Knowledge Discovery of Different Relationship Among Akt, p70S6 and ERK under Different Functions by Phospho-Antibody Microarray

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Abstract

Akt, p70S6 and ERK as very hot and important multifunctional proteins are widely studied. In this paper, the different activation and silencing of Akt/ ERK/p70S6 signaling in treated human cancer cell lines are studied by proteomic analysis of co-localization using western blot, FACS and nano-gold phospho-antibody microarray by reaction with nuclear and cytoplasmic proteins. Our results show that (1) Akt/ ERK signaling co-activation through p-Akt/ p-ERK co-cytoplasmic increase whereas p70S6 signaling inhibition through p-p70S6 by nuclear decrease and cytoplasmic increase (total protein unchanged) in BMP2–induced apoptosis U937 cells (2000ng/ml BMP2 for 3 days); (2) p70S6/ ERK signaling co-activation through p- p70S6/ p-ERK co-cytoplasmic increase whereas Akt signaling inhibition through p-Akt unchanged in BMP2-treated MCF7 cells (100ng/ml BMP2 for 4h); (3) Akt /p70S6 signaling co-inhibition through p-Akt/p-p70S6 nuclear and cytoplasmic co-decrease whereas ERK signaling activation through p-ERK nuclear increase in STI571-treated K562 cells (0.2 uM STI571 for 24h). This study implies that different activations and silencings of Akt/p70S6/ERK signaling reflect different functions.

Keyword: Akt, p70S6, ERK, phospho-Antibody Microarray.

I. Introduction

With the post-genome era proteomics study for direct analysis of a group of protein become more and more important (James et al. 1997). Identification of the type of modification and its location often provide crucial information for understanding the function or regulation of a given protein in biological pathways(Zhu et al. 2003). By classical methods, this would require an extremely large number of time consuming experiments. Antibody array potentially allows the identification of all of the proteins that carry those modifications in a single experiment.

Protein co-localization is fundamentally important to eukaryotic protein function, cell regulation and interactions. It is widely acknowledged that proteins rarely act as single isolated species when performing their functions. The analysis of proteins with known functions indicates that proteins involved in the same cellular processes often interact with each other. Following this observation, one valuable approach for elucidating the function of an unknown protein is to identify other proteins with which it interacts, some of which may have known activities. On a large scale, mapping protein-protein interactions has not only provided insight into protein function but facilitated the modeling of functional pathways to elucidate the molecular mechanisms of cellular processes(Zhu, Bilgin et al. 2003).

Akt, p70S6 and ERK as very hot and important multifunction proteins are widely studied. Although Akt controls cell growth through its effects on the mTOR and p70S6 (in the cytoplasm) kinase pathways, Lehman et al. [4] indicated that a kinase from the MEK/MAPK pathway also plays a role in p70S6K activation by GM-CSF in a hematopoietic cell, the neutrophil. Lorenzini et al. [3] demonstrated that in senescent cells neither ERK nor Akt is able to phosphorylate efficiently their nuclear targets, the relationship among Akt, p70S6 and ERK under different functions have not been elucidated.

In this paper, the different activations and silencing of Akt/ ERK/p70S6 signaling in treated human cancer cell lines are studied by proteomic analysis of co-localization using western blot, FACS and nano-gold phospho-antibody microarray by reaction with nuclear and cytoplasmic proteins. Our results show that Akt/ ERK signaling co-activation through p-Akt/ p-ERK co-cytoplasmic increase whereas p70S6 signaling inhibition through p-p70S6 by nuclear decrease and cytoplasmic increase (total protein unchanged) in BMP2–induced apoptosis U937 cells (2000ng/ml BMP2 for 3 days); p70S6/ ERK signaling co-activation through p- p70S6/ p-ERK co-cytoplasmic increase whereas Akt signaling inhibition through p-Akt unchanged in BMP2-treated MCF7 cells (100ng/ml BMP2 for 4h); Akt /p70S6 signaling co-inhibition through p-Akt/p-p70S6 nuclear and cytoplasmic co-decrease whereas ERK signaling activation through p-ERK nuclear increase in STI571-treated K562 cells (0.2 uM STI571 for 24h). This study implies that different activation and silencing of Akt/p70S6/ERK signaling reflect different functions.

II. Methods

A. ***Cell Lines, Chemicals, Antibodies, Apparatus***

Leukemia cell line K562 and breast cell line MCF-7 were kindly provided by Dr Joachim Clement. Leukemia cell line U937 provided by Frau Dagmar Haase. STI571 was kindly provided by Prof. Pachmann and prepared as a 10 mM stock solution in sterile DMSO (Merck Darmstadt, Germany). BMP2 was kindly provided by Dr. Clement stored at -20°C, and dissolved in sterile water as a 1mg/ml stock solution before use. Stock solutions were then diluted in RPMI medium to achieve the desired final concentration. In all of the cases, final concentrations of DMSO were < 0.1% and did not modify responses of cells to STI571. Biotinamidocaproate N-hydroxysuccinimide ester was purchased from SIGMA D2643; Streptavidin-Gold EM.STP5 and LM/EM Silver Enhancement Kit SEKL15 were purchased from British BioCell; dist.PLANO, Germany. BSA from SIGMA; Milk Powder from Roth GmbH, Germany. Cy 3 and Cy 5 mono-reactive dyes were purchased from Amsham, Germany. p-p70S6 kinase (Thr389), p-Akt (Ser473), p-STAT3 (Ser727), p-Tyrosine (Tyr100), p-SAPK/JNK (Thr183/Tyr185), p-p42/44 MAPK (Thr202/Tyr204), p-p38 (Thr180/Tyr182) and p38 MAPK purchased from Cell Signalling Technology; c-Myc, p-Smad1 (Ser463/465) and p-Smad2/3 (Ser433/435), β-actin, secondary antibodies antigoat, mouse, rabbit purchased from Santa Cruz Biotechnology.

The Clondiag ArrayTube™ platform (AT) was selected for the preparation of antibody arrays. In the AT platform the array chip is positioned at the bottom of a standard 1.5 ml micro reaction tube.

This allows us to use all the standard laboratory equipment for heating, cooling and shaking, as well as centrifugation of 1.5 ml reaction tubes. Together with conventional microliter pipetors everything needed to perform the experiment. The only additional equipment required is a dedicated ArrayTube™ reader. If, as presented here, silver staining is used for detection, the costs for the reader are fairly low, in particular when compared with fluorescence readers used for other array platforms with fluorescence labelling. The ArrayTubes™ are prepared and setup by Clondiag. Clondiag offers two choices for chip preparation, spotting of substances or in situ synthesis of oligomers. For the preparation of antibody arrays commercially available antibodies (Cell Signalling Technology, Beverly, MA; Santa Cruz Biotechnology, Santa Cruz, CA) were used and the spotted micro arrays were taken to set up the ArrayTubes™.

For spotting an equal volume of antibody-spotting-buffer (Clondiag) was added to the PBS antibody preparations. All antibodies were obtained at concentrations of at least 1 µg/µl (or higher). We also included a second set of spots containing a 1:5 dilution for each antibody. The antibodies were spotted using a conventional split-pin micro-arrayed (BioRobotics MicroGrid II) onto glass based array substrate containing a three dimensional epoxy activated surface. After spotting, fresh prepared arrays were left in humid environment at room temperature to allow for covalent binding of the antibodies on the activated substrates. The quality of spotting (spots) was monitored by spot size analysis. Arrays were then mounted in ArrayTubes™ and sealed under inert gas atmosphere.

B. Cell Culture

K562, MCF-7 and U937 human cancer cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT). They were maintained in a 37°C, 5% CO_2, fully humidified incubator, passed twice weekly, and prepared for experimental procedures when in log-phase growth (4 x 10^5 cells/ml).

C. Cell numbers and Experimental format

Logarithmically growing cells were placed in sterile plastic T-flasks (Corning, Corning, NY) to which the designated drugs were added and the flasks placed back in the incubator for intervals ranging from 4 to 72 h. 50 µl cell sample was collected and added into 20 ml of 0.9% NaCl tube for counting of cell numbers in cell count reader. At the end of the incubation period, cells were transferred to sterile centrifuge tubes, pelleted by centrifugation at 400 x *g* for 10 min at room temperature, and prepared for analysis as described below.

D. Cytoplasmic and Nuclear protein Extracts

K562, MCF-7 and U937 human cancer cellular proteins were obtained from cell culture cells as cytoplasmic or nuclear fractions using a buffer system that allows lysis of cells in two steps. In the first step only the plasma membrane is lysed, leaving nucleus intact. The nuclei are pelleted by centrifugation. The supernatant contains the cytoplasmic protein lysate. To obtain nuclear proteins, the nuclei are washed repeatedly and then lysed using the nuclear lysis buffer B which described in details as follows:

K562, MCF-7 and U937 human cancer cells (10^5 to 10^6) were collected from culture cells and washed with 10 ml PBS by centrifugation with 1500 x *g* for 5 min. The cell pellet was resuspended in 1 ml PBS and transfered to 1.5 ml tube by centrifugation for 15 sec, buffer removed. The cell pellet was resuspended in 400 µl ice cold buffer A (cytoplasmic lysis buffer) and left on ice for 15 min (cells should swell). 25 µl of Np-40 (10%solution) was added and vortexed for 10 sec by centrifugation for 30 sec in 9000 rpm. Supernatant in 1.5 ml tube for cytoplasmic proteins was added

0.11 volume of ice cold buffer C and mixed thoroughly by centrifugation for 15 min at maximum speed.

Nuclear pellet was washed in 500 µl of ice cold buffer A and 20 µl Np-40 and votexed for 10 sec followed by a centrifugation with 9000 rpm for 30 sec. The pellet was resuspended in 50 µl of buffer B and rotated or shaken for 15-20 min at 4°C. Samples were centrifuged for 5 min and supernatants collected and frozen in aliquots of 10 µl (-70 C). Buffer A (lysis buffer) including 10 mM Hepes pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF. Buffer B (nuclear extract buffer) including 20 mM Hepes pH 7.9; 0.4 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF. Buffer C (cytoplasmic extract buffer) including 0.3 M Hepes pH7.9 ; 1.4 M KCL ; 0.03 M MgCl2 .

E. Determination of Protein Concentration

Protein concentration was determined according to Bradford (1976). Several dilutions of protein standards (BSA) containing from 1 to 100 µg/ml were prepared. 0.1 ml of standard samples and appropriately diluted samples were placed in dry test tubes. 0.1 ml sample buffer was used as a negative control. 1.0 ml diluted dye reagent was added to each tube and mixed several times by gentle inversion. After 15 min, OD595 values versus reagent negative control were measured. OD595 versus concentration of standard was plotted. The protein of interest was calculated from the standard curve using the Microsoft Excel5 software.

F. Western Analysis

Equal amounts of protein (20 µg) were boiled for 10 min, separated by SDS-PAGE (5% stacker and 10% resolving), and electroblotted to nitrocellulose. After blocking in PBS-T (0.05%) and 5% milk for 1h, the blots were incubated in fresh blocking solution with an appropriate dilution of primary antibody for 4h. The source and dilution of antibodies were as follows: p-Akt 1:200, p-STAT3 (1:100), p-Tyrosine (1:100), p-p42/44 MAPK (1:200), p-p38 (1:200), p-Smad1 (1:100) and β-actin (1:1000). Blots were washed 3 x 5 min in PBS-T and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody for 1h. Blots were again washed 3 x 5 min in PBS-T and then developed by ECL chemiluminescence.

G. Cell cycle Analysis

After treatment, cells were pelleted at 500 x *g* and resuspended in 70% ethanol. The cell pellets were incubated on ice for 1h and resuspended in 1 ml of cell cycle buffer (0.38 mM sodium citrate, 0.5 mg/ml RNase A, and 0.01 mg/ml propidium iodide (all Sigma Chemical Co.) at a concentration of 10^6 cells/ml. Samples were stored in the dark before analysis at 4°C (generally within 24 h) and analyzed on a Becton Dickinson FACScan flow cytometer (Cambridge, MA) using a commercially available software program (ModFit LT 2.0; Verity Software, Topsham, ME).

H. Silver staining in Arraytube

50 µg of cellular proteins are diluted to a final volume of 25 µl in buffer (extraction buffer or PBS). If other buffers than indicated below are used for protein extraction, a change of buffers may be necessary before biotinylation (Tris-based buffers can not be used for biotinylation!). For biotinylation 1 µl of NHS-succinimid-Biotin (SIGMA) (100 µg/µl in ultra pure DMSO; water free) was added and left at room temperature for 1h. Reaction was stopped adding 2%BSA. Protein preparations were left at room temperature for another 15 minutes to ensure complete consumption of the biotinylation reagent. Finally, the volume was adjusted to 100 µl with PBS (2% milk powder

or 2% BSA). Biotinylated proteins are then ready to add to blocked ArrayTubes™ for binding, according to our method [14].

Before incubation with protein extracts, antibody arrays must be blocked. After spotting most of the activated surface is still freely accessible and has a high capacity for protein binding. For this reason, our arrays were blocked with 5% milk powder in PBS for at least 5 minutes at 30° C shaking at 750 rpm (Eppendorf Thermomixer™). Not all milk powder can be used, so we recommend tests with various milk powders (Sigma). In this case best results were obtained with a milk powder that fully dissolved into a clump free white colored solution at 5% in PBS. Blocking solution is then replaced by the biotinylated protein extract in PBS (supplemented with 2% milk powder or BSA). Arrays are incubated with the protein extracts for 2h at room temperature. Alternatively, we also obtain good results with an overnight incubation at 4°C. After incubation arrays are washed 3 times for 5 min with 500 µl PBS.

The bound biotinylated proteins are detected in a two step detection process. In the first step, streptavidin gold nanoparticles (British BioCell, Plano) are bound to the biotin groups. In the second step, a silver precipitate is formed around the gold particles. This step is monitored online in the ArrayTube™ Reader, which allows to detect the onset of sliver precipitation. In this way errors resulting from saturation effects from final point measurements can be avoided.

Before incubation with the streptavidin gold particles tubes are again blocked with PBS (5% milk powder) for 15 min. Blocking solution is replaced by 100 µl of streptavidin gold particles in PBS and tubes are incubated for 30 min at 30° C shaking at 350 rpm. Excess streptavidin gold is removed in 3 wash steps. with 200 µl of PBS-tween (0.1%) for 10 min at 20° C with 750 rpm three times. For silver staining 100 µl silver developing solution is added. The silver developing solutions contains equal amounts of silver enhancer and developer, which are combined directly before use (here we used the reagents from British BioCel, distributed in Germany by Plano. Other reagents may work equally well). Tubes are placed into the ArrayTube™ Reader and recording of pictures is started. Pictures are taken every minute for 40 minutes to 1 h.

50 µl of solution A and 50 µl of solution B from silver enhancement kit were combined immediately before use and added to ArrayTube to start silver development, collect images for 40 min to 1h at 1 min interval and analysis of images with appropriate software e.g. Partisian IconoClust from Clondiag.

For each experiment, 40 exposures are obtained and all are evaluated by the IconoClust software. This software can automatically produce data including mean, background, Sigma etc. To compare results between each experiment, all values are normalized by the median method. The obtained pictures were analyzed using the PARTISAN IconoClust image analysis software from Clondiag. This software automatically recognises the arrays and overlays a grid to measure the intensities for each spot. All pictures were combined to generate time curves for the increasing signals of all samples (spots). This time course was then used to assign differences in signal intensities. In comparison with pictures taken after a given period of staining, this procedure eliminates the assignment of the wrong intensity due to saturation effects.

III. Results

We used 2000ng/ml BMP2 for 3 days treatment in U937 cells, 0.2 µM STI571 for 24h treatment in K562 cells, 100ng/ml BMP2 for 4h treatment in MCF7 cells. Our result showed that in BMP2 treated U937 cells, p-ERK (thr202/tyr204) displayed the increase in cytoplasm and unchanged in nucleus. Total protein (sum of cytoplasm and nucleus) level of p-ERK is higher than in control cells; p-Akt (ser473) displayed the increase in cytoplasm and the slight decrease in nucleus**.** Total protein (sum of cytoplasm and nucleus) level of phospho-Akt is higher than in control cells. P-p70S6

(thr389) displayed the increase in cytoplasm and the decrease in nucleus in BMP2 treated U937 cells. Total protein (sum of cytoplasm and nucleus) levels of phospho-p70S6 was not altered compared with control.

In STI571 treated K562 cells, p-ERK(thr202/tyr204) displayed the decrease in cytoplasm and the increase in nucleus. Total protein (sum of cytoplasm and nucleus) level of p-ERK is higher than in control cells. p-Akt(ser473) and p-p70S6(thr389) displayed the decrease in cytoplasm and nucleus. Total protein (sum of cytoplasm and nucleus) level of phospho-Akt and p-P70S6 are lower than in control cells.

In BMP2 treated MCF7 cells, P-Akt displayed unchanged in cytoplasm and nucleus, p-ERK and p-P70S6 displayed the increase in cytoplasm and unchanged in nucleus . Total protein (sum of cytoplasm and nucleus) level of p-ERK and p-P70S6 were increased compared with control (Fig. 3-4).

Compared with cell number, FACS and western blotting, antibody array results are consistent with the experiments of cell number, FACS and western blotting (Fig. 1-2).

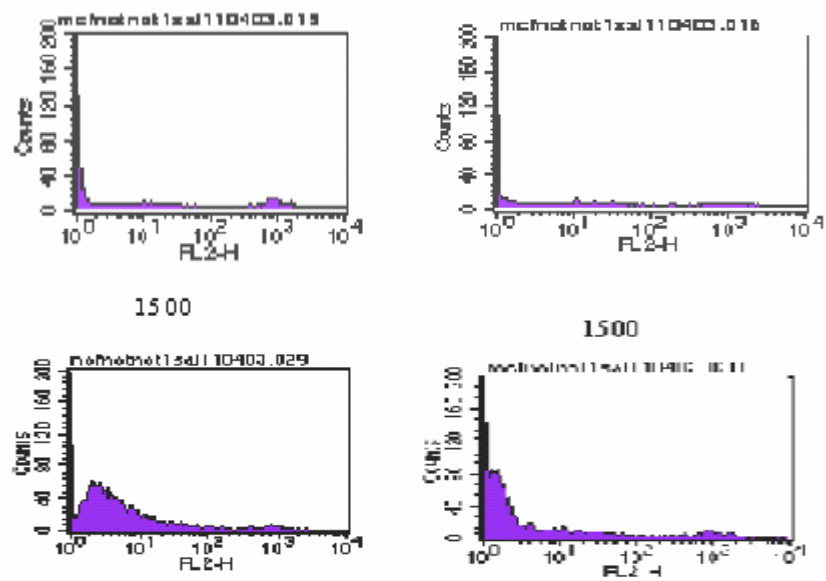

Fig. 1. Examination of Dead cells under untreated and treated bmp2 U937 cells for 3 days by FACS. Different BMP2 concentrations were selected from 50 ng/ml, 100 ng/ml, 300 ng/ml, 500 ng/ml, 1000 ng/ml, 1500 ng/ml to 2000 ng/ml for 3 days to examine dead cells. Positions of control and different concentration BMP2 samples were shown in each picture below, each sample repeating two times. 2000 ng/ml BMP2 treatment is the best condition for the induction of apoptosis in U937 cells from dead cells.

IV. Discussion

Protein localization data provide valuable information in elucidating eukaryotic protein function[2]. Protein trafficking between nucleus and cytoplasm fundamentally important to cell regulation. As such, the nuclear import and export are pivotal in orchestrating the activities of the key regulators of the cell cycle[2].

Our result showed that p-Akt and p-ERK co-cytoplasmic increase appear in BMP2 treated U937 cells. Lorenzini, et al. (2002)[3] demonstrated that in senescent cells not ERK nor Akt is able to phosphorylate efficiently their nuclear targets, however, total protein Akt and ERK no significant change. Our FACS result also showed apoptosis in 2000ng/ml BMP2 treated U937 cells compared with control (Fig.1). From this point it can be reflected the apoptosis state in BMP2 treated U937 cells. Our study demonstrated that Phospho-p70S6 under total protein (sum of cytoplasm and nucleus) level of phospho-p70S6 was not altered in control and BMP2 treated cells displayed an

increase of cytoplasmic phosphorylation and decrease of nucleus in BMP2 treated U937cells (Fig 3-4). From this result, it can be deduced that spatial control of cell cycle is through the retention of p-p70S6 in the cytoplasm, thereby preventing them from physical contact with their substrates or partner by p-p70S6 cytoplasmic inhibition through nuclear decrease and cytoplasmic increase whereas total protein un-changed.

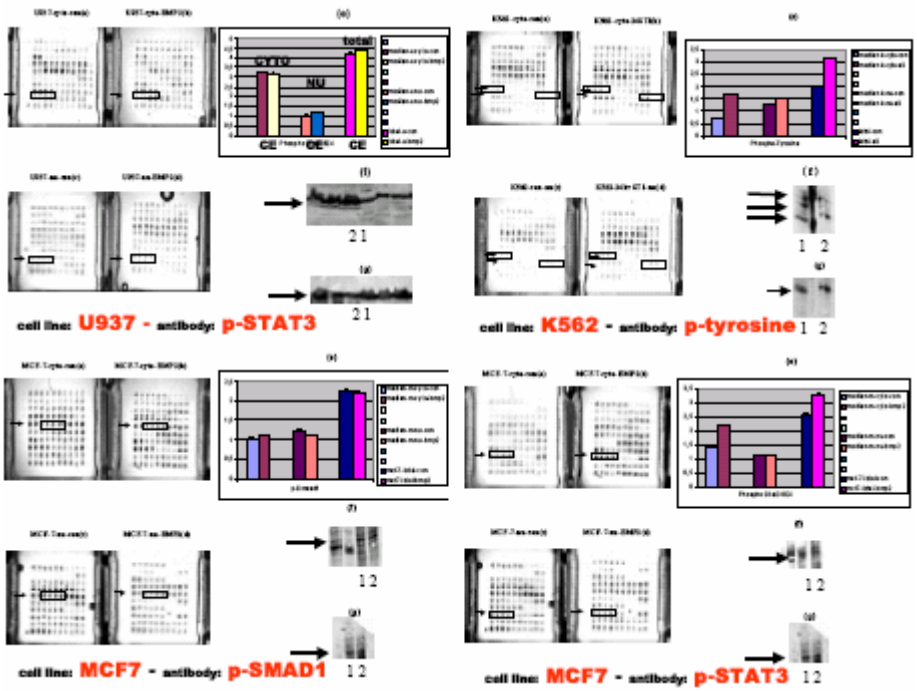

Fig. 2. Western blotting comparison with antibody microarray. (a-d) Antibody micro-array analysis, untreated (a,c) and treated (b,d) with 100ng/ml BMP2 for 4h. (a,b) Cytoplasm; (c,d) Nucleus. Position of antibody is marked with frame and arrow. (e) Graphic display of numerical value analysis. Each column indicates a mean of four measurements with standand deviations. (f) Western blotting analysis is consistent with antibody microarray.

Our study demonstrated p-ERK and p-p70S6 co-cytoplasmic increase in BMP2 treated MCF7 cells (Fig. 3-4). Edelmann, et al (1996) [10] using synchronized Swiss mouse 3T3 fibroblasts that p70 S6 kinase (p70S6k) and mitogen-activated protein kinases (p42MAPK/p44MAPK) are not only activated at the G0/G1 boundary, but also in cells progressing from M into G1. p70S6k activity increases 20-fold in G1 cells released from G0. Throughout G1, S, and G2 it decreases constantly, so that during M phase low kinase activity is measured. The kinase is reactivated 10-fold when cells released from a nocodazole-induced metaphase block enter G1 of the next cell cycle. From this point it can be reflected the G1 state in BMP2 treated MCF7 cells. Our study showed the p-Akt unchanged in BMP2 treated MCF7 cells. Akt controls cell growth through its effects on the mTOR and p70S6 (in the cytoplasm) kinase pathways. p70S6 kinase (p70S6K) belong to multifunctional kinases downstream of phosphatidylinositol 3 kinase (PI3K), such as the 70-kDa ribosomal protein S6 kinase (p70S6K) is itself a dual pathway kinase, signalling cell survival as well as growth through differential substrates which include mitochondrial BAD and the ribosomal subunit S6, respectively[12]. From this point it can be reflected the growth inhibition and cell survival state in BMP2 treated MCF7 cells. From all the above it can be concluded that the net effect of p-Akt unchanged whereas p-ERK and p-p70S6 co-cytoplasmic increase induced cell survival and differentiation in 100ng/ml BMP2 treated MCF7 cells for 4h. ERK is involved in BMP2-induced osteoblastic differentiation in mesenchymal progenitor cells and ERK protein level is up-regulated under BMP2 inducement [11]. Co-increase expression in cytoplasmic phosphorylation of ERK and p70S6 are maybe involved in the cooperative signallings. Subsequent induction of cell survival and differentiation led us to propose a co-operative model, which is

consistent with other study such as: Lehman et al.[4] indicated that a kinase from the MEK/MAPK pathway also plays a role in p70S6K activation by GM-CSF in a hematopoietic cell, the neutrophil.

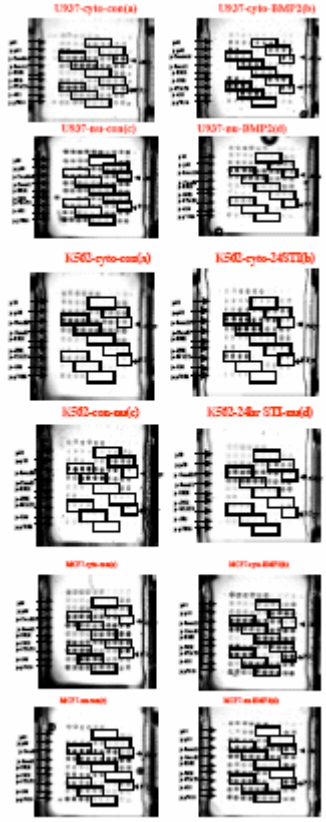

Fig. 3. Antibody micro-array analysis of phosphorylation and localization in control and BMP2 treated U937 cells. Untreated (a,c) and treated (b,d) with 2000ng/ml BMP2 for 3 days. In control and STI571 treated K562 cells. Untreated (a,c) and treated (b,d) with 0.2 μM STI571 for 24h. In control and BMP2 treated MCF7 cells. Untreated (a,c) and treated (b,d) with 100ng/ml BMP2 for 4h. (a,b) Cytoplasm; (c,d) Nucleus. Positions of phospho-p70S6 and phospho-Akt, phospho-ERK antibodies are marked with frame and arrow separately.

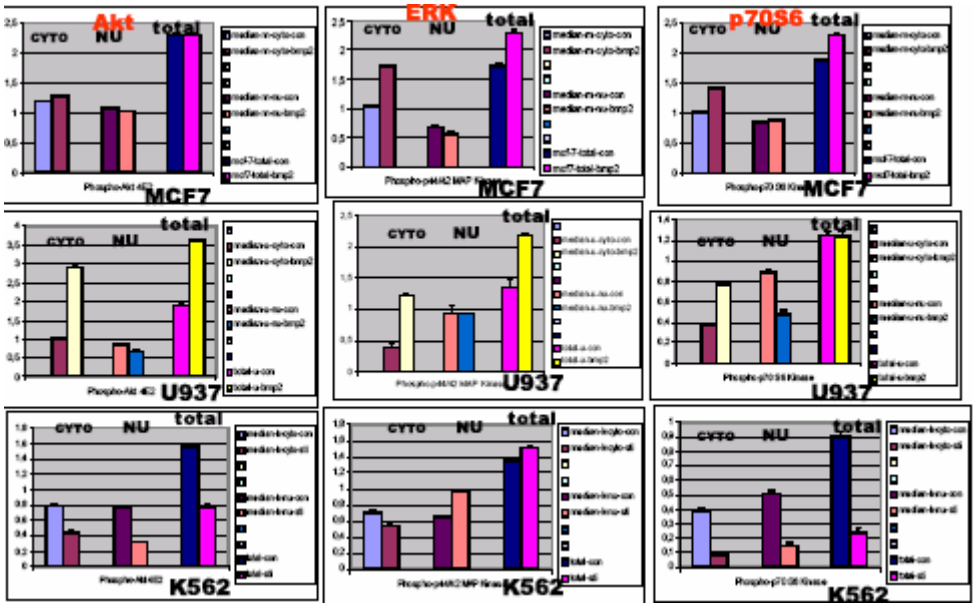

Fig.4. Graphic numerical value analysis of phosphorylation and localization modulation. In BMP2 treated U937 cells, p-ERK (thr202/tyr204) displayed the increase in cytoplasm and unchanged in nucleus. Total protein (sum of cytoplasm and nucleus) level of p-ERK is higher than in control cells; p-Akt(ser473) displayed the increase in cytoplasm and the slight decrease in nucleus**.** Total protein (sum of cytoplasm and nucleus) level of phospho-Akt

is higher than in control cells. P-p70S6(thr389) displayed the increase in cytoplasm and the decrease in nucleus in BMP2 treated U937 cells. Total protein (sum of cytoplasm and nucleus) levels of phospho-p70S6 was not altered compared with control; In STI571 treated K562 cells, p-ERK(thr202/tyr204) displayed the decrease in cytoplasm and the increase in nucleus. Total protein (sum of cytoplasm and nucleus) level of p-ERK is higher than in control cells. p-Akt(ser473) and p-p70S6(thr389) displayed the decrease in cytoplasm and nucleus. Total protein (sum of cytoplasm and nucleus) level of phospho-Akt and p-P70S6 are lower than in control cells; In BMP2 treated MCF7 cells, P-Akt displayed unchanged in cytoplasm and nucleus, p-ERK and p-P70S6 displayed the increase in cytoplasm and unchanged in nucleus . Total protein (sum of cytoplasm and nucleus) level of p-ERK and p-P70S6 were increased compared with control. Each column indicates a mean of four measurements with standand deviations. Different antibodies have different affinities, arrow indicating each antibody quantity value 1 position.

Our finding showed p-ERK nuclear increase in 0.2 uM STI571 treated K562 cells. ERK-1 and –2 nuclear translocation triggers cell proliferation in vitro models [7]. From this point it can be reflected the proliferation state in 0.2 uM STI571 treated K562 cells. This result is consistent with other finding that treatment of Bcr-Abl-expressing cells with STI571 elicits a cytoprotective MAPK activation response [13]. Our study showed p-Akt and p-p70S6 nuclear and cytoplasmic co-decrease in 0.2 uM STI571 treated K562 cells. Akt controls cell growth through its effects on the mTOR and p70S6 (in the cytoplasm) kinase pathways. Therefore, Akt and p70S6 nuclear and cytoplasmic co-decrease reflect the growth inhibition in 0.2 uM STI571 treated K562 cells. In sum, the net effect from ERK nuclear increase and Akt and p70S6 nuclear and cytoplasmic co-decrease reflect the growth inhibition and proliferation state in 0.2 uM STI571 treated K562 cells.

In conclusion of this paper, the different activation and silencing of Akt/ ERK/p70S6 signaling in treated human cancer cell lines are studied by proteomic analysis of co-localization using western blot, FACS and nano-gold phospho-antibody microarray by reaction with nuclear and cytoplasmic proteins. Our results show that Akt/ ERK signaling co-activation through p-Akt/ p-ERK co-cytoplasmic increase whereas p70S6 signaling inhibition through p-p70S6 by nuclear decrease and cytoplasmic increase (total protein unchanged) in BMP2–induced apoptosis U937 cells (2000ng/ml BMP2 for 3 days); p70S6/ ERK signaling co-activation through p- p70S6/ p-ERK co-cytoplasmic increase whereas Akt signaling inhibition through p-Akt unchanged in BMP2-treated MCF7 cells (100ng/ml BMP2 for 4h); Akt /p70S6 signaling co-inhibition through p-Akt/p-p70S6 nuclear and cytoplasmic co-decrease whereas ERK signaling activation through p-ERK nuclear increase in STI571-treated K562 cells (0.2 uM STI571 for 24h). This study implies that different activations and silencings of Akt/p70S6/ERK signaling reflect different functions.

References

[1] James P., “Protein Identification in the Post-genome Era: The Rapid Rise of Proteomics,” Q Rev Biophys, 1997, Vol.30, No.4, pp. 279-331.

[2] Zhu H., Bilgin M., Snyder M, “Proteomics,” Annu Rev Biochem, 2003, Vol. 72, pp. 783-812.

[3] Lorenzini A., Tresini M., Mawal-Dewan M., et al, “Role of the Raf / MEK/ERK and the PI3K/Akt (PKB) Pathways in Fibroblast Senescence,” Exp Gerontol, 2002, Vol. 37, No.10-11, pp.1149-1156.

[4] Lehman J. A., Calvo V., Gomez-Cambronero J., “Mechanism of Ribosomal p70S6 Kinase Activation by GM-CSF in Neutrophils: Cooperation of a MEK-related, T421/S424-kinase and a Rapamycin-sensitive, mTOR-related, T389-kinase,” J Biol Chem, 2003, Vol.278, pp. 28130-28138.

[5] Borgatti P., Martelli A. M., Bellacosa A., et al. “Translocation of Akt/PKB to the Nucleus of Osteoblast-like MC3T3-E1 Cells Exposed to Proliferative Growth Factors,” FEBS Lett, 2000, Vol. 477, No.1-2, pp. 27-32.

[6] Fleckenstein D. S., Dirks W. G., Drexler H. G., et al, "Tumor Necrosis Factor Receptor-associated Factor (TRAF) 4 is a New Binding Partner for the p70S6 Serine/ Threonine Kinase," Leuk Res, 2003, Vol.27, No.8, pp. 687-694.

[7] Tarnawski A.S., Pai R., Wang H., et al, "Translocation of MAP (Erk-1 and -2) Kinases to Cell Nuclei and Activation of c-fos Gene during Healing of Experimental Gastric Ulcers," J Physiol Pharmacol, 1998, Vol.49, No.4, pp. 479-488.

[8] Wang X., Martindale J. L., Holbrook N. J., "Requirement for ERK Activation in Cisplatin-induced Apoptosis," J Biol Chem, 2000, Vol. 275, No.50, pp.39435-39443.

[9] Valovka T., Verdier F., Cramer R., et al, "Protein Kinase C Phosphorylates Ribosomal Protein S6 Kinase BetaII and Regulates its Subcellular Localization," Mol Cell Biol, 2003, Vol. 23, No. 3, pp.852-863.

[10] Edelmann H. M., Kuhne C., Petritsch C., et al, "Cell Cycle Regulation of p70 S6 Kinase and p42/p44 Mitogen-activated Protein Kinases in Swiss Mouse 3T3 Fibroblasts," J Biol Chem, 1996, Vol. 271, No. 2, pp. 963-971.

[11] Xing X., Manske P. R., Li Y. Y., et al, "The Role of Sp1 in BMP2-up-regulated Erk2 Gene Expression," Biochem Biophys Res Commun, 2002, Vol. 297, No.1, pp.116-124.

[12] Harada H., Andersen J. S., Mann M., et al, "p70S6 Kinase Signals Cell Survival as Well as Growth, Inactivating the Pro-apoptotic Molecule BAD," Proc Natl Acad Sci U S A, 2001, Vol. 98, No.17, pp.9666-9670.

[13] Yu C., Krystal G., Varticovksi L., et al, "Pharmacologic Mitogen-activated Protein / Extracellular Signal-regulated Kinase Kinase / Mitogen-activated Protein Kinase Inhibitors Interact Synergistically with STI571 to Induce Apoptosis in Bcr/Abl-expressing Human Leukemia Cells," Cancer Res, 2002, Vol. 62, No.1, pp.188-199.

[14] Wölfl, S., Dummer, A., Pusch, L., Pfalz, M., Wang, L., Clement, J.H., Leube, I., Ehricht, R. Analyzing Proteins and Protein Modifications with ArrayTube Antibody Microarrays. In *Protein Microarrays* (M. Schena, ed.), Jones and Bartlett Publishers (Sudbury, MA), 2004.

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