Chapter 5

Profile of phosphorylation events controlled by hyper-activation of the MAPK signaling pathway in zebrafish liver cells

We have shown that that hyper-activation of the mitogen-activated protein kinase (MAPK) signaling cascade leads to mitogenic transformation of zebrafish liver cells. To dissect the signaling events involved in this transformation, we profiled the kinase activity and the phosphorylation events caused by hyper-activation of this pathway, using newly developed serine/threonine peptide microarrays. We found that phosphorylation of 7 peptides was strongly affected. Related proteins include radixin (RADI), vasodilator-stimulated phosphoprotein (VASP), cystic fibrosis transmembrane conductance regulator (CFTR), liver glycogen phosphorylase isoform 1 (PYGL), chorionic gonadotropin beta polypeptide (CGHB), forkhead box O3 (FOXO3) and neutrophil cytosolic factor 1 (NCF1). We have discussed the possible functions of these peptides in signal transduction. The findings have validated the application of peptide microarray based kinomics approaches in zebrafish cells, and confirmed the cellular alterations predicted by previous transcriptome study.

Keywords: serine/threonine, phosphorylation, Raf/MEK/ERK, ZFL

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Introduction

Protein phosphorylation is an essential mechanism of eukaryote cellular signaling [1]. It controls the activity, localization, stability and interactions of many proteins. At least 30% of all human proteins can be phosphorylated [2]. The phosphorylation events are catalyzed by protein kinases, which act as regulators of almost all cellular processes by phosphorylation of tyrosine, serine and threonine residues in target proteins, and are regulated by specific phosphatases, which remove the phosphate group added bythe kinases. The existence of hard-wired, cell type–specific kinase requirements has been demonstrated in normal healthy cells [3]. Deregulation of the kinases and phosphorylation events often leads to diseases, especially cancer. A large number of kinases are established proto-oncogenes whereas several phosphatases are established tumor suppressors. Currently, protein kinase targets are subjects in 20-30% of the drug discovery programs in pharmaceutical industry [4]. It is estimated that over half of the 518 human protein kinases will be found to be essential in at least one cancer cell line [3]. Hyperactive signaling by kinases can drive cancer growth as well as metastasis. The specific downstream target events of most kinase and their regulation mechanism remain largely unknown, especially those *in vivo* events leading to cell transformation.

In this study we have investigated the specific protein targets of the mitogen-activated protein kinase (MAPK) signaling pathway. This pathway was chosenbecause its aberrant activation is found in most types of human as well as zebrafish tumors and the MAPK pathway provides a broad target area for cancer drug intervention [5, 6]. However, the downstream signaling events of MAPK pathway remain elusive. Since the recent discovery of all MAPK homologs in the zebrafish, it is considered as a very versatile model to study this protein family. Previous research revealed a high level of genetic and functional conservation of this cascade between zebrafish and human [7, 8]. The serine/threonine kinase Raf and its downstream MEK/ERK cascade play essential roles in cell proliferation, differentiation and apoptosis. To obtain more insights in this pathway, we generated a ZFL-ΔRaf1-ER stable cell line where the inducible activation of human oncogenic Raf-1 activates zebrafish MEK/ERK cascade (He *et al.*, submitted). The hyper-activation of the ΔRaf1/MEK/ERK pathway resulted in a number of survival and growth advantages in the zebrafish liver cells, including the self-sufficiency with respect togrowth factors.. Unconstrained proliferation and impaired apoptosis are both key hallmarks of cancer, suggesting that the zebrafish liver cells were (partially) transformed by the hyperactive MAPK signaling.

In order to dissect the complex MAPK signaling network and to identify the direct/indirect protein targets regulated via phosphorylation by the MEK/ERK cascade, we took an innovative approach to analyze *ex vivo* kinase activity profiling directly in cell lysates.Pamchip serine/threonine peptide microarrays were applied to profile the phosphorylation events in the zebrafish liver cells transformed by the hyperactive Raf/MEK/ERK signaling. This is a novel non-radioactive technology to profile kinase activity using peptides encoding known phosphorylation sites as substrates spotted onto porous chips (Figure 2) [9, 10]. Application of cell or tissue lysates containing active kinases allows the peptides to be phosphorylated *ex vivo*, which can be detected by fluorescent antibody against the phorsphorylated tyrosine or serine/threonine residues. It is a convenient and powerful technology, allowing

detection of kinase activity profiles in the cells and direct identification of the phosphorylated protein targets of the kinase of interest, which facilitate investigation of pathway specific phosphorylation patterns and prediction of the downstream kinase targets for drug discovery. As a novel non-radioactive technology allowing high throughput analysis of kinase reactions, the PamChip® peptide microarrays have been applied to analyze activity of synthetic kinases or lysates from mammalian cell cultures and tissues. It was also used to profile endogenous phosphotyrosine signaling in zebrafish embryos [10]. This study, presents the first time that this technology was applied to zebrafish cell cultures.

Results

4-hydroxytamoxifen-dependent ΔRaf1 activation leads to hyper-activation of the MEK/ERK cascade in the ZFL-ΔRaf1-ER stable cell line

1 μM 4-hydroxytamoxifen (4HT) was applied to the ZFL-ΔRaf1-ER cells to activate ΔRaf1. The ΔRaf1 activation significantly induced phosphorylation of zebrafish MEK1/2 and ERK1/2 in serum-starved ZFL-ΔRaf1-ER cells (Figure 1). The 4HT administration was performed at the time points 1, 3, 6, 12 and 24 hours, where the most significant hyper-activation of the ERK1/2 was induced by 1 hour 4HT administration. Addition of the MEK inhibitor U0126 to the ZFL-ΔRaf1-ER cells completely abolished the induction of the ERK1/2 phosphorylation, indicating that the ERK1/2 phosphorylation induced by ΔRaf1 activation is dependent on the MEK1/2 activity (Figure 1).

Figure 1. Activation of ΔRaf1 by 4-hydroxytamoxifen induced hyper-activation of the MEK/ERK cascades in the ZFL-ΔRaf1-ER cells.
(A) Phosphorylation of ERK1/2 was detected by western blot analysis. (B) Relative phosphorylation level of ERK1/2 was normalized against the ERK1 expression in each sample.

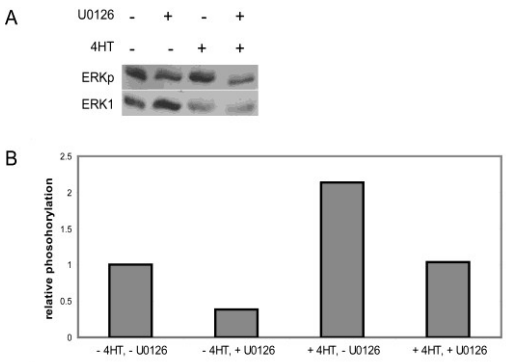
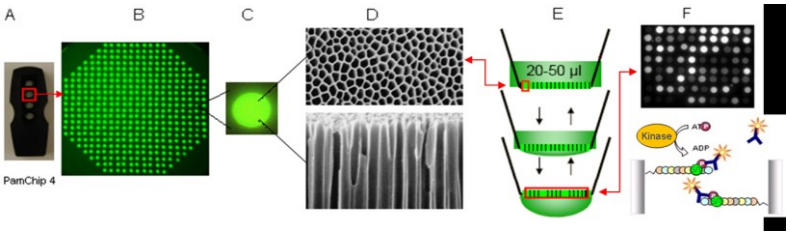


Figure 2. The PamChip® peptide microarray.
The PamChip® serine/threonine peptide microarray (A) contains 144 peptide substrates for serine/theronine kinases (B). Each unique peptide was spotted (C) and coupled to a porous 3D surface made of a microporous material containing millions of 0.2 x 60 micron pores (D). After sample application, cell lysates containing kinases were actively "pulsed" backwards and forwards (E) through these pores where they interact by phosphorylating the immobilized peptides. Phosphorylation of the peptides on the chip is detected by a cocktail of fluorescently labelled anti phosphoserine/threonine antibodies and monitored by CCD camera (F).



PamChip serine/threonine kinase microarrays were applied to profile the kinase activity and phosphorylation events in zebrafish cell lysates

In order to measure the cellular phosphorylation events upon the hyper-activation of the MEK/ERK cascade induced by the ΔRaf1 activation at the kinome level, the PamChip® peptide microarrays were applied using the zebrafish liver cell lysates. The PamChip® peptide microarrays are aligned in multi-well plates (Figure 2A). Each microarray contains 144 peptide substrates serine/theronine kinases (Figure 2B). It incorporates a micro-fluidic system where unique peptides coupled to a porous 3D surface are spotted (Figure 2C). This microporous material contains millions of 0.2 x 60 micron pores in parallel orientation connecting the top and bottom surfaces (Figure 2D). Peptides are pre-coupled to the large internal surfaces of these pores. For kinase activity measurement, samples containing kinases are actively "pulsed" backwards and forwards through these pores where they interact by phosphorylating the immobilized peptides (Figure 2E). Such "pulsing", combined with minimization of diffusion distances, results in significantly reduced assay times. Due to the high efficiency of this method, usually 1 μg of total protein is sufficient to monitor 144 phosphorylation reactions. Phosphorylation of the peptides on the chip is detected by fluorescently labeled anti phospho-tyrosine or a cocktail of anti phosphoserine/threonine antibodies and monitored by a CCD camera (Figure 2F). To establish the assay condition, lysates of zebrafish liver cells with various amount of protein contents were loaded on the PamChip® peptide microarrays in the presence or absence of ATP, and the resulted signals were kinetically measured (Figure 3). The results showed that 0.5 μg protein is optimal, provides significant signal without early saturation and allows kinetic measurement of kinase activities in zebrafish liver cell lysates.

7 peptides were phosphorylated by hyper-activation of the MAPK signaling cascade in the ZFL-ΔRaf1-ER stable cell line

In order to measure the cellular phosphorylation events upon the hyper-activation of the MEK/ERK cascade induced by the ΔRaf1 activation at kinome level, lysates of the ZFL-ΔRaf1-ER cells untreated and treated for 1 hour with 4HT were loaded on PamChip® serine/threonine kinase array. Data processing of the PamChip peptide microarrays using Bionavigator software showed that 7 peptides were specifically hyper-phosphorylated due to 4HT administration and ΔRaf1 activation (Figure 4). Information on these peptides RADI, NCF1, CGHB, VASP, PYGL, FOXO3 and CFTR is summarized in Table 1.. All of these targets were inhibited by application of the MEK inhibitor U0126, indicating that the hyper-phosphorylation was dependent on the MEK/ERK cascade downstream of the ΔRaf1 activation (Figure 4). The identified targets are involved in cellular processes such as cell adhesion and motility, cellular transport, apoptosis and metabolism, indicating that these processes can be regulated via phosphorylation events controlled by the MAPK signaling. It also suggests that the activation or suppression of these target proteins can be involved in cell transformation.

Figure 3. Optimization of applying the PamChip® peptide microarray to zebrafish cell lysates.

ZFL cell lysates with different amount of protein content were loaded on PamChip® serine/threonine peptide microarrays. Background signal on the arrays was identified using conditions without applying ATP to the lysates.

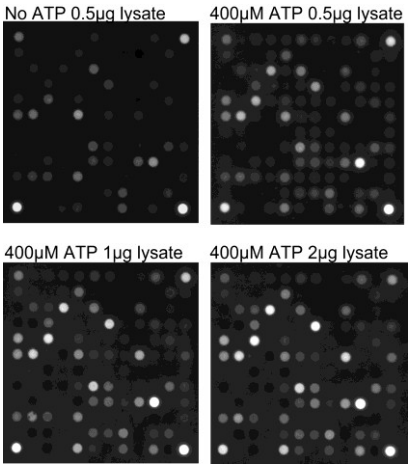


Figure 4. Hyper-phosphorylated of 7 peptides upon ΔRaf1/MEK/ERK activation in the ZFL-ΔRaf1-ER stable cell line.

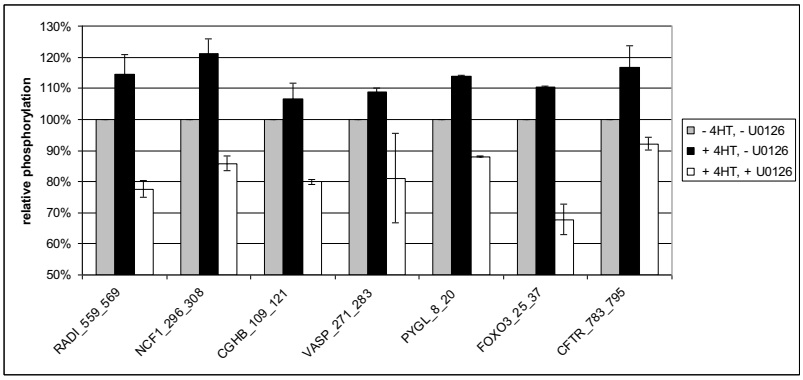


Table 1:

Gene	Full name	Phosphorylated residue	
RADI	radixin	559-569	rdkykTlrqir
NCF1	neutrophil cytosolic factor 1	296-308	rgapprrSSirna
CGHB	chorionic gonadotropin beta polypeptide	109-121	qcalcrrSTtdcg
VASP	vasodilator-stimulated phosphoprotein	271-283	larrrkaTqvgek
PYGL	liver glycogen phosphorylase isoform 1	8-20	qekrrqiSirgiv
FOXO3	forkhead box O3	25-37	qSrprScTwplqr
CFTR	cystic fibrosis transmembrane conductance regulator	783-795	ihrkttaStrkvS

Description of the proteins represented by the identified target peptides

Radixin (RADI) is a cytoskeletal protein that belongs to the ezrin-radixin-moesin (ERM) protein family. ERM proteins function as cross linkers between the membrane proteins and actin filaments and they are involved in many processes such as cortical cytoskeleton organization, cell survival, adhesion, motility and proliferation [11]. Radixin is the dominant ERM protein in the liver, essential for the formation of the hepatocyte microvilli and maintenance of apical canalicular membrane structure and function in rat hepatocytes [12]. Deregulation of radixin has been associated to liver diseases such as cholestasis [12]. It is known that the activation of radixin is dependent on the phosphorylation of the Thr^{564} in the C-terminal domain, which leads to the conformational alterations of radixin and its binding to the cellular cytoskeleton [11].
In the ZFL-ΔRaf1-ER cell lysate, the hyper-activated MAPK signaling cascade led to the phosphorylation of Thr^{564}, which suggests that radixin can be activated by the MAPK cascade in zebrafish liver cells. Thus the MAPK signaling could affect the cytoskeleton organization via regulation of radixin, which in turn affects cell adhesion, motility and vesicular transport.

Vasodilator-stimulated phosphoprotein (**VASP**) is a member of the Ena-VASP protein family. It was initially characterized as a proline-rich substrate of protein kinases A and G in human platelets and later shown to be a scaffold protein associated with filamentous actin formation and involved in cell adhesion and motility [13]. The hyper-activated MAPK cascade in zebrafish liver cells phosphorylated Thr^{278}, which is located in the EVH2 domain of VASP. This phosphorylation may have an inhibitory role on F-actin binding/bundling by VASP, which in turn affects cellular adhesion to extracellular matrix [14].

Cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ATP-binding cassette (ABC) transporter super family, which belongs to the MRP subfamily that is involved in multi-drug resistance. CFTR functions as a chloride channel and controls the regulation of other transport pathways. Its activity is controlled by a series of phosphorylation events, mainly in the central regulatory (R)-domain [15]. In the zebrafish liver cell lysate, phosphorylation of Ser^{790} and Ser^{795} was detected, which are known to be functionally involved in activation of CFTR and modification of its activity. It was reported that the phosphorylation of Ser^{795} (by PKA) was involved in activation of CFTR and the phosphorylation of Ser^{790} (by PKC) might function to modulate the activation [15]. Besides PKA and PKC, other serine/theronine kinases may also phosphorylate and regulate CFTR activity [15]. Our result indicates that the MAPK signaling can induce the chloride influx and affect many signaling events controlled by the chloride. Further experiments are required to investigate whether the MAPK cascade phosphorylate CFTR in a direct manner.

Liver glycogen phosphorylase isoform 1 (PYGL) is a homodimeric protein that catalyses the cleavage of alpha-1,4-glucosidic bonds to release glucose-1-phosphate from liver glycogen stores. It is known that PYGL is switched from inactive phosphorylase B state to active phosphorylase A state by the phosphorylation of Ser^{15} [16, 17], which was phosphorylated by the hyper-activated MAPK cascade in zebrafish liver cells. It strongly indicates the essential role of the MAPK signaling

in hepatic glycogen metabolism and support to elevated proliferation of the liver cells.
Deregulation of liver metabolism is often associated with human diseases, especially liver tumorigenesis. In zebrafish liver tumor, many genes involved in metabolism were transcriptionally deregulated, indicating that the cells undergo mitogenic transformation without differentiating into functional hepatocytes [6]. Similar genetic alterations were found in the zebrafish liver cells with hyper-activation of the MAPK signaling (Chapter 4). The phosphorylation profiling in the same cell line has found that a key player of liver glycogen metabolism can be activated by the MAPK signaling.

Chorionic gonadotropin beta polypeptide (CGHB) is a member of the glycoprotein hormone beta chain family and encodes the beta 3 subunit of chorionic gonadotropin (CG). Ectopic expression of CGHB is frequently found in many common epithelial carcinomas and is often associated with high aggressiveness of the cancer and the resistance to therapy [18]. Various studies have shown that the free CGHB may stimulate tumor growth or inhibit its apoptosis [19]. The free CGHB can be phosphorylated on Ser^{116} and Thr^{117} [20], which were detected in the zebrafish cell lysate. But the function of these phosphorylation events remains unclear, which might be further characterized in the ZFL-ΔRaf1-ER cell line.

Forkhead box O3 (FOXO3) belongs to the forkhead family of transcription factors. It is involved in the transcriptional activation of proapoptotic molecules [21]. Inhibition of the transcriptional activity of FOXO3 can promote cell transformation, tumor progression, and angiogenesis during tumor development [21]. In response to growth factor and insulin stimulation, FOXO3 can be phosphorylated by kinases including AKT, IKK, and ERK. The three kinases phosphorylate FOXO3 at different phosphorylation sites, which all lead to FOXO3 translocation from the nucleus to the cytoplasm and the subsequent degradation [21]. It is known that Thr^{32} of human FOXO3 can be phosphorylated in an AKT-dependent manner, which enhances FOXO3/14-3-3 interaction and promotes FOXO3 nuclear export to the cytoplasm, resulting in the repression of FOXO3 transcriptional function [21]. In the zebrafish liver cells, the hyper-activated MAPK cascade also phosphorylated Thr^{32} of FOXO3, resulting in suppression of its function. It is not clear yet weather the phosphorylation was dependent on AKT. Previous study has shown that the activation of ΔRaf1 inhibited apoptosis in the zebrafish liver cells (Chapter 4). The phosphorylation of FOXO3 identified in this study indicates that the anti-apoptotic role of the ΔRaf1 activation in the zebrafish liver cells might (partially) rely on the suppression of FOXO3.

Neutrophil cytosolic factor 1 (NCF1) also named as $p47^{phox}$, is a cytosolic subunit of the NADPH oxidase which produces superoxide anion. NADPH is a large membrane bound multi-protein enzyme consisting of six subunits. The NCF1 subunit is the key controlling the assembly and activation of the NADPH complex. In the inactive state, NCF1 stays in an auto-inhibited conformation which prevents its interaction with other subunits [22]. When NCF1 are phosphorylated on Ser^{304} and Ser^{304}, it can be released from the auto-inhibited conformation and lead to the NADPH activation [22]. The detected phosphorylation of these two serine residues suggests that the NADPH oxidase can be activated by the hyper-activated MAPK cascade in the zebrafish liver cells. It is known that deregulated activation of the

NADPH oxidase can lead to overproduction of reactive oxygen species and inflammatory responses [22]. Thus the phosphorylation of NCF1 and activation of the NADPH oxidase may contribute to the transcriptional alteration of inflammatory genes in neutrophils. Besides the anti-apoptotic function, previous transcriptome study also revealed that the ΔRaf1 activation in zebrafish liver cells led to deregulated expression of many genes involved in inflammation (Chapter 4) perhaps providing a link with this protein.

Discussion

Because deregulated activity of selected kinases is an important feature of cancer cells, kinase activity profiling can provide mechanism-based cancer biomarker identification. To this goal the zebrafish cell model and the activated MAPK signaling pathway was selected for the case study in the search for cancer drug targets using the PamChip peptide microarray application. This kinomics approach provides the unique ability to kinetically profile *ex vivo* kinase activity in cell lysates. It allows rapid assessment of changes of protein activity and signal transduction associated with biological alterations and transformation, providing more insights for drug target candidates.

Our previous study showed that ΔRaf1 was activated by 4HT administration in the ZFL-ΔRaf1-ER cells, which in turns activated the MEK/ERK cascade. The hyper-activation of the MAPK cascade inhibited apoptosis and promoted cell survival and growth. In order to identify the downstream protein targets causing the partial mitogenic phenotype, the kinase activity of these cells were analyzed using the PamChip® serine/threonine kinase array.

The results showed that 7 peptides were specifically phosphorylated by the cell lysates. It indicates that the activity of proteins containing these peptide sequences can be regulated by the hyper-active ΔRaf1/MEK/ERK cascade in zebrafish liver cells. Cellular processes affected by regulation of these protein activities include cell adhesion, motility, vesicular transport, glycogen metabolism, apoptosis suppression and inflammation response. The same biological alterations were revealed by previous transcriptome study using the same cell line, showing that analysis at different level using different approaches can be bridged together and point to the same direction.

The identified phosphorylation events strongly suggest that the related proteins should be involved in mitogenic transformation of zebrafish liver cells. Furthermore, it was discovered using the same kinase array that phosphorylation of $Ser^{303/304}$ of NCF1, Ser^{795} of CFTR and Thr^{32} of FOXO3 was suppressed by ERK knockdown in zebrafish embryos (data not shown). The fact that these phosphorylations were induced by active MAPK signaling and suppressed by inactivated MAPK signaling shows that these three proteins indeed participate in the signaling network downstream of MAPK in zebrafish liver cells, with high potential as direct protein targets of ERK.

It is also noted that 4 out of the 7 peptides were reported to be phosphorylated by synthetic PKA on the same serine threonine kinase array, including $Ser^{303/304}$ of NCF1, Ser^{15} of CGHB, Thr^{278} of VASP and Ser^{15} of PYGL [23]. It had been suggested that Ser^{795} of CFTR can be phosphorylated by PKA [15], but this phosphorylation was not detected *in vitro* on the PamChip. The detection of phosphorylation of PKA substrates in the cell lysate containing activated MAPK cascade can be due to the fact that multiple serine/theronine kinases share the same target. It can also due to the activation of PKA by MAPK signaling, suggesting the crosstalk of the ERK and PKA signaling pathways.

Similarly it was observed that the Thr^{32} of FOXO3 was phosphorylated upon hyper-activation of the ΔRaf1/MEK/ERK cascade. FOXO3 can be phosphorylated by ERK, AKT and IKK, all leading to inhibition of the transcriptional activity of FOXO3. In human, it is known that ERK, AKT and IKK phosphorylate FOXO3 at different specific sites: ERK on Ser^{294}, Ser^{344}, and Ser^{425}, AKT on Thr^{32}, Ser^{253} and Ser^{315}, and IKK on Ser^{644} [21]. The detected phosphorylation of FOXO3 Thr^{32} might be due to the activation of AKT by MAPK signaling. Alternatively, it is possible that the molecular structures/mechanisms of FOXO3 are evolutionally different in zebrafish and in human.

Taken together, we showed that it is feasible to effectively profile kinomic events of zebrafish cells using the PamChip peptide microarray approach. We profiled the kinase activity and phosphorylation events caused by hyper-activation of the MAPK signaling cascade in the zebrafish liver cell line. The results indicated activation of proteins involved in cell adhesion, motility, cellular transport and metabolism, together with suppression of transcription activator of apoptotic factors. The same alterations were observed in the previous transcriptional study on the same cell line, suggesting that the regulation of these proteins can play a role in cell transformation, which can be further dissected using zebrafish liver tumor models. It suggested that the peptide microarray based kinomics approach can be combined with widely used transcriptomics approaches to better analyze signal network involved in cell transformation and malignancies and to select candidate drug targets. In the future, responsible genes and protein targets will be validated using gene knockdown technology or chemical inhibitors of selected protein targets identified by the kinomics and transcriptomics approaches. Such pathway specific mechanistic studies will help to improve our understanding of the basic kinase biology and contribute to drug discovery.

Material and Methods

Cell culture

See chapter 3.

Sample preparation and analysis

The ZFL-ΔRaf1-ER stable cells were seeded in 12-well plate and grew into confluence after 24 hours. The cells were starved for 24 hours in plain medium. After 30 minutes pretreatment in 30 μM U0126, the cells were incubated in plain medium supplemented with 1μM 4-hydroxytamoxifen (Sigma) in absence or presence of 30 μM U0126. The cells were washed twice with ice-cold PBS and lysed with 100 μl lysis buffer supplemented with phosphatase inhibitor and protease inhibitor according to manufacture's instruction (Pamgene). The lysates were stored at -80°C prior to measurement. Proteins electrophoresis and Western blot protocols are described in chapter 3.

In vitro phosphopeptide profiling

The phosphopeptide profiles were identified using the PamChip® peptide microarrays containing 144 peptide substrates for serine/theronine kinases, according to manufacture's instruction (PamGene)[23]. 0.5 μg protein of each cell lysate and 400 μM ATP were loaded on the array. Incubations and kinetic reading of the PamChip peptide microarrays were performed on a PamStation 4 instrument (PamGene), which allows fully automated incubation, washing, and imaging of 4 microarrays simultaneously. Each sample was loaded on two independent arrays for technical duplicates.

Data analysis

Data generated on PamChip peptide microarrays were processed with Bionavigator software (PamGene) [23]. Because most peptides were over-exposed at exposure time of 50 ms while only 1~2 pre-phosphorylated control peptides were over-exposed at 20 ms, the data at exposure time of 20 ms was used for analysis. The average MedBg signal is 361 with a CV of 9% and a STDEV of 32. The peptides with signal above 64 (2x STDEV) were used for comparisons across conditions.

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