HT Multi-Detection Microplate Reader

(Bio-TEK, USA) with the excitation and emission wavelength at 480 nm and 520 nm, respectively. Migration was calculated by dividing the relative fluorescence value of invading cells with that of total cells plated in triplicate wells without the filter inserts. Migration of the control was set at 100%. The experiments were repeated 3 times with at least triplicate per experiment. The percentage of inhibition was determined relative to control.

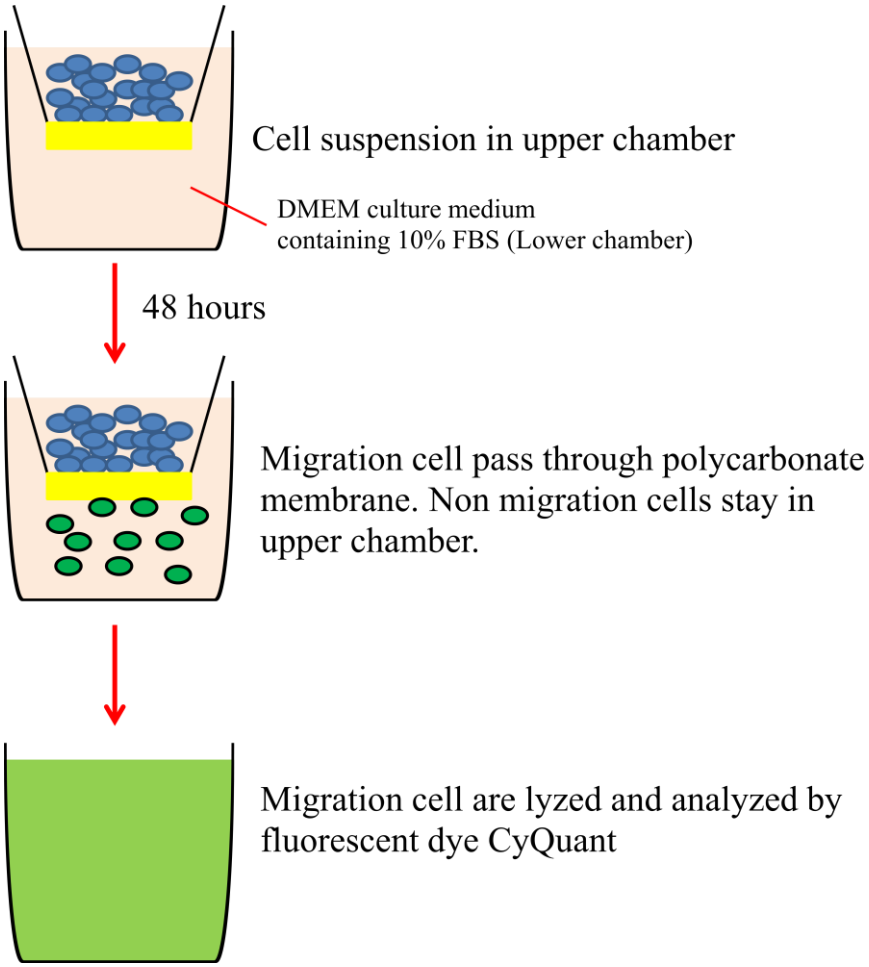

Figure 9 *In Vitro* Cell Migration Assay

2.3.2 *In vitro* Matrigel Invasion Assay

As shown in **Figure 10**, polycarbonate 8 μm pore-sized Multiscreen MIC 96-well was precoated with 50 μg/mL Matrigel (BD Bioscience, USA). MDA-MB231 transfectant cells ($2x10^4$ cells) in serum free medium were added to the upper wells of the filter plate in which the lower wells were loaded with culture medium containing 10% FBS. Cells in a duplicate 96 well plate without inserts were prepared as controls for cell proliferation and/or death during the assay period. After 48 h of incubation at 37°C in 5% CO_2 incubator, the medium in the bottom of each well was carefully removed by aspirating. The adherent cells were measured by staining with fluorescent dye CyQuant as previously described in the migration assay. Invasion is calculated by dividing the relative fluorescence value of invading cells with that of total cells plated in triplicate wells without Transwell inserts. Invasion of the control was set at 100%. The experiments were repeated 3 times with at least triplicate per experiment. The percentage of inhibition were further determined.

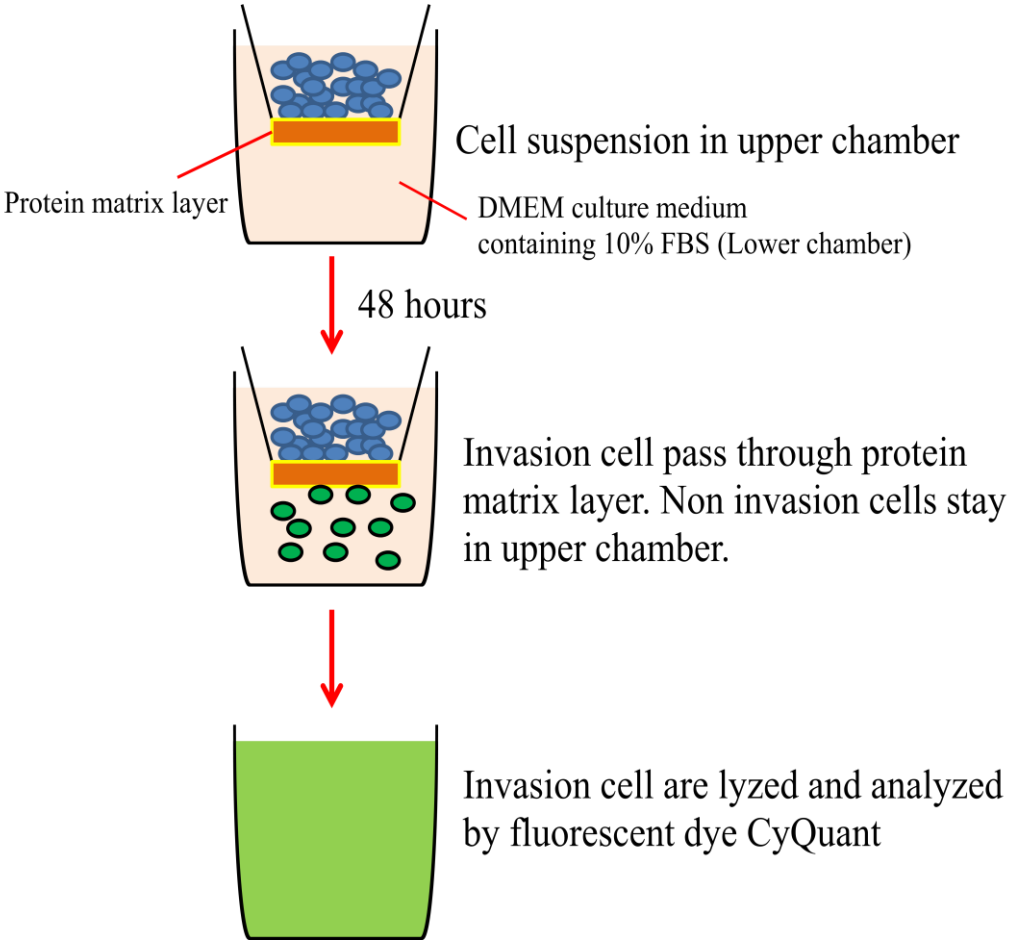

Figure 10 *In Vitro* Matrigel Invasion Assay

2.3.3 *In vitro* Cell-fibronectin Adhesion Assay

MDA-MB-231 transfectant cells were harvested using 2 mM EDTA in phosphate buffered saline (PBS), and resuspended in serum-free DMEM. Next, they were plated at a density of 2×10^4 cells/well and allowed to attach for 1 h at 37 °C on fibronectin-coated 96-wells. Following washing with PBS, the adherent cells were stained with 0.2% crystal violet in 10% ethanol. The excess stain was removed by gently washing three times with PBS. The attached cells were solubilized with a 1:1 mixture of 0.1 M NaH_2PO_4 (pH 4.5) and 50% ethanol. Cell attachments were determined by measurement of dye color at 550 nm on Synergy™ HT Multi-Detection Microplate Reader (Bio-TEK, USA). The experiments will be repeated 3 times with three to five replicates per experiment.

2.4 20S PROTEASOME ACTIVITY ASSAY

Cellular chymotrypsin-like activity of 20S proteasome was assayed in a 96-well plate according to the manufacturer's protocol (Millipore, USA). The assay is based on detection of the fluorophore 7-Amino-4-methylcoumarin (AMC) after cleavage from the labeled peptide substrate LLVY-AMC. MDA-MB-231 transfectant cells were harvested and homogenized in ice-cold mammalian protein extraction buffer (GE Healthcare Lifescience, USA) without addition of protease inhibitors. Then, total protein concentration was determined using Bio-Rad Protein Assay as described in section 2.2.4.2. Assay mixtures in a 96-well fluorometer plate were prepared according to **Table 1**. The assay buffer contained 25 mM HEPES pH 7.5, 0.5 mM EDTA, 0.05% NP-40 and 0.001% SDS. Fifty µg of lysate protein were added to assay mixture of test sample with a final volume of 90 µL. After pre-incubation for 5

min at 37˚C, the proteasome substrate were added into each reaction and further incubated for 2 h at 37˚C. Finally, fluorescence was measured using a fluorescent microplate reader Synergy™ HT Multi-Detection Microplate Reader (BioTEK, USA) with Ex: 380 nm and Em: 460 nm filters. The experiments were performed with at least triplicate per experiment.

Table 1 Assay Mixture for 20S Proteasome Activity Assay

Sample	**Assay Mixture (µL)**				
	10X Assay Buffer	**Proteasome Sample**	**DI H_2O**	**Proteasome substrate**	**Total Volume**
Buffer Blank	10	0	90	0	100
Substrate Blank	10	0	80	10	100
Test Sample	10	X	80-X	10	100

2.5 PROTEOMIC ANALYSIS

2.5.1 Two-Dimensional Gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a form of gel electrophoresis in which proteins are separated and identified in two dimensions oriented at right angles to each other. In this technique, proteins are separated by two different physical properties. The first dimension, isoelectric focusing (IEF), separates proteins on the basis of pI. The second dimension, SDS-PAGE, further separates the proteins by their size.

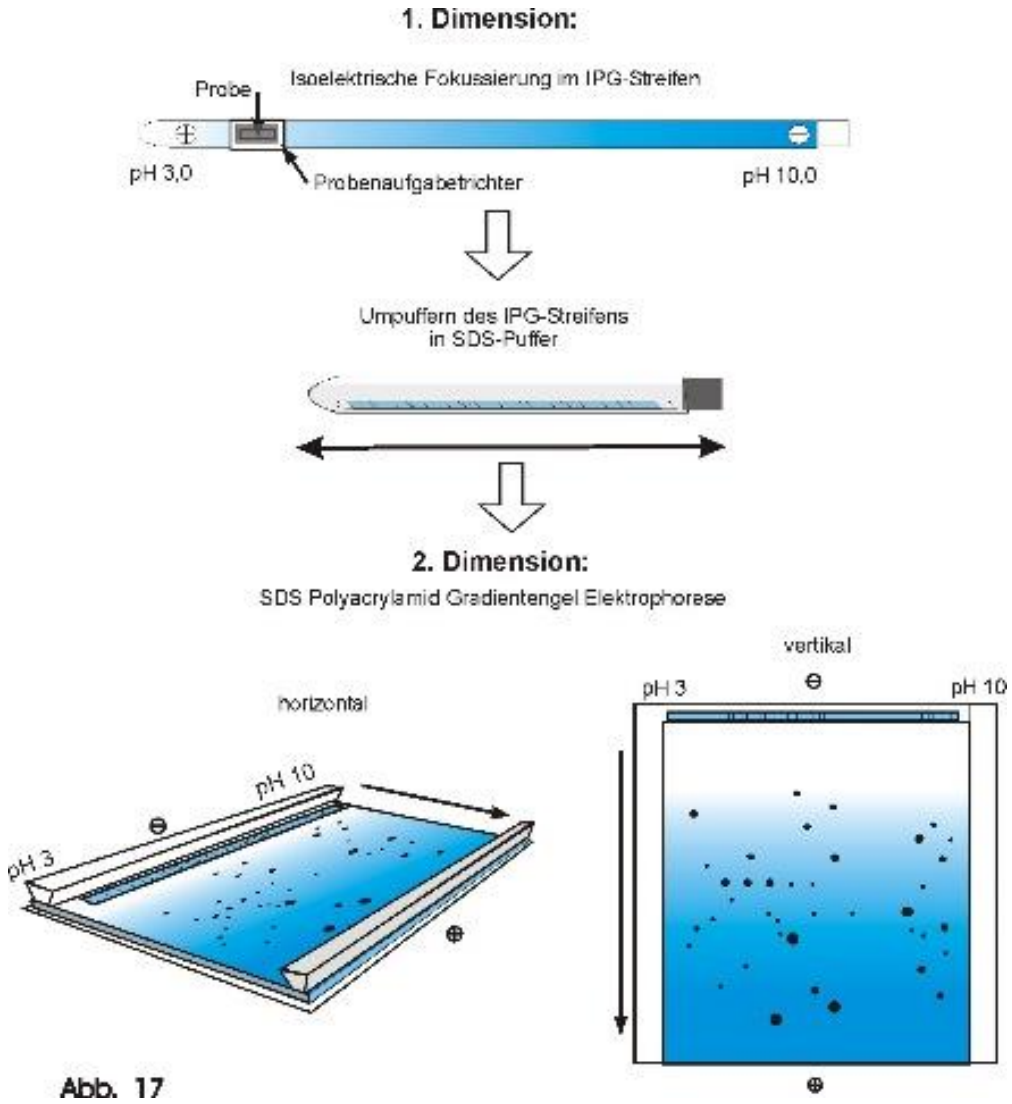

Figure 11 2D-Gel Electrophoresis Workflow

(http://www.electrophoresis-development-consulting.de/html/2delpho.html)

Mixture of total cell lysate of the MBA-MB-231 transfectants was separated by two-dimensional electrophoresis using Ettan IPGphor 3 platform (GE Healthcare, USA). The protein extracts were clean-up by acetone precipitation and resuspended in 50 µl of rehydration loading solution with IPG buffer (8M urea, 2% CHAPS, 60mM DTT, 0.5% IPG buffer, 0.002% bromophenol blue) for first dimension, isoelectric focusing (IEF). Next, the protein sample was loaded by rehydration procedure (125 uL) onto 7 cm Immobilized pH gradient (IPG) strips (pH 3-10). The isoelectric focusing was conducted by Ettan IPGphor 3 System using the following running conditions

Step 1: Step and Hold	300 V	0:30 h	200 Vh
Step 2: Gradient	1000 V	0:30 h	300 Vh
Step 3: Gradient	5000 V	1:20 h	4000 Vh
Step 4: Step and Hold	5000 V	0:25 h	2000 Vh
Total		2:45 h	6500 Vh

After IEF, the strips were subsequently equilibrated for 15 min with solution A (1 M Tris-HCl, pH 8.8, 6 M urea, 30 % glycerol, 2% SDS, 20 mM DDT) and then with solution B (1 M Tris-HCl, pH 8.8, 6 M urea, 30 % glycerol, 2% SDS, 240 mM iodoacetamide) for 15 min. In the second dimension the proteins will be separated in a 12 % polyacrylamide gel. Next, gels will be stained using Coomassie Brilliant Blue G250, followed by destaining solution (5% (v/v) acetic acid, 22.5% (v/v) methanol, 72.5% (v/v) in deionized water). Differences in the protein expression profile of the

2-D gels were identified by use of the image analysis software (ImageMaster 2D Platinum 7).

2.5.2 Shotgun proteomics

Shotgun proteomics refers to the use of bottom-up proteomics techniques in identifying proteins in complex mixtures using a combination electrophoresis and high performance liquid chromatography combined with mass spectrometry as illustrated in **Figure 12**. The most common method of shotgun proteomics starts with the proteins in the mixture being digested and the resulting peptides are separated by liquid chromatography. Then, tandem mass spectrometry is used to identify the peptides.

2.5.2.1 Prefractionation of Protein by SDS-PAGE

Proteins were fractionated on SDS-PAGE mini slab gel (8 x 9 x 0.1 cm, Hoefer miniVE, Amersham Biosciences, UK). The polyacrylamide gel was prepared according to the standard method described by Laemmli (51). The separating gel used for the fractionation of soluble proteins from mammalian cells contained 12.5% acrylamide. The equal amount of protein samples in 20μL were mixed with 5 μL of 5X sample buffer (0.125M Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 0.2M DTT, 0.02% bromophenol blue), boiled at 95^{o}C for 10 min before loading onto the 12.5% SDS-PAGE. To estimate size of polypeptides, low molecular weight protein standard marker (Amersham Biosciences, UK) was used. Electrophoresis was performed in SDS electrophoresis buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS)

until the tracking dye reached the bottom of the gel. After the electrophoresis finished, gels were silver stained according to Blum (52).

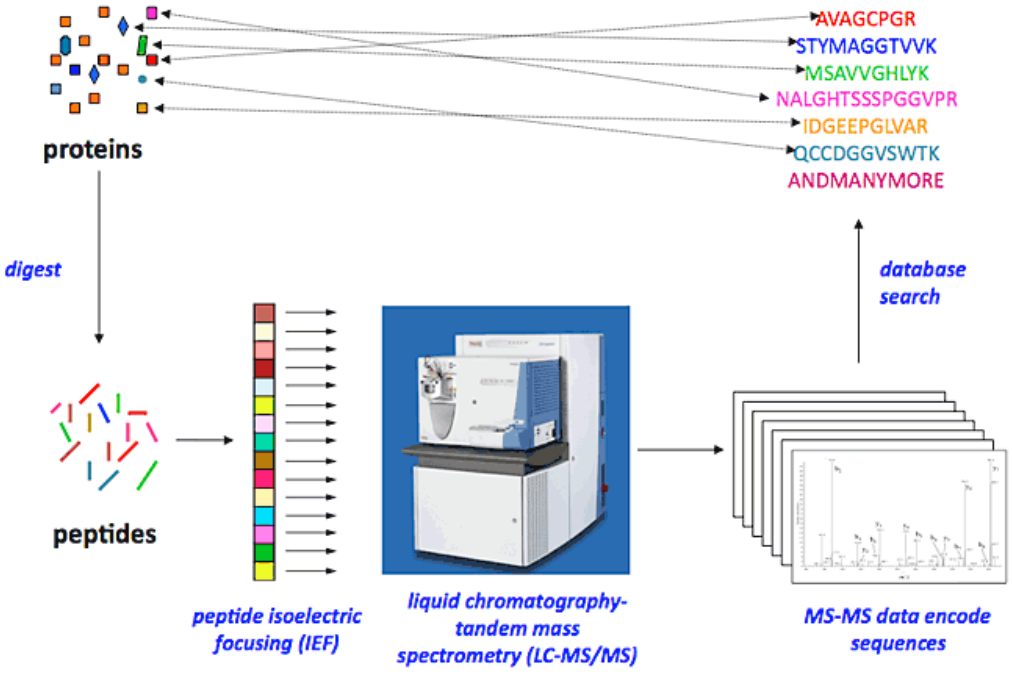

Figure 12 Shotgun Proteome Analysis Platforms

(http://www.vicc.org/jimayersinstitute/technologies/)

2.5.2.2 In-gel Digestion

After protein bands were excised, the gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10mM dithiotheritol (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 100mM 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 of trypsin in 50% ACN/10mM 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, and then the gels were incubated at room temperature for 10 min in a shaker. 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.

2.5.2.3 LC-MS Analysis

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 nano ACQUITY UPLC system. Four µL 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.

Mass lists in the form of Mascot generic files were created and used as the input for Mascot MS/MS Ions searches of the National Center for Biotechnology Information nonredundant (NCBI nr) database (www.matrixscience.com). Default search parameters used were the following: Enzyme = trypsin, max, missed cleavages =1; fixed modifications = carbamidomethyl (C); variable modifications = oxidation (M); peptide tolerance ±1.2 Da; MS/MS tolerance ±0.6 Da; peptide charge = 1+, 2+ and 3+; instrument = ESI-QUAD-TOF.

2.5.2.4 Proteins Quantitation and Identification

DeCyder MS Differential Analaysis software (DeCyderMS, GE Healthcare) was used for protein quantitation. Acquired LC-MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ions signal intensities in MS mode. The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software (Matrix Science, London, UK). The data was searched against the NCBI database for protein identification. Database interrogation was; taxonomy (Human or Eucaryote); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1 Da); fragment mass tolerance (±0.4 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages (51). Proteins

considered as identified proteins had at least two peptides with an individual mascot score corresponding to $p<0.05$ and $p<0.1$, respectively.

Data normalization and quantification of the changes in protein abundance between the control and treated samples were performed and visualized using *MultiExperiment Viewer* (Mev) software version 4.6.1 (53). Briefly, peptide intensities from the LC-MS analyses were transformed and normalized using a mean central tendency procedure. They performed statistical tests of variance of differences (ANOVA) for these data sets that statistically significant proteins ($p <0.05$).

2.6 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Reverse Transcription-PCR (RT-PCR) is a method used to amplify cDNA copies of RNA by generating large cDNA libraries from cellular mRNA. This method can measure the strength of gene expression when the amounts of available mRNA are limited and/or when the RNA of the interest is expressed at very low levels.

The first step of RT-PCR is enzymatic conversion of RNA to a single-stranded cDNA template. An oligodeoxynucleotide primer is hybridized to the mRNA and then extended by an RNA-dependent DNA polymerase to create a cDNA copy. Depending on the purpose of the experiment, the primers for first-stand cDNA synthesis can be specifically designed to hybridize to a particular target gene or it can bind randomly to all mRNAs. Amplification of the desired portion of target cDNA can be achieved in PCRs using forward and reverse oligonucleotide primers corresponding to specific sequence in particular cDNAs.

2.6.1 RNA Extraction

Total mRNA was extracted using Trizol reagent (Invitrogen, USA), according to the manufacturer's instruction. In brief, the cell monolayer was directly lysed in a culture dish by adding 1 mL of Trizol reagent to 35 mm diameter dish, and passed several times through a pipette. The cleared homogenate solution was transferred to a fresh tube. The homogenized cells were incubated for 5 min at RT to allow the complete dissociation of nucleoprotein complexes. Next, 0.2 mL of chloroform was added per 1 mL of Trizol reagent used in each tube. The tubes were then vigorously shaken for 15 sec and incubated at RT for 3 min. After centrifugation at 12,000xg for 15 min at 4 ˚C, the mixture was separated into a lower red, phenol-chloroform phase, an inter-phase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol, incubated at -20 ˚C for 10 min and centrifuged at 12,000xg for 10 min at 4 ˚C. After removing the supernatant, the RNA pellet was washed once with 75% ethanol, mixed by a brief vortex and centrifuged at 7,500xg for 5 min at 4 ˚C. At the end of the procedure, the RNA pellet was air dried for 5-10 min and then dissolved in diethylpyrocarbonate (DEPC)-treated water. The quantity and quality of total RNA were assessed by the ratio of OD_{260}/OD_{280}. The concentration of RNA was determined by GeneQuant pro (Amersham Biosciences, Germany). An OD of 1.0 at the wavelength of 260 nm corresponds to approximately 40 µg/mL. The obtained RNA was diluted 50 times in DEPC-treated water before measuring the absorbance. The concentration of RNA is calculated by using the formula below:

Concentration of RNA (µg/mL) = OD_{260} x 40 x dilution factor

The quality of the RNA is essential to overall success of the analysis. OD_{260} is frequently used to measure RNA concentration and OD_{280} is used to measure protein concentration. For further analysis, it is imperative that the RNA extract should have high purity with the ratio of OD_{260}/OD_{280} values 1.7 to 1.9. Smaller ratio usually indicates contamination of protein or organic chemicals.

2.6.2 cDNA Synthesis by Reverse Transcription

In reverse transcription reaction, 1.0 µg of total RNA was reverse-transcribed into cDNA by oligo-$(dT)_{18}$ primer and AMV reverse transcriptase using RevertAid™ First Stand cDNA synthesis kit (Fermentus, Germany) according to the manufacturer's instructions. Briefly, the 20 µL reaction mixture contained 1 µg of extracted RNA, 0.5 µg of oligo-$(dT)_{18}$, 1 mM dNTPs, 20 unit of ribonuclease inhibitor, 200 units of reverse transcriptase, and adjusted volume to 20 µL with DEPC-treated water. The reaction mixture was incubated at 70 ˚C for 5 min, 4 ˚C for 1 min, and then 42 ˚C for 60 min.

2.6.3 Quantitative Real Time Polymerase Chain Reaction (Q-PCR/qPCR)

Real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (Q-PCR/qPCR) or kinetic polymerase chain reaction, now becomes a routine laboratory PCR technique used to amplify and simultaneously quantify a targeted DNA molecules. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample. To

confirm the results from RT-PCR experiment, real time qPCR is used to examine the mRNA levels of target protein. For determination of target genes expression, the qPCR reactions were performed in an ABI 7500 Real-time PCR system (Applied Biosystems, USA) using Maxima^{TM} SYBR Green qPCR Master Mix (Fermentas, Germany). The sequence primers of target genes are shown in **Table 2**. Relative expression levels for targets genes were normalized to the expression of GAPDH by the $2^{-\Delta CT}$ method (54).

Table 2 Primer Sequences for qPCR

Primer Name	**Sequence**
Epithelial cell transforming 2	
ECT2: Forward	5'-ATTTTCATGTCGCCCGTTGT-3'
ECT2: Reverse	5'-CCCATGTGATGGACCAATGTC-3'
Rho GDP-dissociation inhibitor 2	
RhoGDI2: Forward	5' -TTTATGGTTGGCAGCTATG-3'
RhoGDI2: Reverse	5'-GAGGTGGTCTTGCTTGTC-3'
Proteasome subunit beta type 3	
PSMB3: Forward	5'-ATCTTTCCCATGGGTGACCG-3'
PSMB3: Reverse	5'-CTGCCGACCTTCCTTCAACT-3'
Proteasome subunit alpha type-1	
PSMA1: Forward	5'-ATACTTTCGGCAGCACCTCC-3'
PSMA1: Reverse	5'-AGACCAACTGTGGCTGAACC-3'

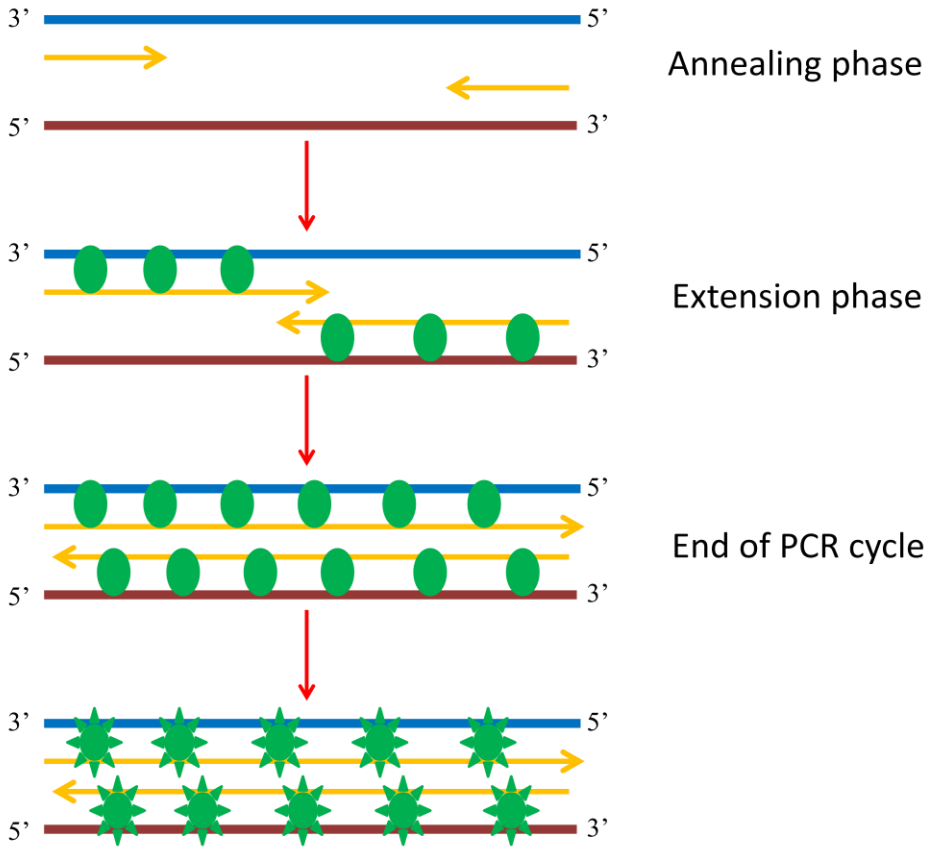

Figure 13 The Principle of SYBR Green Detection in Real-time PCR

The 25 µL reaction mixture contained 5 µL of cDNA (dilute 1:5). 1X Maxima^{TM} SYBR Green qPCR Master Mix, 10 nM ROX solution and 0.3 µM of primers. Each reaction mixture was then placed on an ABI 7500 machine. The temperature profile was as follows: initial denaturation at 95 ˚C for 10 min, followed by 45 cycles of the amplification process, which are denaturation at 95 ˚C for 15 sec, annealing and extension at 60 ˚C for 60 sec.

2.7 STATISTICAL ANALYSIS

All values were given as mean ± standard derivation (X ± SD) from at least triplicate samples of three independent experiments. Overall differences among the transfectant groups were determined using one-way analysis of variance (ANOVA), and differences between individual transfectant using the Student-t-test by SPSS 16.0 software package. *P* values < 0.05 are regarded as significant.