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Comparative Proteomics:
An Approach to Elucidating the
Function of a Novel Gene Called BRE

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1. Introduction

Proteomics was developed in the early 1990s to allow proteins expressed by cells and tissues to be systematically studied (Celis et al., 1999; Arrell et al., 2001). The word proteome was coined by Marc Wilkins et al (Wilkins et al, 1996) from the words "protein and genome". It is therefore defined as protein equivalent of the genome. Generally, unique spectrum of proteins is only synthesized by specific cell types, for example amylase is secreted by the parotid gland, insulin by the pancreas and thyroxin by thyroid follicles. Protein synthesis is a complicated process formed by the different combination and length of the 20 unique amino acids found in our body (Arnstein, 1965). For example, following the transcription of genes encoded in the DNA, the mRNAs translocate into the cytoplasm where they are translated into a specific type of protein by the ribosomes (Lengyel, 1966). This is then followed by post-translational modification of the peptide chain to configure the protein so that it becomes biologically active. Post-translational modifications of proteins involve glycosylation, alkylation, methylation and sulfation (Blundell et al., 1993, Fleischer, 1983). The co- and post-translational modifications allow the protein to be transported and secreted during cellular homeostasis (Finnerty et al., 1979; Mao et al., 2011). In this chapter, we have described the comparative 2-dimensional electrophoresis (2-DE) proteomics workflow for protein identification by mass spectometry. Comparative proteomics was used

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to identify proteins that were differentially expressed in the tissues after treatment with various small molecules and siRNAs.

2. Proteomics research and applications

Protein properties are diverse and complex. They are dynamically influenced by physiological change in their environment, such as hormones, factors present in inflammatory response and enzymes activated by the presence of drugs. Proteomics is founded on three basic procedures: (1) the isolation and separation of proteins from cells and tissues, (2) the identification of the proteins by mass spectrometry and (3) the resolution of analyzed protein peptides by bioinformatics. Advancement in proteomic technologies has allowed researchers to investigate the proteome of many diverse biological systems – allowing breakthroughs to be made in biomedical and biological sciences. Proteomics has also enabled the identification of important biomarkers of many human diseases and allows the discovery of novel targets for drugs. In this section, we will to discuss how proteomic technologies have been applied in biomedical sciences research and the limitations encountered.

2.1 History of protein research

Swedish biochemist Pehr Victor Edman first developed the technique called Edman Degradation which allowed the amino acid sequence in peptides to be elucidated (Edman, 1950). Determination of the protein structure could be performed under micro scale. Pehr Victor Edman also developed an instrument, the protein sequenator, which allowed the amino acids sequence to be determined following Edman degradation reaction (Edman and Begg, 1967). This sequenator was commercialized by the company Beckman. The discovery popularized the studying of protein chemistry. However, there are several disadvantages associated with this method. Firstly, the technique can only accurately determine amino acid sequences up to 50-60 residuals after using Edman reagent, phenyl isothiocyanate for degradation. Secondly, the peptide N-terminal, with NH_2-group, has to react with the Edman reagent. Thirdly, sequencing can only work on a single pure peptide and not a protein mixture. Finally, only the primary peptide structure can be determined but not information on the secondary structure, such as the position of disulfide bridge. Nevertheless, it has the advantage that only small quantity (10-100 pico-moles) of peptide is needed for the Edman reaction and can be performed directly from PVDF membranes. For its time, it was a pioneering and sophisticated method for studying protein chemistry, allowing the important amino acid sequence of hormones to be discovered (Niall et al., 1969 and Birr and Frank, 1975).

In the early 1970s, mass spectrometry was used to try and resolve all the peptide sequences derived from a protein mixture (Lucas et al., 1969; Morris et al, 1971). This early work has now developed leaps and bounds and protein mixtures can routinely be analyzed by computer aided high resolution mass spectrometry (MS). Consequently, John Fenn was awarded the 2002 Nobel Prize for his work in developing the electrospray ionization for mass spectrometry which provided a new platform for protein research (Fenn et al., 1989, 2002). The electrospray ionization mass spectrometer can rapidly, accurately and sensitively analyze peptide sequences from recombinant proteins, large biomolecules, protein mixture and body fluids (Chowdhury et al., 1990; Andersen et al., 1996; Bergquist et al., 2002). The parallel development of protein databases, search engines and new softwares has made it

now even easier to conduct proteomic studies. Protein databases are essential tools that allow the matching and identification of peptides from peak spectrums obtained from MS studies. In particular, the Protein Prospector (Chalkley et al., 2005) and Mascot (Perkins et al., 1999) databases are user-friendly and contain many years of interpreted MS data for protein identification.

2.2 New era in studying the protein profile

Protein chemistry has now shifted to studying the proteome which permits a better understanding of interaction between cells, hormones with cells and bioactive molecules with cells. Profiling of protein mixtures is still difficult, despite recent development in using a partial enzyme digestion strategy and advancement in instrumentation - such as electrospray ionization tandem (triple quadrupole) and mass spectrometry (ESI-MS/MS) (Ceglarek et al. 2009), quadrupole ion trap MS (Schwartz and Jardine, 1996) and Matrix-assisted laser desorption/ionization-time of flight mass spectrophotometer (Maldi-TOF MS) (Hillenkamp et al., 1991; Andersen et al., 1996). Studying the proteome also depends on the use of two dimensional electrophoresis (2-DE) (O'Farrell, 1975). This technique allows complex mixture of proteins found in cells to be separated into individual protein spots by isoelectrical focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteinase inhibitors are always added to protein lysates freshly prepared from cells or tissues to prevent protein degradation. Contaminants such as phospholipids, nucleic acid and ionic molecules are also present and can be removed by gel filtration, dialysis and protein precipitation. Although O'Farell improved the IEF procedure, he used non-equilibrium pH gradient electrophoresis which cannot be reproducible from batch to batch - as the pH gradient is difficult to maintain during IEF. However, Bjellqvist et al. (1982) developed the immobilized pH gradients (IPG) method which replaced the use of the carrier-ampholyte. Development of the IPG strip was a milestone in proteomics and is now widely used in resolving individual proteins from complex protein mixtures (Weiss and Görg, 2009). In the IPG strip, proteins migrate under a high electrical field (up to 5000V) but always stop at same pI point. If several protein spots co- exist within the same pI, then a wider range of IPG strip could be flexibly used. SDS-PAGE is used to separate the protein spots according to their molecular weight. The limitation with this method is that it can only resolve proteins ranging from 120 kDa to 10 kDa. The protein spots resolved in the 2-DE gel need to be stained before it can be analyzed. Gels are most commonly stained with coomassie blue because it is inexpensive, and compatible for MS analysis. However, the sensitivity of this staining method is limited and cannot stain-up protein spots lower than 30 ηg. Silver staining is also another method widely used for revealing the resolved protein spots in the gel and only need 1 ηg of protein. Fluorescent dyes (CyDyes) have now been developed to label protein samples for Difference Gel Electrophoresis (DIGE). The DIGE technique is very sensitive, with protein detection range down to 125 pg per spot, giving it high precision in terms of protein quantification and use in comparative proteomics (Conrotto and Souchelnytskyi, 2008; Larbi and Jefferies, 2009). 2-DE/MS is now a well-established technique for large-scale protein expression studies. However, there are drawbacks with the method which hold it back from being developed for clinical diagnosis. Drawbacks such as the high abundance of plasma and albumin present in biofluids which interfere with detection of lower abundant proteins. Resolving hydrophobic, very acidic and basic proteins is also a major deficiency with the 2-DE/MS technique (Altland et al., 1988; Görg et al., 2009).

3. Breakthroughs in proteomics

Proteins are separated according to their isoelectrical points and molecular weights by 2-DE. In addition, their m/s ratio and peptide sequences in MS can resolve up to 2,000-4,000 single protein spots at a time (Görg et al., 2004). Moreover, proteins that cause abbreviated changes in normal tissues may be identified and use as potential biomarkers in medical diagnosis. This is especially important in oncology where early detection of the cancer could be properly treated and not metastasize. In the last decade, advancement in 2-DE, mass spectrometry and bioinformatics has allowed potential cancer biomarkers to be identified in serum and biofluids in the blood (Voss, et al., 2001; Gioia et al., 2011), colon (McKerrow et al., 2000), breast (Sauter et al., 2002; Lau et al., 2007; Galvão et al., 2011), ovaries (Zhang et al., 2004; Tung et al., 2008) and prostate (Ornstein et al., 2004; Ornstein and Tyson, 2006). However, the 2-DE technique still has its limitation – where proteins with extreme isoelectric point and molecular mass are not resolvable and identified. Also, it is very difficult to resolve membrane proteins and non-water soluble proteins by 2-DE. Another approach is to use non-gel based proteomic techniques (for example, ionic exchange affinity, reverse-phase and liquid chromatography) followed by MS/MS provide a novel platform for identifying proteins and therefore it can resolve the disadvantage of 2-DE technique. Now, the development of laser capture micro-dissection and MALDI-MS has allowed proteomics to be performed on a specific cell population isolated from heterogeneous tissues (Marko-Varga, 2003). It is possible to surgically isolate cancer tissue from normal tissues in histological sections of biopsies for proteomic analysis. This will accelerate the discovery of cancer biomarkers as the laser capture micro-dissection will remove "background noise" generated by normal tissues.

4. Comparative proteomics

Comparative Proteomics is the identification of the differentially expressed proteins from comparison of two or more 2-DE protein profiles, for example, isolated from cells that were treated and untreated with a drug. This method allows proteins that are differentially expressed to be identified and quantified. It is a very powerful technique for identifying the molecular targets of drugs and understanding the function of novel genes. The comparative proteomic technique is schematically summarized in Figure 1. Basically, it involves image analysis of 2-DE by matching different sets of gels together; identifying and isolating of proteins which are differentially expressed; mass spectrometry and bioinformatics. The proteome of a wide variety of biological systems can be investigated that includes cells, tissues, organs, fractionated cell lysates, and immuno-precipitated cell lysates. Since the technique only requires micrograms of materials to create a complex protein profile, the proteomes of bacteria, yeast and insect have also been investigated (Chen and Snyder, 2010; Han et al., 2011; Novak et al., 2011; Sirot et al., 2011).

5. As an example of the usefulness of comparative proteomics in identifying gene function, a gene called BRE which has anti-apoptotic properties, was analyzed

We have been interested in genes that are responsive to DNA damage (Li et al., 1995; Dong et al., 2003), and identified a novel human gene which we named BRE in this context. The
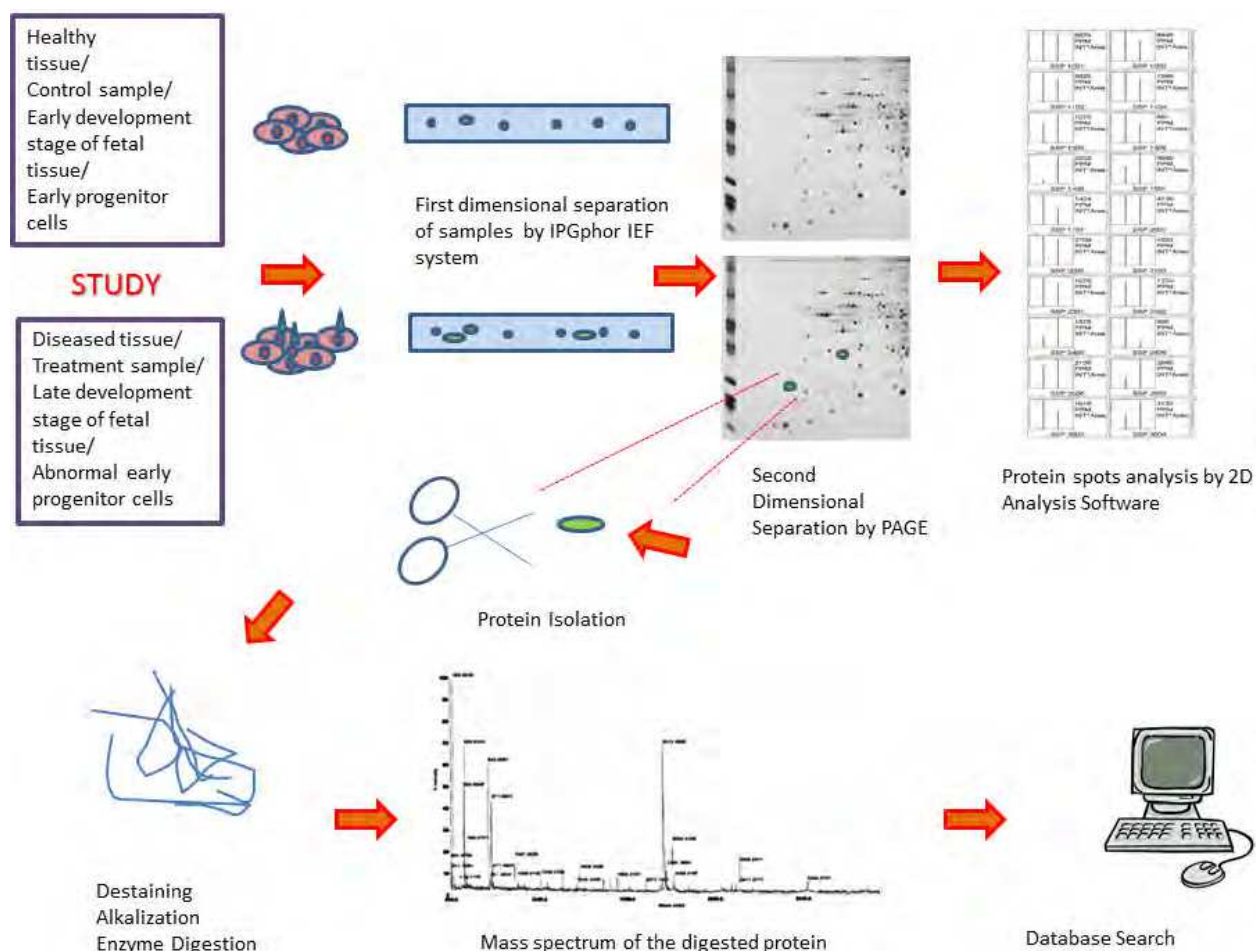
Fig. 1. The principle and workflow involved in comparative proteomics.

gene is highly Expressed in Brain and Reproductive organs and that is why we named it, BRE. The gene is down-regulated after treatment of cells with DNA damaging agents such as ultraviolet light (UV), 4-nitroquinoline-1-oxide and all-trans retinoic acid (Li et al., 1995). The BRE gene encodes a 1.7-1.9 kb mRNA which give rise to a protein with 383 amino acid residues and a molecular weight of 44 kDa. Using the yeast two-hybrid assay, it was reported that BRE interacts with the juxtamembrane (JM) region of p55-TNFR, but has no affinity for the p75-TNFR, Fas or p75-NGFR of the TNFR family (Gu et al., 1998). Meanwhile, over-expression of BRE in the human 293 embryonic kidney cells that was treated with TNF-α could inhibit the activation of the transcriptional factor NF-κB (Gu et al., 1998). Since NF-κB is known to induce the survival pathway associated with TNF receptor, it is likely that BRE can modulate the cell death process. The expression of the BRE gene has been investigated in various biological models including adrenal glands (Miao et al., 2001), testis (Miao et al., 2005) and hepatocellular carcinoma cells (Chan et al., 2008), but the function of BRE has still not been clarified - the protein structure of BRE do not have identifiable functional domain. It has been suggested the BRE contained 2 ubiquitin-conjugating enzyme family-like regions (Hu et al., 2011). However, these regions lacked the critical Cys residues required for ubiquitination but retain the ability to bind ubiquitin. The multifunctional nature of BRE and the lack of positive identifiable functional domains on BRE, make it an ideal candidate for study using proteomics. We therefore used comparative proteomics to examine the function of this novel gene in different cell types and also in vivo.

5.1 Materials and methods
5.1.1 Tissue cultures
All of the cell cultures were maintained at 37°C and 5% CO_2 in a humidified cultured chamber. C2C12 myoblasts (ATCC) and D122 Lewis lung carcinoma cells (gift from Lea Eisenbach) were cultured in DMEM medium supplemented with 10% FBS and penicillin/streptomycin. Two stably transfected cell lines were produced from D122 using a pcDNA3.1 expression vector. D122v3B harbor the empty vector, while D122αa4 cells over-express the full length *BRE* (Chan et al., 2005). D122v3B and D122αa4 were maintained in DMEM plus 10% FBS and 400 mg/mL of G418 (Invitrogen), Immortalized human esophageal epithelial (SHEE) cell line and the malignantly transformed esophageal carcinoma cell line (SHEEC) were cultured in DMEM medium plus F-12 Nutrient Mixture (1:1) supplemented with 10% FBS (GibcoBRL) and penicillin/streptomycin (Shen et al., 2000). Chang cells (ATCC, CCL-13) were cultured in Minimum Essential Medium Eagle plus 10% FBS.

5.1.2 Transgenic mice
The transgenic mice were generated carrying the full-length BRE gene and the transthyretin (TTR) promoter. The TTR promoter is specifically expressed in hepatocytes in the liver (Ching et al, 2001). All mice were maintained in the Laboratory Animal Services Centre, Chinese University of Hong Kong. Ethical approval has been obtained from the animal ethics committee, Chinese University of Hong Kong before performing the animal experiments.

5.1.3 Subcellular fractioning of soluble proteins
SHEE and SHEEC cells were extracted in lysis buffer (8M Urea, 2M Thiourea, 2% CHAPS, 0.01% TBP, 0.01% NP-40) containing protease inhibitors (GE Healthcare). After extraction, the lysates were incubated on ice for 30 min and then centrifuged at 8000 rpm for 15 min to remove all cell debris. The fractions (cytosol, membrane, and nucleoplasm) were obtained using a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) following instructions provided by the manufacturer. The total protein concentration for each fraction was determined using a Bio-Rad Protein Assay kit (Bio-Rad, Richmond).

5.1.4 BRE gene silencing analysis
Two BRE-specific siRNAs were designed corresponding to 5′-TCTGGCTGCACATCATTGA-3′ (nucleotides 124–142, nucleotide position number 1 being the start of the initiation codon), and 5′-CTGGACTGGTGAATTTTCA-3′ (nucleotides 491–509). siRNA sequence 5′-AAGCCUCGAAAUAUCUCCU-dTT-3′ with no known mRNA targets was used as a control.

5.1.5 Semi-quantitative RT-PCR analysis
The total RNA was isolated and purified by using TRIzol solution (Invitrogen Corporation, United States). 1 μg of the total RNA was used for reverse-transcription to synthesize the complementary DNA (cDNA) according to the procedures of ImProm-II™ Reverse Transcription System. cDNA was used as the template for PCR amplification. 20 μl of PCR mixture containing 1 μl of cDNA, 2.5 μl of PCR 10X buffer (Bio-firm, Hong Kong), 0.75 μl of magnesium chloride solution (25 mM, Bio-firm, Hong Kong), 1 μl of dNTP mix (10 mM,

Promega Corporation, United States), 1 μl of forward primer, 1 μl of reverse primer, 0.25 μl of Taq polymerase (Bio-firm, Hong Kong) and DEPC-treated water in a PCR microcentrifuge tube was placed into the thermal cycler for PCR amplification. All of the primers used in this study were manufactured and desalted by Invitrogen Corporation. The primers' sequences and the annealing temperature and duration shown in Table 1 were designed with Primer3

Primers	Sequences	Annealing temp & duration
mouse β-actin	Forward: 5'-TGAGACCTTCAACACCCCAG-3' and	59 °C , 45s
	Reverse: 5'-TTCATGAGGTAGTCTGTCAGGTCC-3'	55 °C , 60s
	or	
	forward: 5'-TGAGACCTTCAACACCCCAG-3'	
	reverse: 5'-TTCATGAGGTAGTCT GTCAGGTCC-3'	
mouse BRE	Forward: 5'-CTAGTCGCCGGTTACTGA-3'	56 °C, 45s
	Reverse: 5'-TTCATGAGGTAGTCTGTCA-3'	
	or	55 °C, 60s
	Forward: 5'-CCACATTCCCACATACCTTCTC-3'	
	Reverse: 5' –GCCATTTCATTTCCATCCCATC-3'	
mouse Mdm4	Forward: 5'-CTCCAAGCAAGAGGTACTG-3'	54 °C, 60s
	Reverse: 5'-AATGACCTGGTCCTCCTAG-3'	
Mouse Akt-3	Forward: 5'- CTGGCACCAGAGGTATTAGA-3'	56 °C, 60s
	Reverse: 5'-AGGAGAACTGAGGGAAGTGT-3'	
Mouse 26S Proteasome	Forward: 5'-TGATCTGTAACCTGGCCTAC-3'	57 °C, 60s
	Reverse: 5'-GTTACCCTCAGTGTCTTGGA-	
mouse Prohibitin	Forward: 5'-TGAGTGATGACCTCACAGA-3	54 °C, 45s
	Reverse: 5'-CAGTCTGCATAGGCACTTG-3'	
mouse p53	Forward:5'-ACTCTCCTCCCCTCAATAAG-3'	54 °C, 60s
	Reverse: 5'-CTGGAGTCTTCCAGTGTGAT-3'	
human β-actin	Forward: 5'-ATGGATGATGATATCGCCGCG-3'	55 °C, 45s
	Reverse: 5'-CTCCATGTCGTCCCAGTTG GT-3'	
human BRE	Forward: 5'-ATCTTGCCTCCTGGAATCCT-3'	57 °C, 60s
	Reverse: 5'-CACGTACTGCACCTTGTTGG-3'	
human Prohibitin	Forward: 5'- CGGAG AGGACTATGATGAGC-3'	57 °C, 60s
	Reverse: 5'- GGTAGGTGATGTTCCGAGAG-3'	
human cyclin A	Forward: 5'-TCCTGTCTTCCATGTCAGTG-3'	57 °C,60s
	Reverse: 5'- TAGGTCTGGTGAAGGTCCAT-3'	
Human TNF-R1	Forward: 5'- ACCAAGTGCCACAAAGGAACC -3'	56 °C, 60s
	Reverse: 5'-TACACACGGTGTTCTGTTTCTCC -3'	
human p53	Forward: 5'-GCCTGACTCAGACTGACATT-3'	54 °C, 60s
	Reverse 5'-GACAGCTTCCCTGGTTAGTA-3'	
mouse TUSC4	Forward: 5'-CTGGTATCC ATCCTCCAGTA-3'	53 °C, 60s
	Reverse: 5'-GTCTTGCAGCAGATCTCATC-3'	
mouse ENO1	Forward: 5'-CTACGAGGCCCTCTAAGAACTCC-3'	58 °C, 60s
	Reverse: 5'-TCCTTCCCGTACTTCTCCTT-3'	
mouse DPF2	Forward: 5'-TCCTTGGCGAGC AATACTAC-3'	53 °C, 60s
	Reverse: 5'-GCTGCCATCCTGAGAGATAA -3'	
mouse HSPA7	Forward: 5'-GCAGTCGGATATGAAGCACT-3'	58 °C, 60s
	Reverse: 5'-CTCCTCCCAAGTGGGTATCT-3'	
mouse HSPA2	Forward: 5'-GACGAATGTCAGGAGGTGAT-3'	58 °C, 60s
	Reverse: 5'-CTAAGTTGTTGCACCTCTCC-3'	

Table 1. Primers used in the study.

software (version 0.4.0, Rozen and Skaletsky; http://frodo.wi.mit.edu). The PCR mixtures were reacted in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) set under the following amplification conditions: initial denaturation at 95°C for 2 min, followed by a total of 35 cycles of denaturation at 95°C for 1 min, annealing at different temperature according to the primer' conditions as shown in Table 1 and extension at 72°C for 1 min. An additional 7 min extension step at 72°C was performed at the end of the last cycle. After the electrophoresis, the PCR products were analyzed on a 1.5% agarose gel with ethidium bromide staining, the intensities of the PCR products were visualized and determined using the GelDoc-It imaging system (UVP, BioImaging System, USA). β-actin was used as a house keeping gene for internal control and normalization. The experiments were repeated three times.

5.1.6 Western blot analysis

Control and treated cells were lysed in 200 µl of lysis buffer (50 mM NaCl, 20 mM Tris, pH 7.6, 1% NP-40, 1 X protease inhibitor mixture) for 60 min. The lysates were cleared by centrifugation at 16 000×g at 4 °C for 10 min. Crude protein concentration was measured by using a protein assay kit (Bio-Rad). 30 to 50 µg of total protein lysate were resolved on 10 to 12% SDS-PAGE, with Rainbow molecular weight markers and electroblotted onto Hybond NC membranes (GE Healthcare). The blots were incubated with Akt-3 (1:100, sc-11521 Santa Cruz Biotechnology), Bre (1:500 to 1000, Chan et al,. 2008), mdmX (1:100, sc-14738, Santa Cruz Biotechnology), prohibitin (1:000, sc-18196, Santa Cruz Biotechnology),p53 (1:000, sc-6243, Santa Cruz Biotechnology) or β-tubulin (1:1000 to 1500, Zymed Laboratories), α-tubulin (1:1500, Zymed Laboratories), cyclin A (1:1000, sc-11521, Santa Cruz Biotechnology), prohibitin (1:600, sc-18196, Santa Cruz Biotechnology), TNF-R1 (1:800, sc-8436, Santa Cruz Biotechnology), CDK2 (M2) (1:800, sc-163 Santa Cruz Biotechnology). Bound antibodies were detected using the appropriate horseradish peroxidase-conjugated secondary antibodies (Southern biotechnology), followed by development with an ECL Western blotting Detection kit (GE Healthcare). The blots were analyzed using Quantity One software (Bio-Rad) and the intensity of the bands produced for each antibody was normalized against the tubulin band (internal control) produced from each sample. Three replicates of each sample were studied.

5.1.7 In situ hybridization

All of the procedures performed were according to Lee et al. (2001). The liver samples were fixed in 4% paraformaldehyde (w/v, Sigma, United States) for 24 hrs.   The fixed samples were washed in Dulbecco's Phosphate Buffered Saline (DPBS, Invitrogen Corporation, United States) for 15 min with three changes. The samples were then dehydrated, cleared and embedded in paraffin wax. Finally, the specimens were sectioned at 7 µm and mounted onto TESPA treated slides. The riboprobe was prepared from pGEM-T plasmid containing 1,205 bp encoding BRE sequence. The plasmid cDNA was linearized by EcoRI and in-vitro transcribed to generate digoxigenin (DIG)-labeled sense and antisense BRE riboprobe using a DIG RNA labeling kit (Roche Applied Science, United States). After dewaxing the paraffin sections, the specimens were rehydrated and equilibrated in DPBS for 10 min. The sections were digested with 10 µg/ml of proteinase K (Fermentas Life Science, Canada) for 7 min and post-fixed in 2% paraformaldehyde for 5 min. After washing in DPBS for 10 minutes twice, the samples were incubated in pre-hybridization buffer (2X SSC, 1X Denhardt's reagent, 5mM EDTA , 0.1% sodium dodecyl sulfate, 10X Dextran sulfate (Chemicon, United

States), 50 µg/ml salmon sperm DNA and 50% formamide) for 2 hrs. The samples were then added and hybridized in 0.5 µg/ml of DIG-labeled antisense riboprobe. The sense probe was used as a negative control. The hybridization temperature was 55°C and the incubation time was 16 hrs. Following hybridization, the samples were washed in 2X SSC at 42°C for 20 mins with two changes, 0.1% SDS (w/v) in 0.2X SSC buffer for 15 min and then 0.2X SSC buffer for 10 mins. The alkaline phosphatase-conjugated digoxigenin antibody (1:50, Roche Applied Science, United States) was added to the specimens for 2 hrs and then washed in DPBS for 10 min with four changes. Nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP, Roche Applied Science, United States) were used as the color substrates. After color development, the sections were mounted in 50% glycerol (v/v, USB, United States). The experiment was performed in triplicates.

5.1.8 BrdU (Bromodeoxyuridine) labeling assay

Chang liver cells were cultured in 8-well glass slide (Nalge Nunc international, Naperville) with Minimum Essential Medium Eagle plus 10% FBS. After 80% confluent, the cultures were transfected with Ctl-siRNA or BRE-siRNA respectively according to maufacturers' instructions. Forty-eight hours after transfection, BrdU was added into the cultures to a final concentration of 20 µM and incubated at 37°C for 4 hrs. The treated cultures were then fixed with 2% paraformaldehyde for 24 hr. The fixed cultures were processed for immunohistochemistry by using mouse BrdU antibodies (1:1000, Sigma-Aldrich, United States). The BrdU positive and negative cells were counted and analysed by Spot Digital Camera & Carl Zeiss Microscope Axiophot 2 Integrated Biological Imaging System.

5.1.9 First dimensional separation of samples – Isoelectric focusing

The cell lysate for the first DE was performed on an IPGphor IEF system using 11-cm long IPG electrode strip with 4-7 pH gradient (Amersham Biosciences, United Kingdom) and an Ettan IPGphor Strip Holder (Amersham Biosciences, United Kingdom). 150 µg of protein was applied for each IPG strip. The total volume of protein sample and rehydration buffer (8M Urea, 2% CHAPS (w/v), 1% IPG buffer (v/v, Amersham Biosciences, United Kingdom), 40 mM DTT loaded onto the strip holder was 210 µl. 1ml of IPG Cover Fluid (Amersham Biosciences, United Kingdom) was applied to each strip so as to minimize evaporation and urea crystallization. The rehydration step was done under voltage and followed by a separation process. The electrophoresis condition for step 1 was 30 V for 13 hrs; step 2 was 500 V for 1 hr; step 3 was 2000 V for 1 hr and step 4 was 5000 V for 20 hrs. The program was stopped when the total volt-hours reached 40000.

5.1.10 Second dimensional separation – Sodium dodecyl sulphate polyacrylamide-gel

After first DE was completed, the IPG strips were removed from the strip holders. Each strip was then treated with 1% DTT in 6.5 ml of equilibration buffer (50 mM Tris, 6M of urea, 30% glycerol, 2% SDS, 0.1% bromophenol blue) for 30 min. The strips were further treated with 1% iodoacetamide (IAA, w/v, Sigma-Aldrich, United States) dissolved in the 6.5 ml of the same equilibration buffer. The strips were treated in the solution for 30 min. The equilibrated strips were then loaded on the 12% SDS-acrylamide separating gels. The 2-DE was performed in an ISO-DALT apparatus (Hoefer Scientific Instruments). Prestained protein molecular weight marker (Fermentas Life Science, Canada) with the range of 20 to 120 kDa was used to determine the sizes of the proteins on the gel.

5.1.11 Gel to gel matching

The gels were stained and scanned by using a GS 800 Densitometer (Bio-Rad Laboratories, United States) and images were captured for further analysis. The protein spots on the gel were analyzed by the discovery series, PDQuest 2D Analysis Software (Bio-Rad Laboratories, United States) version 7.13 PC. The experiment was performed in triplicate.

5.1.12 Protein identification by mass fingerprinting

All protein spots of interest were isolated from the gel and processed for destaining. The gel pieces were first washed in MilliQ water, immersed in 200 µl of destaining solution (15 mM potassium ferricyanide and 50 mM sodium thiosulphate) and then incubated at room temperature until they turned into colorless. Each gel piece was then washed with 400 µl of MilliQ water for 15 min, three times. The destained gel pieces were equilibrated in 200 µl of 10 mM ammonium bicarbonate/50% acetonitrile each for about 15 min. The solution was discarded and the equilibrated gel pieces were dehydrated by incubating in 200 µl of acetonitrile for 15 min. The solution was then poured off and the spots were dried in an incubator at 30ºC for 5 min. Fifteen µg/ml trypsin working solution in 40 mM ammonium bicarbonate/50% acetonitrile (v/v) was used for in-gel digestion. Twelve µl of the working solution was added to each gel sample. The samples were then incubated at 35ºC for 16 hrs. After trypsinization, 3 µl of extraction solution (50% acetonitrile (v/v) and 5% trifluoroacetic acid (Fluka Chemika, Switzerland) were added to each gel piece to stop the reaction. They are then centrifuged at 3,000 rpm for 2 min at room temperature. Three µl of reaction mixture from each sample was mixed with α-cyano-4-hydroxycinnamic acid matrix and then spotted onto a sample plate (Applied Biosystems, United States) for the MALDI-TOF mass spectroscopy. The mass spectrums generated were analyzed using the software Data Explorer Version 4.0.0.0 (Applied Biosystems, United States) and by mass fingerprinting search using the search engine provided by Protein prospector (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). To determine the significance of variance in the experiments, data were analyzed using the two-tailed, paired student's t-test. P<0.05 was considered to be statistically significant. All statistical analysis was performed using the SPSS software.

5.2 Results and discussions of the comparative proteomic analysis of BRE
5.2.1 Comparative proteomic analysis reveals BRE regulates prohibitin and p53 expression

BRE gene encodes a highly conserved stress-modulating protein. To gain further insight into the function of this gene, we used comparative proteomics to investigate the protein profiles of C2C12 and D122 cells resulting from small interfering RNA (siRNA)-mediated silencing as well as overexpression of BRE. It was found that silencing BRE expression in C2C12 cells would up-regulate Akt-3 and carbonic anhydrase III expression. In contrast, 26S proteasome regulatory subunit S14 and prohibitin expressions were down-regulated as shown in Figures 2 (2-DE gel) and 3 (semiquantitative RT-PCR and Western blot analyses). It has been reported that prohibitin is normally expressed in different cellular compartments involved in regulating cell proliferation, mitochondrial activities and protein processing (Mishra, 2010). Prohibitin can apparently directly interact with p53 in response to stress (Fusaro et al., 2003; Joshi et al., 2007). We established that cell proliferation was significantly increased after silencing BRE expression and this was accompanied by a reduction in p53 and
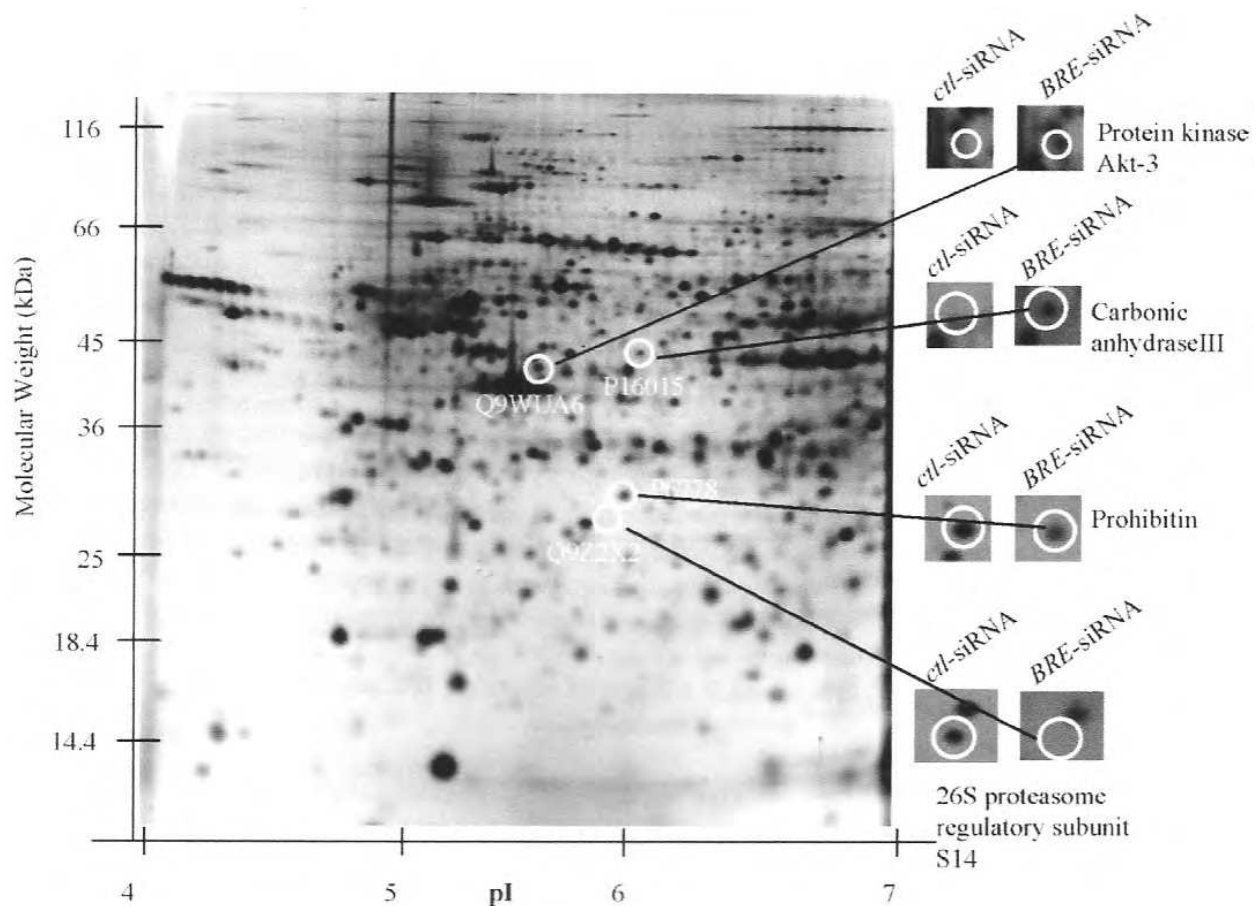
Fig. 2. Representative 2-DE gel of protein extracts from C2C12 cells that had been transfected with CTL- or BRE-siRNAs. Four differentially expressed proteins were identified (Swiss-Prot accession number provided). Silencing BRE expression up-regulated protein spots Q9WUA6 and P16015, but P6778 and Q9Z2X2 were down-regulated. pI 4–7 (x-axis) and MW in kDa (y-axis) (Tang et al., 2006).
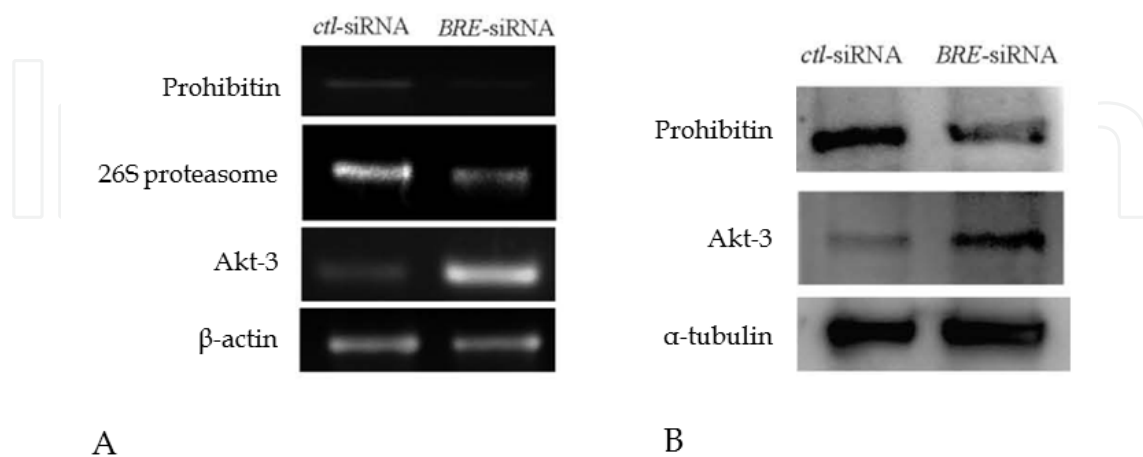

A   B

Fig. 3. Semiquantitative RT-PCR (A) and Western blots (B) analyses confirming the comparative proteomic results that silencing *BRE*, down-regulated p*rohibitin* and *26S proteasome regulatory subunit S14* expression, while *Akt-3* expression was up-regulated. *β-actin* and α-tubulin serve as internal controls (Tang et al., 2006).

prohibitin expression. We also identified Akt-3 that was affected by BRE silencing which suggests BRE might be involved in the P13/AKT signaling pathway (Madhunapantula et al., 2009). We observed that cell proliferation was suppressed when BRE was overexpressed in the D122αa4 cell line as shown in Figure 4. This was accompanied by an increase in p53 and prohibitin expression as shown in Figure 5. It has been reported that in the nucleus BRE is
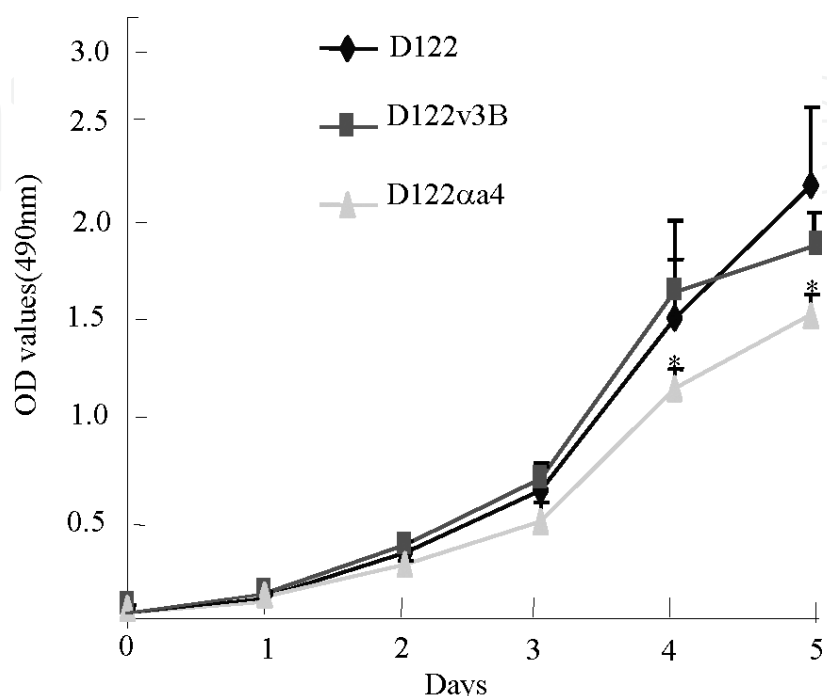
Fig. 4. MTT assay of D122, D122v3B and D122αa4 cell lines. The chart shows *BRE* overexpression in D122αa4 inhibited cell proliferation. Values = means $\pm$SEM, *P*, ≤0.01, * D122αa4 significantly different from D122 and D122v3B (Tang et al., 2006).
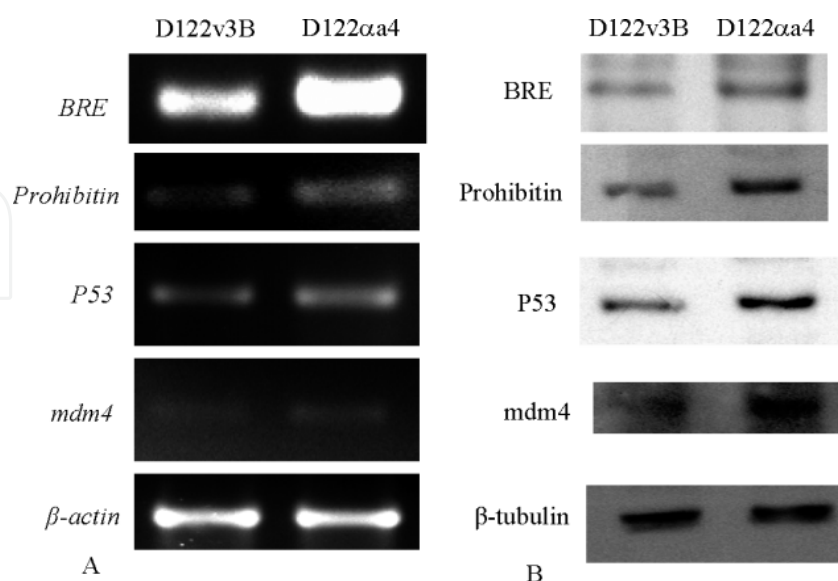
Fig. 5. Semiquantitative RT-PCR (A) and Western blot (B) showing that D122αa4 cells overexpressed *prohibitin*, *p53* and *mdm4*. β-*actin* and α-tubulin serve as internal controls (Tang et al., 2006) .

one of the components of BRCA1 A complex that is essential for tumor suppression (Harris and Khanna, 2011). BRE peptide has an ubiquitin E2 variant domain which has been determined to bind ubiquitin in co-immunoprecipitation experiments (Hu et al., 2011; Li et al., 2004). Coincidently, a 26S proteasome regulatory subunit S14 was one of the proteins found to be down-regulated by BRE over-expression. It is now known that the ubiquitin-proteasome pathway plays an important role in regulating the proteolytic processes that occur during signal transduction, transcriptional regulation and cell-cycle progression (Clague and Urbé, 2010). In this context, we speculate that BRE participates in the ubiquitin-proteasome pathway to regulate protein turnover within cells. In the 2-DE profiling of D122α4 cells, where BRE was stably overexpressed, we identified five proteins that were up-regulated. They were granulin precursor, TNF receptor associated factor 6 (TRAF6), mitogen protein kinase 8, Mdm4 and baculoviral IAP repeat-containing protein 4 as shown in Figures 6 (2 DE gel) and 7 (semiquantitative RT-PCR and Western blot analyses).
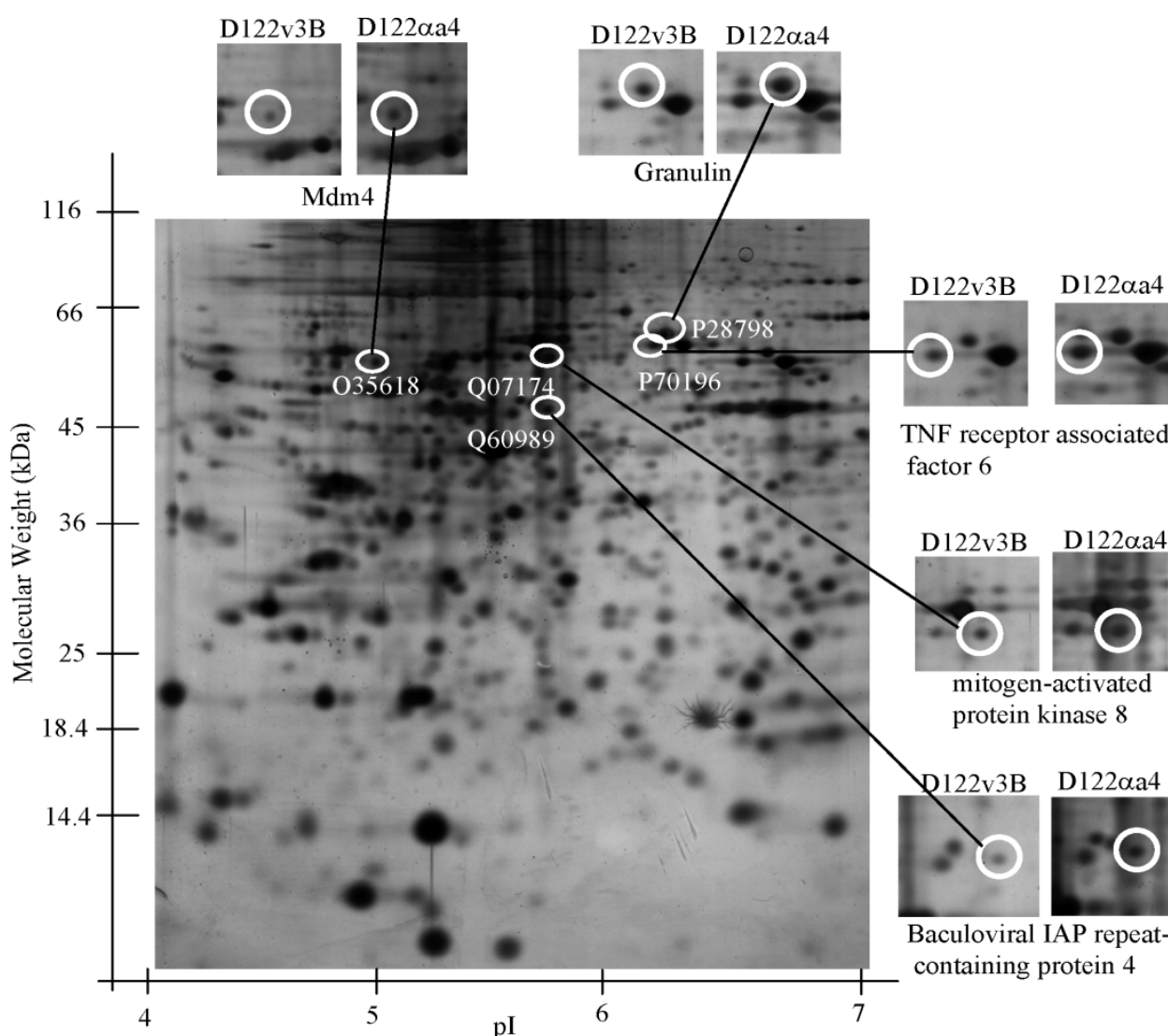

Fig. 6. Representative 2-DE gel of protein extracts from D122v3B and D122αa4 cell lines. Five protein spots (O35618, P28798, Q07174, P70196 and Q60989) were up-regulated in D122αa4 cells (Swiss-Prot accession number provided) (Tang et al., 2006).
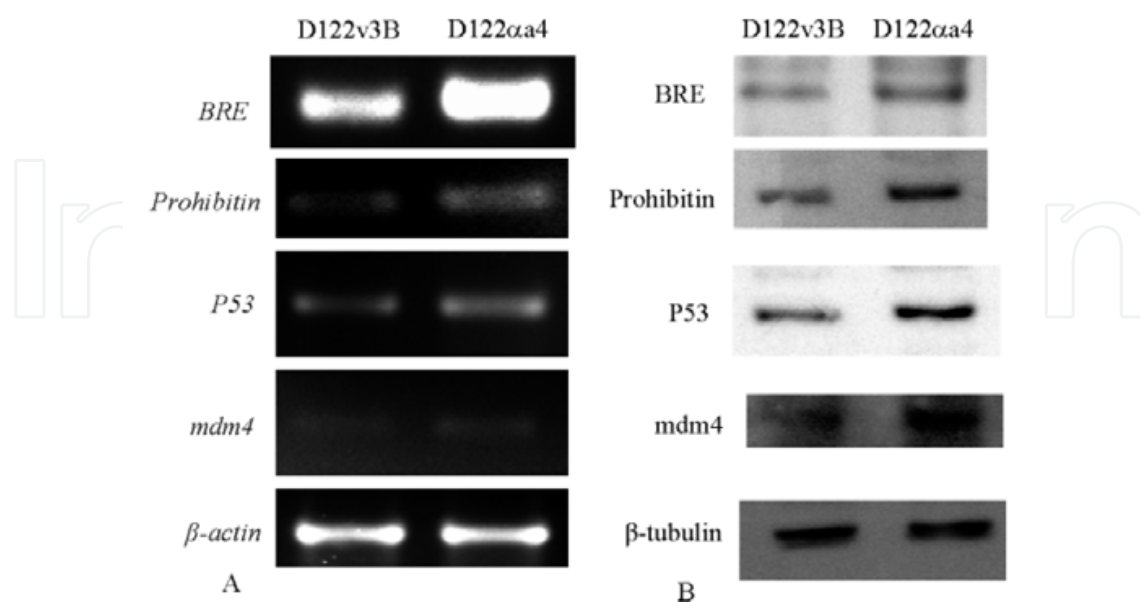
Fig. 7. Semiquantitative RT-PCR (A) and Western blot (B) showing that D122αa4 cells overexpressed *prohibitin*, *p53* and *mdm4*. β-*actin* and α-tubulin serve as internal controls (Tang et al., 2006).

Interestingly, TRAF6 is a unique member of the TRAF family of adaptor protein. It is associated with a diverse range of cellular responses to pathogens, growth factors or intracellular stress (Chung et al., 2007). Recent finding also showed that TRAF6 was involved in the RANK-TRAF6-NF-κB pathways during osteoclastogenesis (Inoue et al., 2007). Overexpression of BRE in human 293 embryonic kidney cells has been reported to inhibit NF-κB activation in response to TNFα (Gu et al., 1998). This finding suggests that BRE indirectly cross-talk with TRAF6 and NF-κβ, where it may play a central role in regulating cell proliferation, differentiation and survival. BRE may also mediate in post-translational sumoylation, similar to the action of PML and MO25α proteins (Kretz-Remy and Tanguay, 1999). Our results established a crucial function for BRE in regulating key proteins of cellular stress-response and provided an explanation for the multifunctional nature of BRE.

5.2.2 Comparative proteomic analysis reveals differentially expressed proteins regulated by a potential tumor promoter, BRE, in human esophageal carcinoma cells

Esophageal cancer is one of the most common malignancies that cause high mortality. Esophageal carcinogenesis is a complex and cascading process that involve the interaction of many genes and proteins (Kuwano et al., 2005). In this study, we have used comparative proteomic approaches to identify proteins that maybe involved in esophageal carcinogenesis. Two dimensional electrophoresis (2-DE) and MALDI-TOF-MS analyses of esophageal carcinoma, SHEEC and control cells SHEE revealed 10 proteins that were up-regulated as shown in Figure 8 of the 2-DE. Additional 10 proteins were down-regulated as shown in Figure 9. Interestingly, BRE, prohibitin, cyclin A and p53
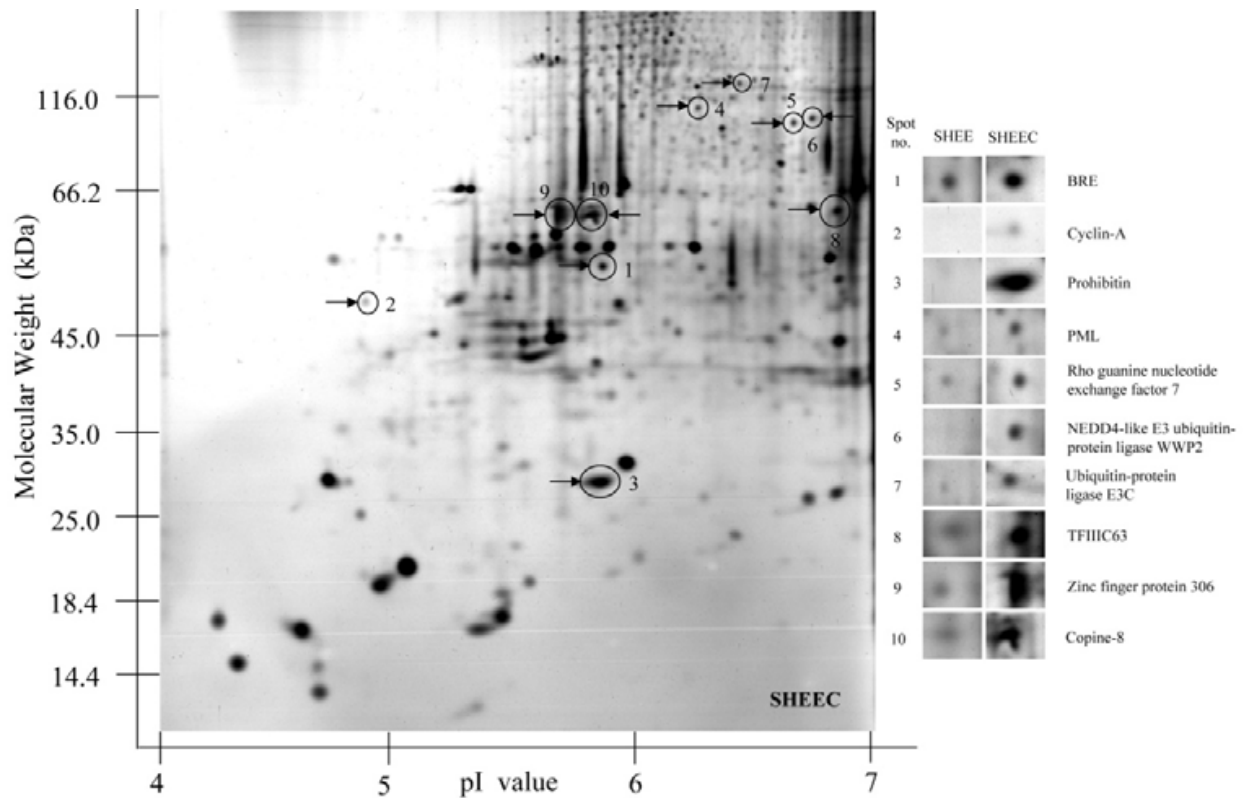
Fig. 8. Representative 2-DE gel of nucleic proteins extracted from SHEE and SHEEC cells. Ten silver-stained protein spots were found to be up-regulated in SHEEC cells (Chen et al., 2008).
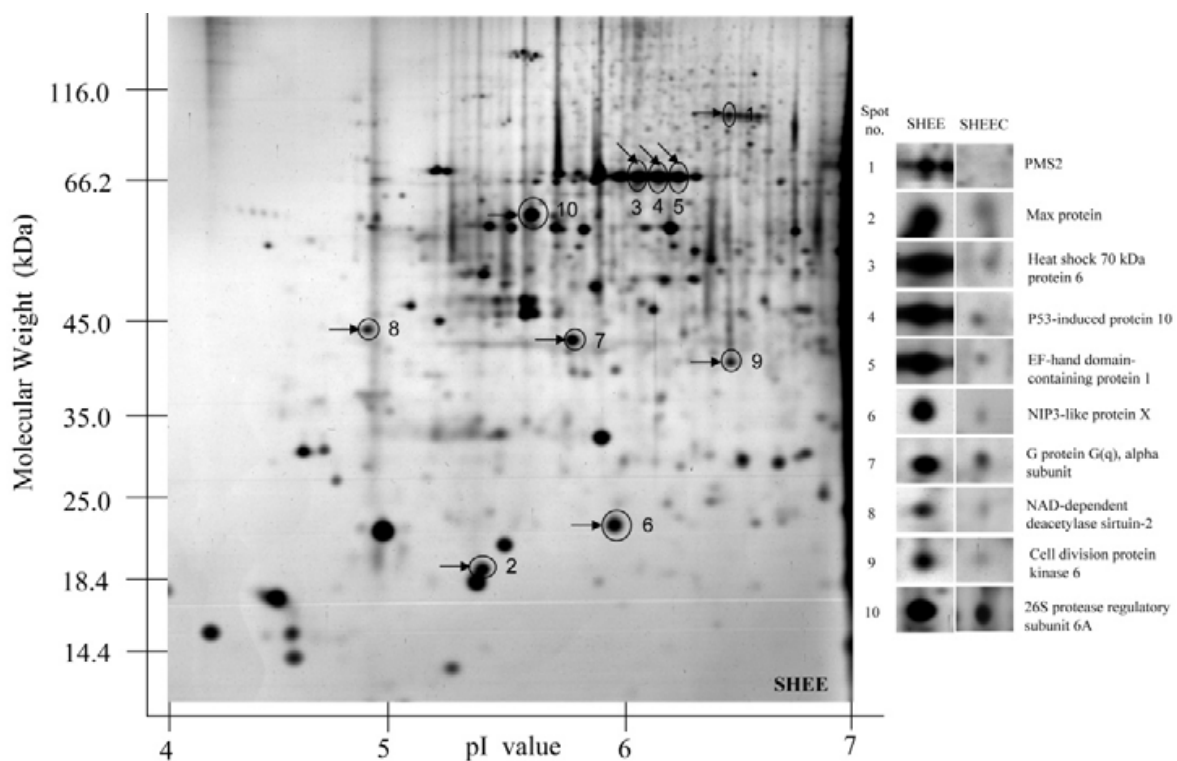

Fig. 9. Representative 2-DE gel of nucleic proteins extracted from SHEE and SHEEC cells. Ten silver-stained protein spots were found to be down-regulated in SHEEC cells (Chen et al., 2008).
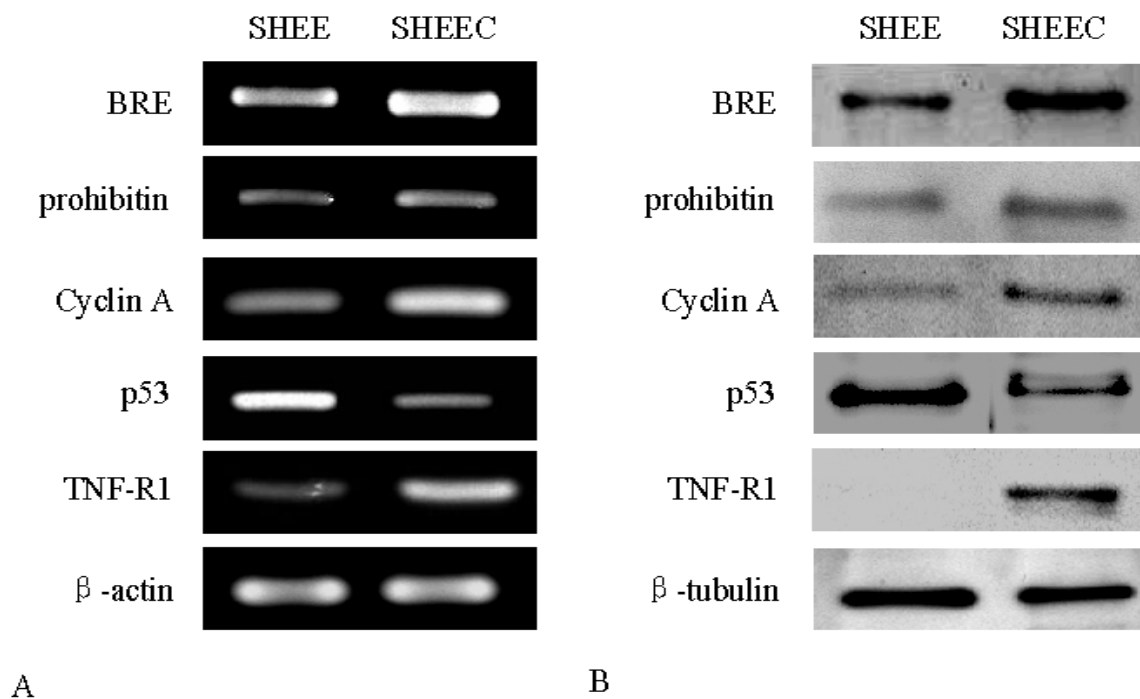
Fig. 10. Semiquantitative RT-PCR (A) and Western Blot (B) analyses of SHEE and SHEEC cells. The results confirmed the proteomic data that BRE, prohibitin and cyclin A were highly expressed in SHEEC cells. The SHEEC cells also expressed relatively higher levels of TNF-R1 but lower levels of p53, when compared with SHEE cells. *β-actin* and α-tubulin serve as internal controls (Chen et al., 2008).

expression were up-regulated in the cancer cells and this was confirmed by both semiquantitative RT-PCR and western blot analyses (Figure 10). Among these 20 differentially expressed proteins, BRE protein was identified as a potential tumor promoter. Furthermore, we have also determined p53 expression was down-regulated; whereas TNF-R1 expression was up-regulated in SHEEC cells (Figure 10). It has been reported that BRE can interact with the intracellular juxtamembrane domain TNF-R1 and inhibit the TNF-α induced activation of NF-κB (Gu et al., 1998). Therefore, we propose that BRE plays an anti-apoptotic role in SHEEC cells. To gain more insight into BRE's function, we silenced BRE expression in esophageal carcinoma cells using BRE-specific small interference RNA. It was found that silencing BRE expression corresponds to down-regulated prohibitin expression but up-regulated tumor-suppressor gene, p53 as shown in Figure 11. These findings contradicted the results with previous data (Tang, et al., 2006) that may due to multifunctional nature of BRE. Besides BRE, cyclin A and CDK2 expressions were suppressed in the SHEEC cells. Cyclin A is an important regulator of the cell cycle that rises in early S phase and falls in mid M phase (Parwaresch and Rudolph, 1996). Recent finding showed the cyclin A might be a prognostic marker in early breast cancer (Ahlin, et al. 2007). In summary, these results imply that BRE may be a survival factor and plays a proliferative role in esophageal carcinoma.
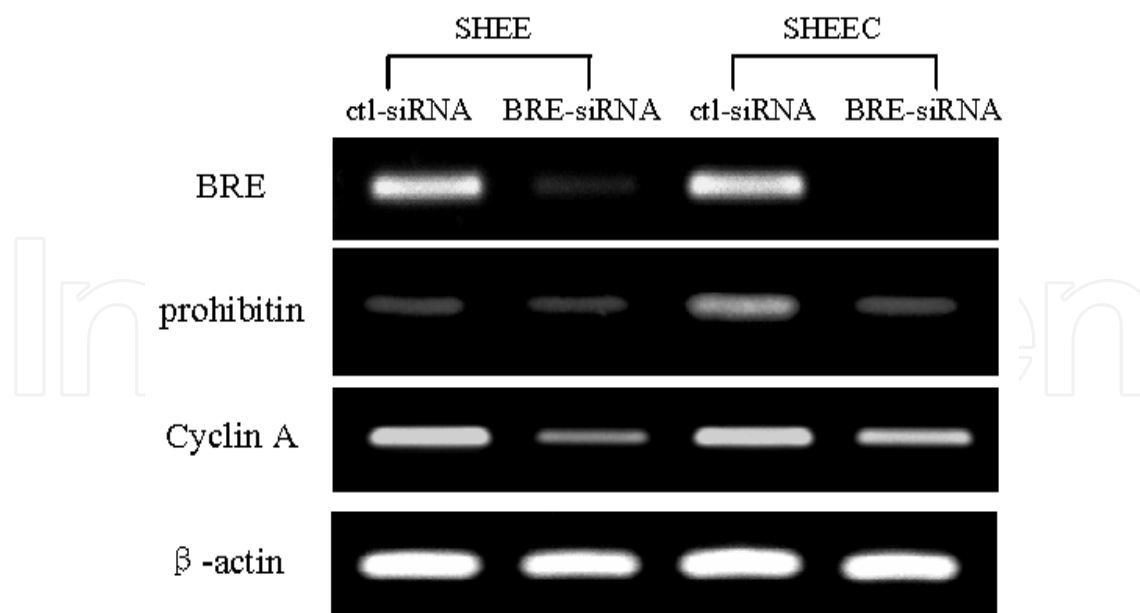
Fig. 11. Semiquantitative RT-PCR analysis of SHEE and SHEEC cells transfected with CTL- and BRE-siRNAs. The results showed that our BRE construct can silence BRE expression, as well as suppressed prohibitin and cyclin A expressions. *β-actin* served as an internal control (Chen et al., 2008).

5.2.3 Livers over-expressing BRE transgene are under heightened state of stress-response, as revealed by comparative proteomics

BRE is normally expressed at very low levels in the liver (Chan, et al., 2008). It binds to TNF-R1 and Fas, and modulates the actions of these cytokines (Li, et al., 2004; Chan et al., 2010). In this study, we demonstrated that BRE expression was rapidly induced when the liver was insulted with carbon tetrachloride (CCl_4) or in human hepatocellular carcinoma (HCC) as shown in Figure 12. We produced transgenic mice that specifically over-expressed BRE in the liver to determine the effect of high levels of BRE in the liver. The livers of these transgenic mice were determined to be histologically normal. Because of the lack of a phenotype, we conducted comparative proteomics to determine whether there were any differences at the protein level (Figure 13). The 2-DE revealed four up-regulated protein spots and nine down-regulated protein spots as summarized in Table 2. It was established that several stress responsive proteins were up-regulated in the BRE-transgenic liver including: Alpha enolase (ENO 1), Heat shock-related 70 kDa protein 2 (HSPA2), Putative heat shock 70 kDa protein 7 (HSPA7), Zinc-finger protein Ubid 4 (DPF2) and Tumor suppressor candidate 4 G21 protein (TUSC4) as shown in Figure 14. Recently, it has been reported that HSPA7 is a biomarker for early detection of HCC (Park, 2011). In addition, we have silenced BRE expression in Chang liver cells and inversely demonstrated that it did not affect cell proliferation rate as confirmed by BrdU Labelling assay (Table 3). We have previously reported that BRE is not only expressed in the cytoplasm but also in the nuclei of HCC cells. BRE also accumulates in the nuclei of esophagus cancer SHEEC cells (Chen, et al., 2008). Since BRE is one of the components of BRCA1 A complex, it could be involved in DNA repair, as well as responding to environmental stress. We propose that the livers in our BRE transgenic mice were under a heighten state of stress response and this may explain why the transgenic mice was more resistant to liver toxic drugs.
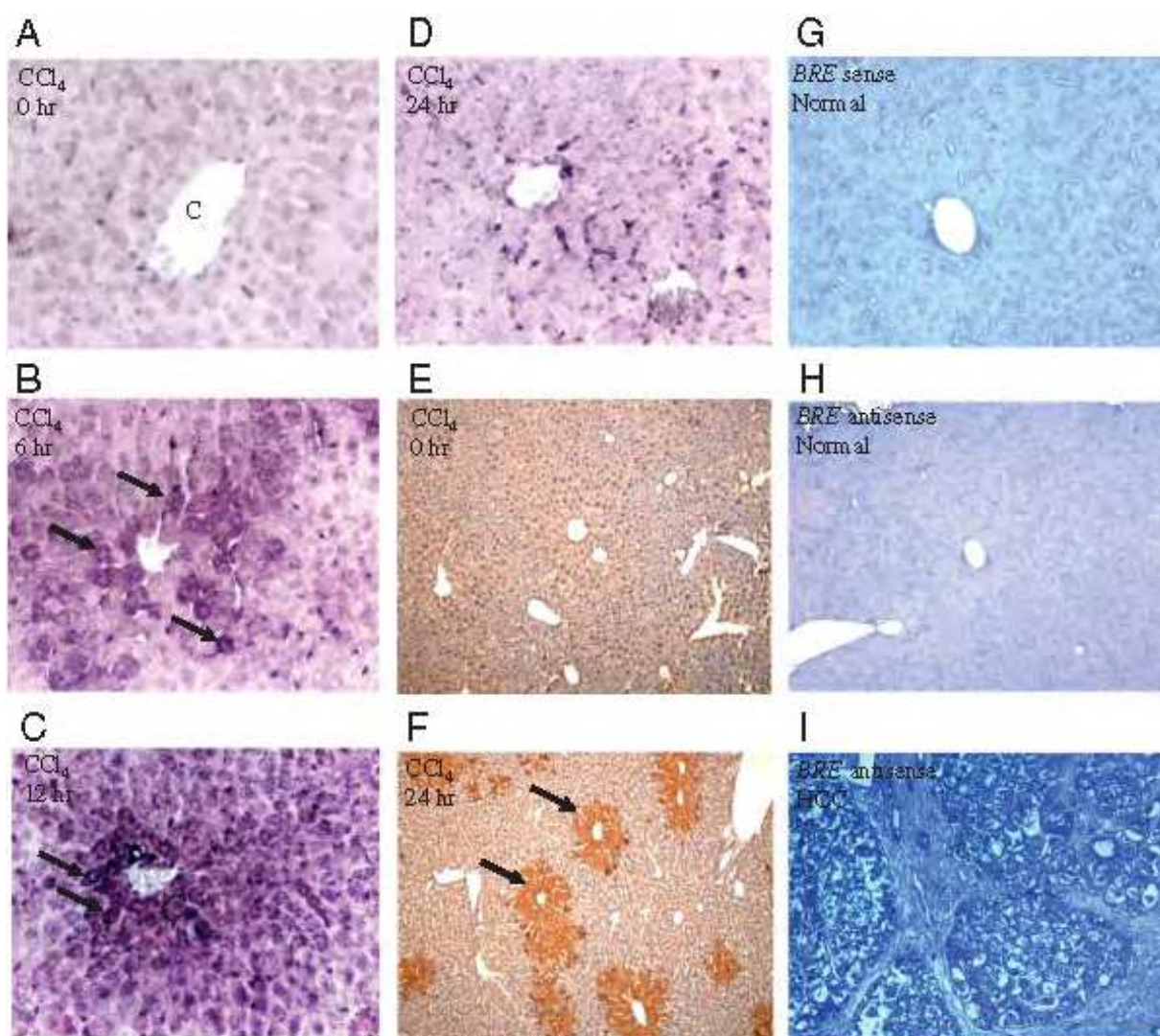
Fig. 12. In situ hybridization (A–D and G–I). BRE is normally expressed at very low levels in normal mouse liver (A). CCl4 insult induced increased BRE expression in the affected hepatocytes at 6 h (B) and 12h (C). Twenty-four hours after CCl4 insult, BRE expression declined. This was probably the result of the affected hepatocytes starting to die off (D). Immunohistological staining revealed that BRE expression was strongly induced in the affected hepatocytes by CCl4 (E, F). BRE expression remained low in the unaffected cells. We also examined BRE expression in HCC cells. BRE was expressed at low levels in non-tumor human liver tissues (H). In HCC tissues, all the cells strongly expressed BRE (I). Sense control (G). Arrows, hepatocytes overexpressing BRE. C, liver central veins (Tang et al., 2009).
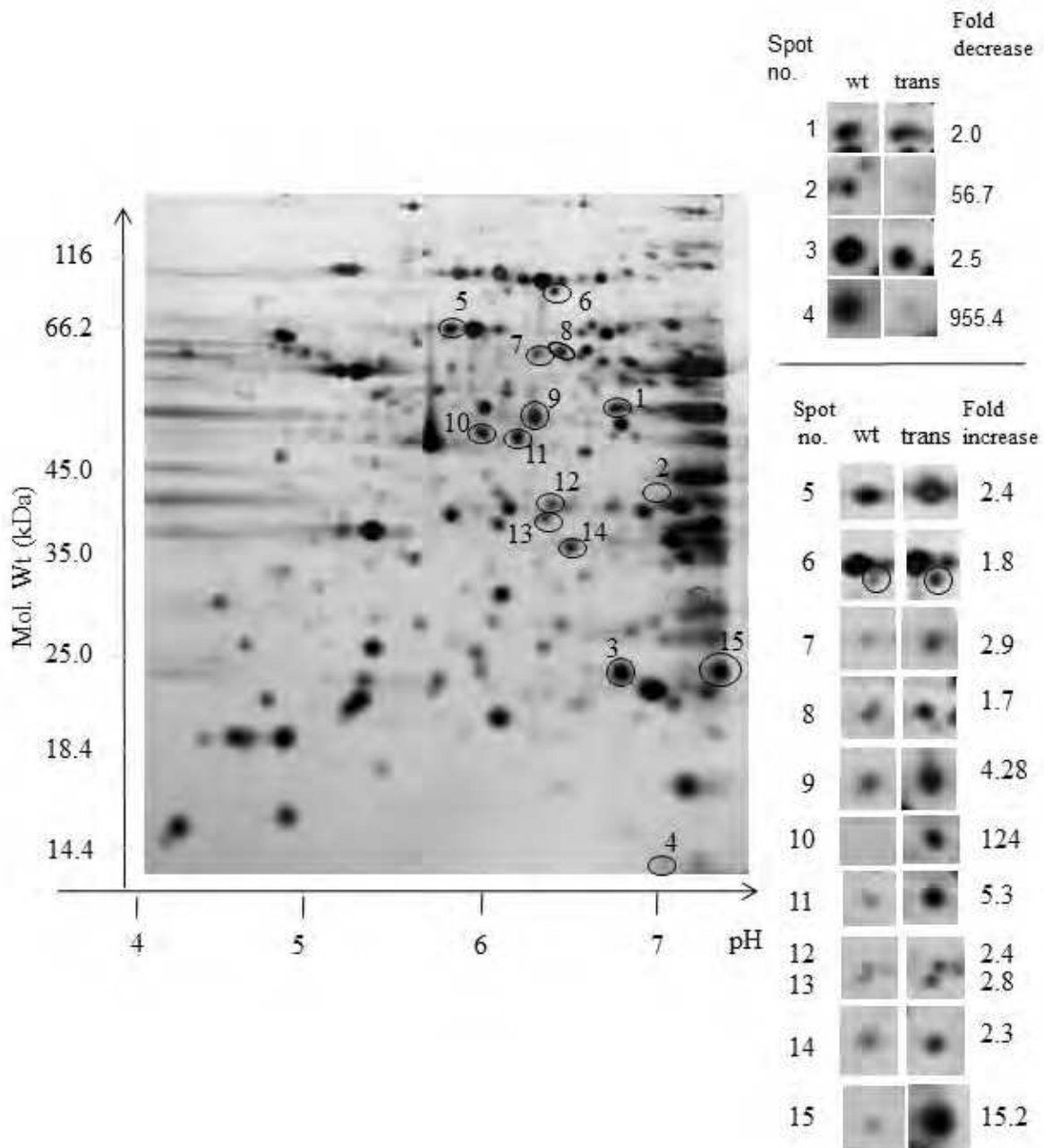
Fig. 13. A representative 2-DE gel of BRE transgenic liver. Protein spots 1–15 were identified to be differentially expressed when compared with control gels. Protein spots 1–4 were downregulated in the transgenic (trans) liver, while protein spots 5–15 were upregulated in the wild type (wt) liver. These results were acquired from three independent liver samples and 2-DE was correspondingly performed three times (Tang et al., 2009).
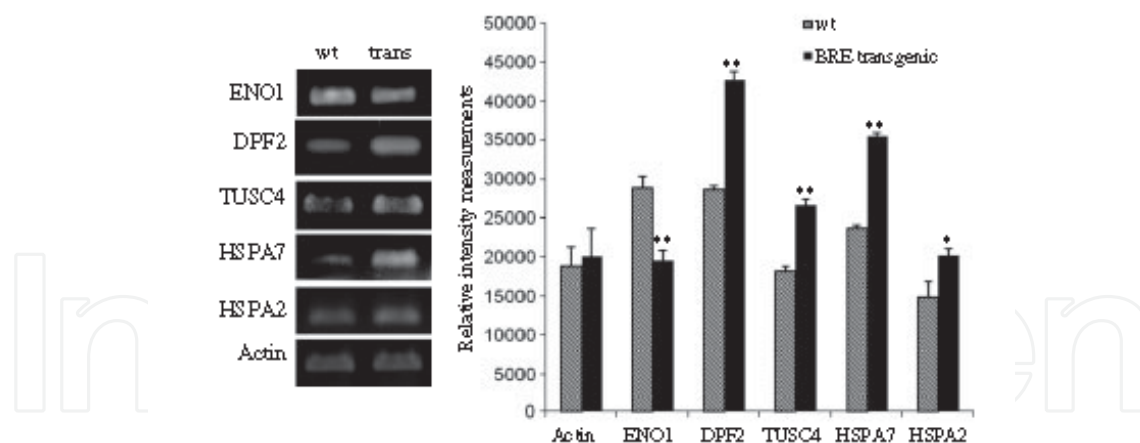
Fig. 14. Semi-quantitative RT-PCR revealed that the proteins identified were differentially expressed in BRE transgenic livers were also correspondingly affected at the transcriptional level. *p<0.05, **p<0.01, denote significant difference in the staining intensity of wt and BRE transgenic PCR bands (Tang et al., 2009).

Spot no.	Proteins identified	Swissprot accession no.	Mowse Score	Actual MW (kDa)/pI	No. of peptide mass match (%)	Functions
1	Alpha enolase	P17182	2.65e+008	47.1/6.37	18/165 (10%)	Multifunctional enzyme involved in various processes such as growth control, hypoxia tolerance and allergic responses
2	Zinc-finger protein	Q9QX66	6.74e+003	44.2/6.73	8/121 (6%)	May play a role as a neurospecific transcription for developing neurons by participating in regulation of cell survival
3	ARF-related protein	Q8BXL7	1.07e+006	22.7/6.17	16/272 (5%)	Possibly involved in plasma membrane-related signaling events
4	Hepatocellular carcinoma associated antigen 127	Q9NQZ6	4.22e+003	26.2/6.68	9/77 (11%)	Unknown
5	Heat shock-related 70kDa Protein 2	P54652	4.77e+008	70.0/5.56	28/274 (10%)	Cooperate with other chaperones, it stabilizes preexistent proteins against aggregation and mediates the folding of newly translated polypeptides in the cytosol or within organelles
6	Tumor suppressor candidate 4 (G21 protein)	Q9WUE4	1.01e+005	43.6/6.23	9/119 (6%)	May act as a tumor suppressor
7	Nuclear receptor ROR-alpha	P35398	1.23e+006	63.0/5.97	9/119 (6%)	Might be an orphan nuclear receptor that binds DNA as a monomer to hormone response elements
8	Leucine-rich repeat LG1 family member 2	Q8K4Z0	1.61e+008	63.0/6.10	10/165 (6%)	Might be a potential secreted protein
9	Mitogen-activated protein kinase 12	P53778	7.17e+008	41.9/5.98	28/197 (14%)	Responds to activation of environmental stress or pro-inflammatory cytokines by phosphorylating downstream targets
10	Ankyrin repeat domain protein	Q99LW0	7.81e+005	44.0/5.85	15/205 (5%)	Plays a wide variety of roles in protein–protein interaction and in the signal pathways
11	Caspase-2 precursor	P29594	1.12e+003	48.9/5.80	5/68 (7%)	Might be involved in regulation of proteins in cell death
12	Protein FAM54A	Q8VED8	6.66e+004	40.8/6.05	11/149 (7%)	Unknown
13	CD209 antigen-like protein B	Q8CJ91	4.77e+004	37.1/6.10	8/121 (6)	Might be a pathogen-recognition receptor and mediate the endocytosis of pathogens
14	Zinc-finger protein Ubid4	Q61103	4.75e+006	44.2/6.06	16/280 (5%)	Might be a transcription factor in apoptosis response; it also plays a role in the development and maturation of lymphoid cells
15	Heat shock 70 kDa protein	P48741	1.52e+006	25.9/6.97	4/37 (10%)	May act as chaperones in presenting tumor antigens

Table 2. Proteins that are differentially expressed in BRE transgenic liver (Tang et al., 2009).

Treatments	BrdU⁻ cells	BrdU⁺ cells	Total	Percentage Brdu⁺ cells
Ctl-siRNA	293±30	293±32	585±36	50.04±0.02
BRE-siRNA	276±32	265±16	541±24	49.09±0.03

Table 3. Effects of silencing BRE expression on Chang liver cell proliferation (Tang et al., 2009).

6. Future perspective of proteomics

Conventional "gel-based" electrophoresis and improved mass spectrometry have provided useful tools for revealing molecular changes in cells and tissues that otherwise maybe missed by morphological observation alone (Vercauteren et al., 2007). Nevertheless, the 2-DE protocol is still to be refined and improved so that 2-DE is more reproducible and sensitive. Therefore, it has still some distance to go before it can be adopted as a standard "diagnostic tool" in the 21st century (Colucci-D'Amato et al., 2011). The "shotgun" methodology has been used as a high-throughput screen to identify proteins that are differentially expressed in cells or tissues, as a result of some experimental procedure or changes in environmental condition (Lill, 2003; Zhu et al., 2010). Liu et al. (2011) recently described the SELDI-TOF-MS technology that could be used to screen and detect differentially expressed proteins in the serum of patients with cancer. Liquid chromatography interfaced plasma mass spectrometry has now been developed for absolute quantitation of proteins (Esteban-Fernández et al., 2011). Furthermore, latest development of computational tools for analyzing high-throughput 'shotgun' proteomic data also play a vital role in moving proteomic research forward (Dowsey et al., 2010). All of these improvements will allow proteomics to be rapidly developed as a practical, robust, accurate and inexpensive analytical tool for routine use in the clinical setting. The proteomics will also allow many novel disease biomarkers to be discovered and also lead to the discovery of new drugs.

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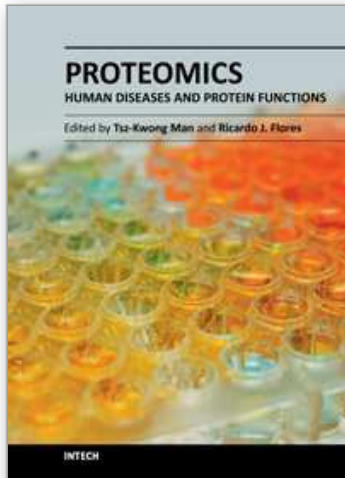
Proteomics - Human Diseases and Protein Functions

Edited by Prof. Tsz Kwong Man

Biomedical research has entered a new era of characterizing a disease or a protein on a global scale. In the post-genomic era, Proteomics now plays an increasingly important role in dissecting molecular functions of proteins and discovering biomarkers in human diseases. Mass spectrometry, two-dimensional gel electrophoresis, and high-density antibody and protein arrays are some of the most commonly used methods in the Proteomics field. This book covers four important and diverse areas of current proteomic research: Proteomic Discovery of Disease Biomarkers, Proteomic Analysis of Protein Functions, Proteomic Approaches to Dissecting Disease Processes, and Organelles and Secretome Proteomics. We believe that clinicians, students and laboratory researchers who are interested in Proteomics and its applications in the biomedical field will find this book useful and enlightening. The use of proteomic methods in studying proteins in various human diseases has become an essential part of biomedical research.

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