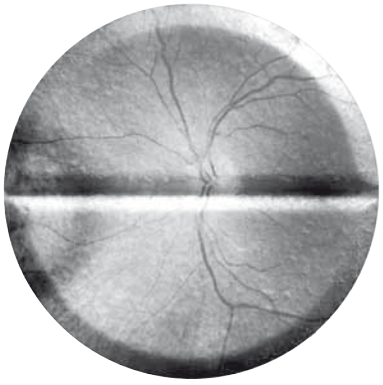
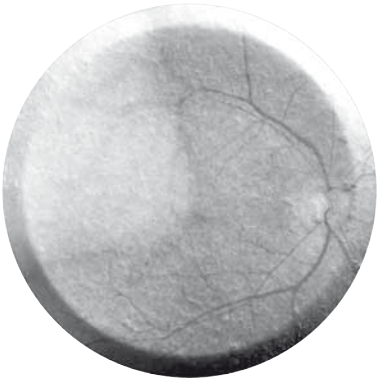
CHAPTER 9

EPISODIC SRC ACTIVATION IN UVEAL MELANOMA REVEALED BY KINASE ACTIVITY PROFILING

W. Maat, M. Filali, A. Dirks-Mulder, G.P.M. Luyten, N.A. Gruis, L. Desjardins, P. Boender, M.J. Jager, P.A. van der Velden

Br J Cancer. 2009 Jul 21;101(2):312-9





ABSTRACT

The RAS/RAF/MEK/ERK pathway is involved in the balance between melanocyte proliferation and differentiation. The same pathway is constitutively activated in cutaneous and uveal melanoma and related to tumor growth and survival. Whereas mutant BRAF and NRAS are responsible for activation of the RAS/RAF/MEK/ERK pathway in most cutaneous melanoma, mutations in these genes are usually absent in uveal melanoma. We set out to explore this pathway and used mitogen-activated protein kinase profiling and tyrosine kinase arrays and identified Src as a kinase that is associated with ERK1/2 activation in uveal melanoma. However, low Src levels and reduced ERK1/2 activation in metastatasis cell lines suggest that proliferation in metastases can become independent of Src and RAS/RAF/MEK/ERK signaling. Inhibition of Src led to growth reduction of primary uveal melanoma cultures and cell lines and thereby identified Src kinase as a potential target in primary uveal melanoma treatment. Metastasis cell lines displayed a more resistant phenotype and indicate that in metastases a different approach may be required.

INTRODUCTION

Uveal melanoma (UM) is a rare neoplasm, which arises from melanocytes in the eyes. It usually affects people in their sixties with an incidence rate of approximately 6-8 new cases per million per year among Caucasians [1,2]. Little is known about the molecular pathogenesis of UM as compared to cutaneous melanoma (CM). CM and UM share the same embryonic origin and similar histological features, but mutations that regulate proliferation and cause loss of cell cycle control in CM can hardly be found in UM. Whereas p16-regulated cell cycle control is targeted by deletion of chromosome 9p or mutation of CDKN2A in CM, most of UM cell lines posses a wildtype p16-encoding gene that is, however, not expressed due to epigenetic modification of the CDKN2A gene [3]. The same may be true for activation of the RAS-RAF-MEK-ERK, or the classical mitogen-activated protein kinase (MAPK) pathway. MAPK activation is crucial in the development of melanocytic neoplasia and constitutive activation of this pathway has been associated with many different types of cancer [4,5]. In CM, activation of the MAPK pathway has been shown to occur by a variety of mechanisms, including autocrine growth factor stimulation and mutation of the NRAS (20% of cases) and BRAF (60% of cases) genes [6-8]. BRAF mutations have only rarely been reported in UM and activating mutations in NRAS, which are found in 25% of all cancers, have never been reported [9-14]. However, we and others have found that UM are heterogeneous and that, with more sensitive techniques, the percentage of mutant BRAF-positive UM may be higher [15,16]. The lack of mutations in the majority of cells is in contrast with immunohistochemistry and Western blot analysis that have shown activation of ERK1/2 in most UM [13,17,18]. Still, pharmacological inhibition of MAPK/ERK kinases 1 and 2 (MEK1/2) and genetic targeting of BRAF with siRNA resulted in a reduced proliferation of UM cell lines [19,20]. This indicates that although mutations are absent, the RAS-RAF-MEK-ERK pathway is essential for UM growth and suggests that an upstream factor is involved in autonomous UM proliferation. Recently, c-Kit was shown to be upregulated in uveal melanoma and involved in an autocrine loop that also involved the RAS-RAF-MEK-ERK pathway [20]. An incomplete response to c-Kit inhibition indicates that additional factors are involved [21]. Also the GNAQ gene was shown to be mutated in almost half of the uveal melanoma [22]. GNAQ is part of the G-protein heterotrimer and represents the GTP-binding part that couples GPCR signaling to MAPK activation which marks it as a potential therapeutic target. However, targeting downstream signaling molecules may be just as effective as they may be shared with other mutant pathways. Tyrosine kinase activity profiling in uveal melanoma was used to explore the involved kinases. Based on a UM cell line and two related metastasis cell lines which revealed reduced ERK1/2 activation in metastases, we were able to identify Src as a crucial upstream tyrosine kinase for ERK1/2 activation in primary UM. Unfortunately, metastasis cell lines appeared less dependent on Src and may indicate that metastasis may require an alternative approach for intervention.

MATERIAL AND METHODS

Cell lines and tumor material

Eleven cell lines derived from primary UM (92.1; OCM-1, -3 and -8; Mel-202, -270, -285, -290) and UM metastases (OMM-1, -2.3, and -2.5) were analyzed for kinase activity [23-26]. UM cell lines were cultured in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 3mM L-glutamine (Gibco), 2% penicillin/streptomycin and 10% FBS (Hyclone, Logan, UT). Primary UM were cultured in Amniochrome Pro Medium (Lonza Group Ltd, Basel, Switzerland). All cell cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Cell lysates were obtained by lysing cells in M-PER Mammalian Protein Extract Reagent (Pierce, Rockford, IL), supplemented with 1% Halt Protease Inhibitor Cocktail, EDTA-free (Pierce) and 1% Halt Phosphatase Inhibitor Cocktail (Pierce). Protein concentrations were measured by using the BCA Protein Assay kit (Pierce). Cell lysates were also acquired from three fresh primary UM samples obtained by enucleation and from three liver metastases of three different patients, in which the diagnosis was confirmed.

Phospho-MAPK Array

The Human Phospho-MAPK Array (R&D Systems, Abingdon, UK) was used to simultaneously detect the relative levels of nine MAPkinases and nine other serine/threonine kinases in cell lines, primary UM and liver metastasis. In this array, capture and control antibodies were spotted in duplicate on nitrocellulose membranes. Experiments were carried out according to the manufacturer's guidelines. In short, cell lysates were diluted and incubated with the array. After binding of both phosphorylated and unphosphorylated kinases, unbound material was washed away. A cocktail of phospho-site specific biotinylated antibodies was used to detect phosphorylated proteins via Streptavidin-HRP and chemiluminescence. The X-ray films of the blots were scanned and analyzed using the G-boxHR (Syngene, Frederick, MD). Control spots with mouse, goat and rabbit antibodies were used for background correction.

PamGene Tyrosine Kinase Array

Experiments were performed by using a 4-array semi-automated system (PamStation 4, PamGene, `s-Hertogenbosch, The Netherlands) designed for processing PamChip-4 arrays. The PamChip Tyrosine Kinase Array (PamGene) contains 144 phospho-peptides, immobilized on a porous microarray surface via the peptide N terminus, representing tyrosine kinase substrates. Each array was blocked with 0.2% Bovine Serum Albumin, fraction V (Calbiochem Immunochemicals, Merck KGaA, Darmstadt, Germany) by pumping it through the porous microarray for 30 cycles of 30 seconds. Thereafter, each array was washed three times for 8 seconds with 1x ABL Protein Tyrosine Kinase Reaction Buffer solution (New England Biolabs, Ipswich, MA). Next, incubation was performed at 30°C with the reaction mix, containing 5µg cell lysate, 4µl 100x BSA (New England Biolabs, Ipswich, MA),

0.4µl 10mM ATP (Sigma-Aldrich, Zwijndrecht, the Netherlands), 0.5µl 1mg/ml Monoclonal anti-phosphotyrosine FITC conjugate (clone Py20, Exalpha Biologicals, Maynard, MA), adjusted to 40 µl with distilled H_2O. The sample was pulsed back and forth through the porous material for 45 cycles, which is coupled to the base of a well to maximize reaction kinetics and to reduce analysis time. Every 5 pump cycles, a 16-bit TIFF image was taken with a built-in CCD camera.

Blocking experiments were carried out with Src family-selective tyrosine kinase inhibitors, PP1, PP2 (Biomol international, LP, of Plymouth Meeting, PA) and PP3 (the inactive analogue, Calbiochem), at an end concentration of 10µM in line with a large body of literature. Each particular inhibitor was mixed with lysates of cell lines and tissue together with the reaction mix just before incubation on the array.

Acquired data from the PamStation 4 was captured with the supplied software package BioNavigator (Version 0.3.1; PamGene). For the purpose of finding differentially phosphorylated substrates, the data was imported in the LIMMA package and we applied the empirical Bayes method [27]. Background subtracted data was normalized for differences between experiments and substrates and P-values of 0.05 or less were corrected for multiple testing using Benjamini and Hochberg correction. Substrates with a corrected P-value of 0.05 or less were assumed significant.

Western blot analysis

Cell lysates (10 µg) were separated on 12.5% SDS-PAGE gels and proteins were transferred to Hybond-polyvinyldifluoride membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked with 5% skim milk in PBS-Tween 0.1% solution and probed at room temperature for 1 hour with antibodies specific to each antigen: Phospho-Src (Tyr527; dilution 1:1000), Phospho-Src Family (Tyr416; dilution 1:1000) and Src (36D10; dilution 1:1000) antibody (all from Cell Signaling Technology, Hertfordshire, UK). An antibody against Actin (Abcam, Cambridge, UK) was used as a loading control. Membranes were subsequently incubated at room temperature with horseradish peroxidase-conjugated IgG anti-mouse or anti-rabbit secondary antibodies for 1 hour. Supersignal West Femto ECL (Pierce) was used to visualize protein bands on the membrane.

siRNA treatment

Sub-confluent cell cultures were grown without antibiotics 24 hours prior to transfection in RPMI 1640 medium. A mixture of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and two different siRNA constructs (40 nM) were incubated in standard medium with reduced serum (1%) all in line with the advice of the manufacturer. The siRNA constructs (Stealth) were predesigned and validated (~70% knock down) by the manufacturer (Invitrogen). After 24 and 48 hours the cells were harvested and RNA and protein lysates were prepared.

WST-1 assay

Cell proliferation in response to PP1 (10 and 50μM) was measured by mitochondrial function using the WST-1 proliferation reagent (Sigma-Aldrich) as previously described [28]. This assay measures tetrazolium reductase activity in the mitochondria which serves as a measure of cell viability. In short, 96-well plates were filled with 1250 uveal melanoma cells per well. One (tumor 1-5) or six days (92.1; OCM-1, -3 and -8; Mel-202, -270, -285, -290, OMM-1, -2.3, and -2.5) after treatment, WST-1 reagent was added and absorbance was measured at 450nm on a multiwell spectrophotometer. The median and standard error of 8 wells were taken at each time and dosage point.

QPCR

The cell lines (92.1; OCM-1, -3 and -8; Mel-202, -270, -285, -290, OMM-1, -2.3, and -2.5) were analyzed for Src gene expression. Primers for Src and the reference gene, β-actin, were developed with Beacon Designer software (Premier Biosoft, Palo Alto, CA). Primer sequences for Src: 5'-GCTGCGGCTGGAGGTCAAG-3' (forward) and 5'-AGACATCGTGCCAGGCTTCAG-3' (reverse). Primer sequence for β actin; 5'-CGGGACCTGACTGACTACCTC-3' (forward) and 5'-CTCCTTAATGTCACGCACGATTTC-3' (reverse). The PCR reaction settings were 95°C for 5 min, then 40 cycles at 96°C for 15 s and 60°C for 45 s. DNA melting point of the amplicons were acquired by measuring the fluorescence of SYBR Green during a linear temperature transition from 70°C to 97°C at 0.2°C each 10 seconds with accompanying software (Bio-Rad, Hercules, CA).

RESULTS

ERK1/2 activation in uveal melanoma

An antibody array was applied to investigate the mapk pathway in ten um cell lines, three primary UM and three UM metastasis. We observed uniform HSP27 phosphorylation with the exception of three UM cell lines (OCM1, -3, -8). UM displaying activated ERK1/2 as well as phosphorylated HSP27 were most common whereas signals for phosphorylated ERK1/2 were low in metastasis tissue (MET1-3) and metastatic UM cell lines (OMM1, OMM2.3 and OMM2.5) (Fig. 1). Remarkably, two of the metastatic cell lines (OMM2.3, OMM2.5) are derived from the same patient as cell line Mel270 but contained far less activated ERK1/2.

Differential kinase activity in uveal melanoma

Reduction of ERK1/2 activation in metastatic cell lines compared to the primary UM cell lines provides a model to identify the underlying mechanism of ERK1/2 activation in the absence of BRAF and NRAS mutations.

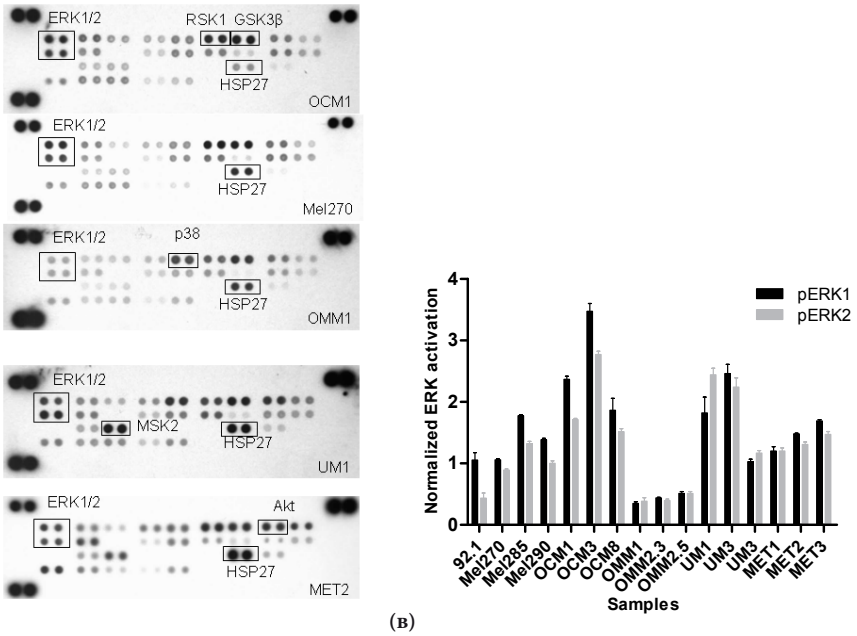


FIGURE 1. MAPK activation in primary UM and UM metastases was studied with a MAPK antibody array. We observed uniform HSP27 phosphorylation in both cell lines and tissue samples except for OCM1, -3 and -8 (A). Activated ERK1/2 was normalized with HSP27 and shown to be low in UM metastases (MET1-3) while metastatic cell lines just passed the background (OMM1, -2.3, -2.5) (B).

To investigate whether a kinase is differentially activated between primary UM cell lines and metastatic UM cell lines we used peptide-based tyrosine kinase arrays [29]. The UM cell lines displayed a high kinase activity while the metastatic UM cell lines displayed a low kinase activity although the same amount of lysate was incubated (Fig. 2A). After normalization, we could analyze the kinase data and identify nine substrates that were significantly differentially phosphorylated between primary and metastatic UM cell lines (Fig. 2B, Tab. 1). Primary UM and metastatic tissue also showed differential phosphorylation of these nine peptides though not as clearly as observed in the cell lines (Fig. 2C).

Candidate kinase: Src

We identified nine peptides derived from eight proteins that were differentially phosphorylated between primary and metastatic cell line lysates. based on a literature search we identified candidate tyrosine kinases for eight out of nine peptides (Table 1) [30-33]. Among the candidates, Src and Src family members were most prominent. In order to validate the candidacy of Src we performed *in vitro* inhibition experiments with the Src-kinase specific inhibitors PP1 and the PP1 analogue, PP2. We added PP1 and PP2 (10μM) to lysates of primary UM tissue and of a primary UM cell line and we measured the inhibitory effect of these Src inhibitors using the kinase array (Fig. 3A). Seven out of nine substrates that identified

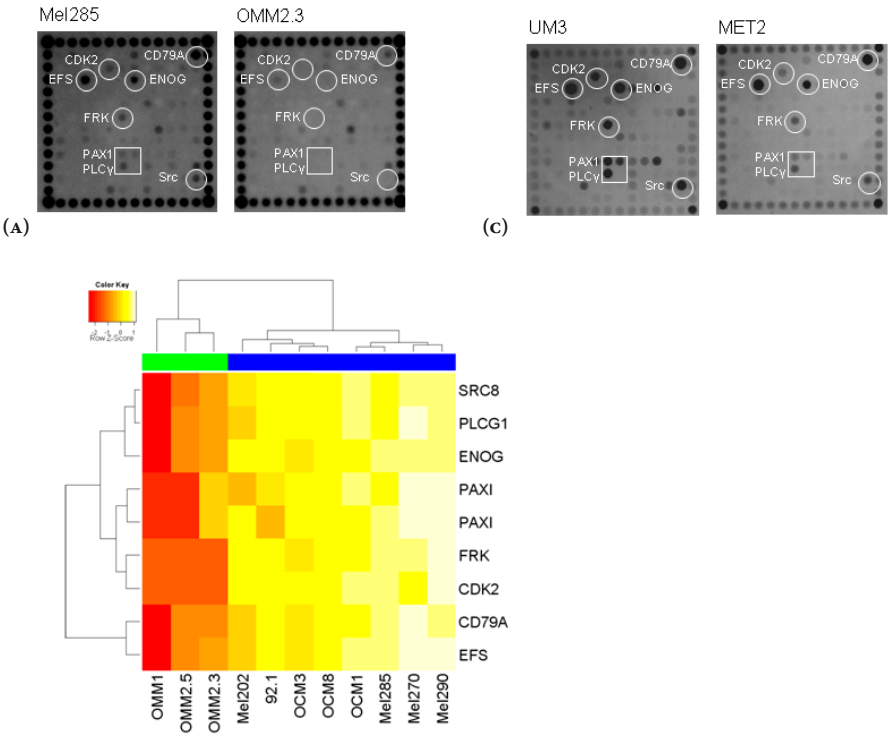


FIGURE 2. Tyrosine kinase activity was measured with an array of peptide substrates.
Two representative examples of a UM cell line and a metastatic cell line (A). Analysis with eBayes identified nine substrates, representing eight proteins, to be significantly (p=0.01) differentially phosphorylated between UM and metastatic cell lines (B). UM (UM1-3) tyrosine kinase activity is high compared to liver metastasis (MET1-3) (two representative arrays are shown) (C).

TABLE 1. Tyrosine kinase substrates on the kinase array that were differentially phosphorylated between primary UM cell lines and metastatic cell lines.

Substrate	UniProt ID	Position	Log Fold Change	Adj. P value	Kinase
CDK2	P24941	T14/Y15	5.3	0.00005	Lyn
FRK	P42685	Y387	6.2	0.0002	unknown
SRC8	Q14247	Y499	4.6	0.006	Src
ENOG	P09104	Y43	4.7	0.01	Src
EFS	O43281	Y253	3.1	0.01	Src
PLCG1	P19174	Y771	4.2	0.01	Syk, Sky, GFRs
CD79A	P11912	Y182/Y188	3.1	0.01	Lyn
PAXI	P49023	Y118	4.0	0.02	FAK, Src, Brk
PAXI	P49023	Y31	4.3	0.03	FAK, Src, Brk

Tyrosine kinase substrate specificities are included.

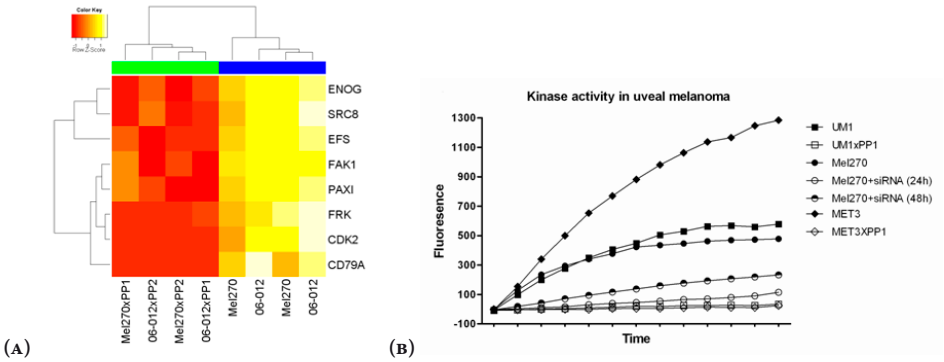


FIGURE 3. UM1 (06-12) and Mel270 treatment with Src inhibitors (PP1/PP2) identified eight substrates with a significant reduction in phosphorylation (A).
Inhibition of EFS peptide phosphorylation by genetic (Src-siRNA) and pharmacological means (PP1) in cell line Mel270 and PP1 treatment of cell lysates of UM (UM1) and metastasis tissue (MET3) (B).

Src in the first screen displayed a significantly reduced phosphorylation when PP1 or PP2 were added to lysates of UM1 and Mel270 (Fig. 3A). The PLCG1 peptide and one of the PAX1 (Y31) substrates did not reach significance but were still phosphorylated at a reduced level after PP1 and PP2 treatment. The peptide representing FAK1 Y576/Y577 is a genuine substrate for Src that was not detected in the UM cell line comparison but phosphorylation was significantly downregulated by PP1 and PP2 treatment. In the control experiment in which we added the inactive analogue of PP1 (PP3) to cell lysates, we did not observe a loss of kinase activity (not shown).

Kinase activity of metastasis tissue and UM tissue differed marginally (Fig. 2C) and incubation with PP1 (10μM) resulted in a decimation of kinase activity similar to the inhibition that we observed in UM tissue (Fig. 3B). In order to validate Src activity, Mel270 was transfected with two siRNA constructs that target Src and reduced kinase activity (Fig. 3B).

Regulation of ERK1/2 and growth

To investigate whether Src contributes to ERK1/2 activation in Mel270 we analyzed the two Src siRNA transfected cell cultures with the MAPK antibody array. Twenty four and 48 hours after transfection with Src siRNA we observed a reduced ERK1 phosphorylation whereas ERK2 phosphorylation was minimally affected (Fig. 4).

Whether Src inhibition and consequently a lowered ERK activation in UM cell lines is associated with a reduced growth was investigated with the WST-1 viability assay (Fig 5). All UM cell lines showed a PP1-dose and time (1 to 6 days) dependent reduction in cell viability but the magnitude of the response differed widely. In general, the metastatic UM cell lines were less affected by PP1. We also determined the growth inhibition rate of PP1 in cultures of five primary UM cell cultures and observed an increased sensitivity to PP1 treatment

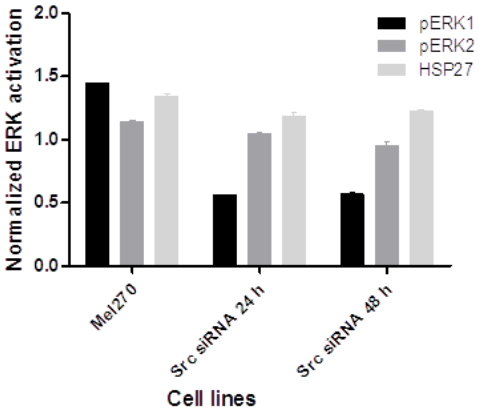


FIGURE 4. ERK1/2 activation in Mel270 24 and 48 hours after transfection with Src siRNA. Phosphorylated HSP27 is included as reference signal.

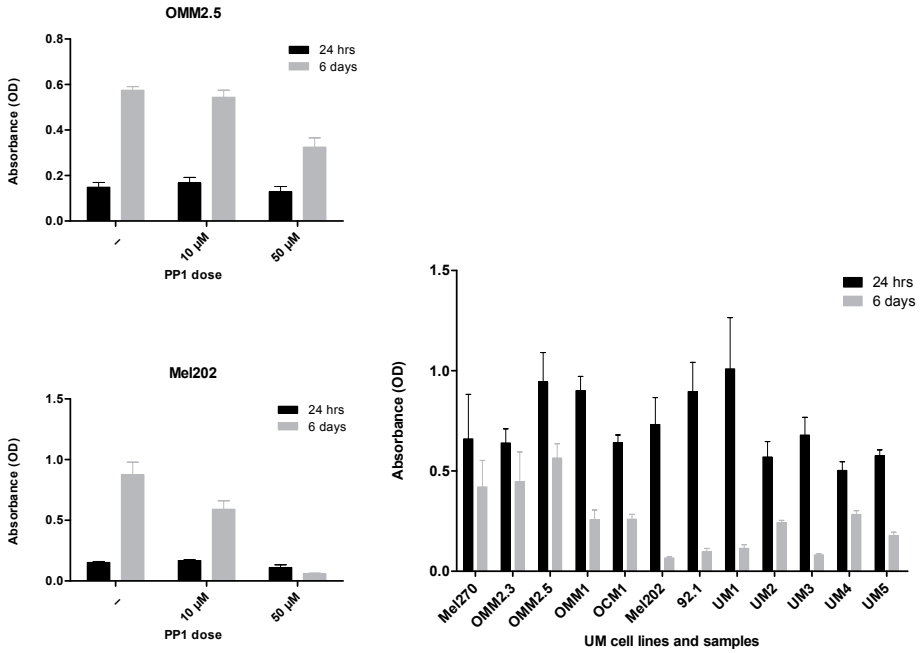


FIGURE. 5. UM cell lines and primary cultures were cultured with PP1 (10μM and 50μM). After 24 hours and 3 days (UM cultures) and 6 days (cell lines) the viability was tested with the WST-1 assay. Two representative cell culture experiments for which all time points and conditions are shown (A). Growth inhibition by PP1 (50 μM) after 24 hours and 3/6 days was normalized to the control culture of each individual cell line (B).

compared to the cell lines. We had to take samples at day 3 of PP1 treatment because hereafter massive cell death occurred (Fig 5B).

Src protein is reduced in metastasis cell line

Src is regulated by phosphorylation of tyrosine residues at position 416 (Y416) and 527 (Y527). Expression of phosphorylated Src Y416 which is associated with an active conformation, was low in the metastatic UM cell lines (Fig. 6A). Surprisingly, phosphorylation of Y527 that is associated with an inactive conformation, was also low and subsequent analysis indicated that Src expression is low in the metastatic UM cell lines. Therefore the difference between kinase activity in metastatic cell lines (OMM1, OMM2.3, OMM2.5) and UM cell lines (OCM1, OCM3, OCM8, Mel202, Mel270, Mel285, Mel290 and 92.1) seems to be the result of a difference in Src expression.

To investigate the origin of lowered Src expression we performed gene expression analysis (Fig. 6B). Src gene expression varied widely in the cell lines and the metastatic cell lines but a correlation between protein and gene expression was not observed in UM cell lines.

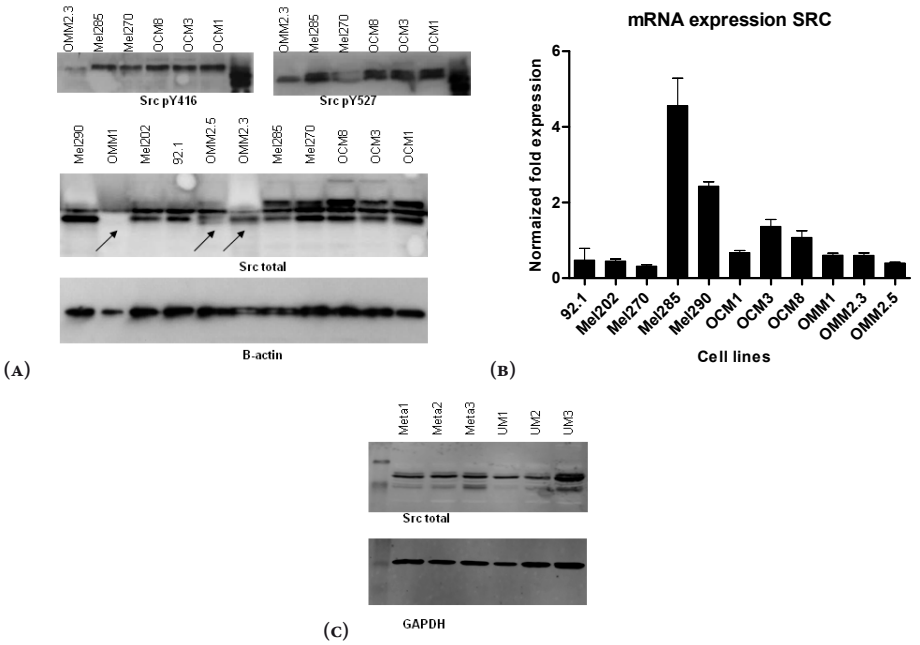


FIGURE. 6. Western analysis of Src in the UM cell lines for activating phosphorylation (Y416) inactivating phosphorylation (Y527) and total Src expression (A).
Src gene expression measured by QPCR varied widely but did not correlate with variation in protein expression (B). UM and metastasis tissue all displayed medium Src kinase expression except for UM3 that presents a high level of expression (C).

Western analysis of Src expression in UM and metastasis tissue revealed in only one out of three primary UM a very high Src expression whereas all three metastasis tissue displayed medium expression of Src protein (Fig. 6C).

DISCUSSION

Constitutive activation of ERK1/2 has often been reported for UM [13,17,18]. Using a more quantitative approach we distinguished a decrease of active ERK1/2 in metastatic cell lines and fresh liver metastasis, suggesting loss of ERK1/2 activation during UM progression. The latter is unexpected since ERK1/2 activation is generally associated with mitogen signaling and is known to determine malignant potential *in vitro*. However, in endometrial and breast cancer, ERK1/2 activation has been associated with a good prognosis [34 35]. A possible explanation is provided by the observation that ERK1/2 is involved in oncogene and stress induced senescence [36,37]. This mechanism is thought to be an important defense for cells that are at risk of neoplastic transformation and needs to be circumvented by tumor cells in order to proliferate. Loss of activated ERK1/2 may relieve the associated inhibitory mechanisms in a direct manner but also requires for alternative mitogenic signals to take over in UM metastasis.

The metastatic and UM cell lines provide a unique model to identify the mechanisms that regulate ERK1/2 activation in uveal melanoma. Previous work already showed that ERK1/2 phosphorylation in UM depends on the MAPK pathway although mutations in the usual suspects (e.g. BRAF and NRAS) are lacking [19]. We investigated the possibility of a tyrosine kinase with differential activities in UM and UM metastasis to be responsible for ERK1/2 activation, using an array of kinase activity assays. Src was revealed as differentially activated tyrosine kinase and this was supported by incubation with Src specific kinase inhibitors PP1 and PP2. Moreover, by treating the cell lysates instead of the cell cultures, we minimized the secondary effects of the inhibitors. However, PP1 and PP2 affect most of the Src-family of tyrosine kinases and the observed reduction of kinase activity therefore does not specifically mark Src. Multi-target inhibitors are a problem in molecular analysis but may be beneficial in the clinical application since in CM a switch from Src to Yes signaling has been reported in brain metastases [38]. In order to specifically inhibit Src we targeted the Src gene expression with a siRNA approach. We detected a reduced kinase activity in Mel270 upon transfection and this was correlated with a reduced ERK1 activation. ERK2 activation appeared unaffected which could be due to the limited efficacy of siRNA treatment or it could indicate the activity of another, yet unidentified, kinase. Low Src protein expression in conjunction with loss of ERK1/2 activation in metastatic UM cell lines however supports the hypothesis that in UM, Src kinase is involved in ERK1/2 activation. Gene expression analysis revealed no significant differences between metastatic and UM cell lines and thereby indicated that

post-transcriptional mechanisms are most likely involved in Src down regulation. Src is both a kinase as well as a client protein for the chaperone HSP90 that is expressed in UM [39]. Whether HSP90 is reduced in metastases and whether treatment with HSP90 inhibitors depends on Src signaling is part of future investigation [40,41]. Inhibition of Src kinase activity resulted in a strong growth reduction in all UM cultures while in UM cell lines the response varied more widely. The genetic background of the cell lines might play a role in the observed variation. However, all UM cell lines displayed Src kinase activity and PP1 sensitivity irrespective of c-kit upregulation (Mel270) or BRAF V600E (OCM1) and GNAQ Q209L (Mel202) mutation status [20,22]. Tissue of UM and UM liver metastasis displayed more or less comparable Src levels. Incubation of the lysates with Src inhibitors resulted in comparable reduction of kinase activity in UM and metastasis tissue. The possibility that there exist Src negative clones in liver metastasis can however not be ruled out on the basis of these data. Clinical trials targeting Src kinase activity in uveal melanoma should therefore anticipate on this potential risk.

In conclusion: we have identified differential ERK1/2 activation in UM and metastatic UM cell lines. Using tyrosine kinase activity profiling we identified Src as a determinant of ERK1/2 activation and showed that Src expression and kinase activity together with ERK1/2 activation is reduced in UM metastases cell lines.

ACKNOWLEDGEMENTS

We would like to acknowledge the technical assistance of Mieke Versluis, Amal F. Abukar, Aabed Baghat and the staff at PamGene. The authors also thank Jolanda Reek for statistical analysis. This study was supported by grant RUL 2001-2472 from the Dutch Cancer Society (KWF).

REFERENCE LIST

1. Egan, K. M., Seddon, J. M., Glynn, R. J., Gragoudas, E. S., and Albert, D. M. *Epidemiologic aspects of uveal melanoma.* Surv Ophthalmol 1988. 32:239-51.
2. Singh, A. D. and Topham, A. *Incidence of uveal melanoma in the United States: 1973-1997.* Ophthalmology 2003. 110:956-961.
3. van der Velden, P. A., Metzelaar-Blok, J. A., Bergman, W., Monique, H., Hurks, H., Frants, R. R., Gruis, N. A., and Jager, M. J. *Promoter Hypermethylation: A Common Cause of Reduced p16(INK4a) Expression in Uveal Melanoma.* Cancer Res 2001. 61:5303-5306.
4. Goding, C. R. *Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage.* Genes Dev. 2000. 14:1712-1728.
5. Reddy, K. B., Nabha, S. M., and Atanaskova, N. *Role of MAP kinase in tumor progression and invasion.* Cancer Metastasis Rev. 2003. 22:395-403.
6. Satyamoorthy, K., Li, G., Gerrero, M. R., Brose, M. S., Volpe, P., Weber, B. L., Van Belle, P., Elder, D. E., and Herlyn, M. *Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation.* Cancer Res. 2003. 63:756-759.
7. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. *Mutations of the BRAF gene in human cancer.* Nature 2002. 417:949-954.
8. van Elsas, A., Zerp, S., van der, F. S., Kruse-Wolters, M., Vacca, A., Ruiter, D. J., and Schrier, P. *Analysis of N-ras mutations in human cutaneous melanoma: tumor heterogeneity detected by polymerase chain reaction/single-stranded conformation polymorphism analysis.* Recent Results Cancer Res. 1995. 139:57-67.:57-67.
9. Edmunds, S. C., Cree, I. A., Di Nicolantonio, F., Hungerford, J. L., Hurren, J. S., and Kelsell, D. P. *Absence of BRAF gene mutations in uveal melanomas in contrast to cutaneous melanomas.* Br.J.Cancer. 2003. 88:1403-1405.
10. Cohen, Y., Goldenberg-Cohen, N., Parrella, P., Chowers, I., Merbs, S. L., Pe'er, J., and Sidransky, D. *Lack of BRAF mutation in primary uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2003. 44:2876-2878.
11. Cruz, F., III, Rubin, B. P., Wilson, D., Town, A., Schroeder, A., Haley, A., Bainbridge, T., Heinrich, M. C., and Corless, C. L. *Absence of BRAF and NRAS mutations in uveal melanoma.* Cancer Res. 2003. 63:5761-5766.
12. Mooy, C. M., Van der Helm, M. J., Van der Kwast, T. H., de Jong, P. T., Ruiter, D. J., and Zwarthoff, E. C. *No N-ras mutations in human uveal melanoma: the role of ultraviolet light revisited.* Br.J.Cancer 1991. 64:411-413.
13. Rimoldi, D., Salvi, S., Lienard, D., Lejeune, F. J., Speiser, D., Zografos, L., and Cerottini, J. C. *Lack of BRAF mutations in uveal melanoma.* Cancer Res. 2003. 63:5712-5715.
14. Soparker, C. N., O'Brien, J. M., and Albert, D. M. *Investigation of the role of the ras protooncogene point mutation in human uveal melanomas.* Invest Ophthalmol.Vis.Sci. 1993. 34:2203-2209.
15. Janssen, C. S., Sibbett, R., Henriquez, F. L., McKay, I. C., Kemp, E. G., and Roberts, F. *The T1799A point mutation is present in posterior uveal melanoma.* Br.J.Cancer. 2008. 99:1673-1677.

16. Maat, W., Kilic, E., Luyten, G. P., de Klein, A., Jager, M. J., Gruis, N. A., and van der Velden, P. A. *Pyrophosphorolysis detects B-RAF mutations in primary uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2008. 49:23-27.
17. Weber, A., Hengge, U. R., Urbanik, D., Markwart, A., Mirmohammadsaegh, A., Reichel, M. B., Wittekind, C., Wiedemann, P., and Tannapfel, A. *Absence of mutations of the BRAF gene and constitutive activation of extracellular-regulated kinase in malignant melanomas of the uvea.* Lab Invest 2003. 83:1771-1776.
18. Zuidervaart, W., van Nieuwpoort, F., Stark, M., Dijkman, R., Packer, L., Borgstein, A. M., Pavey, S., van, d., V, Out, C., Jager, M. J., Hayward, N. K., and Gruis, N. A. *Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS.* Br.J.Cancer 2005. 92:2032-2038.
19. Calipel, A., Mouriaux, F., Glotin, A. L., Malecaze, F., Faussat, A. M., and Mascarelli, F. *Extracellular signal-regulated kinase-dependent proliferation is mediated through the protein kinase A/B-Raf pathway in human uveal melanoma cells.* J.Biol.Chem. 2006. 281:9238-9250.
20. Lefevre, G., Glotin, A. L., Calipel, A., Mouriaux, F., Tran, T., Kherrouche, Z., Maurage, C. A., Auclair, C., and Mascarelli, F. *Roles of stem cell factor/c-Kit and effects of Glivec/STI571 in human uveal melanoma cell tumorigenesis.* J.Biol.Chem. 2004. 279:31769-31779.
21. Hofmann, U. B., Kauczok-Vetter, C. S., Houben, R., and Becker, J. C. *Overexpression of the KIT/SCF in Uveal Melanoma Does Not Translate into Clinical Efficacy of Imatinib Mesylate.* Clin.Cancer Res. 2009. 15:324-329.
22. Van Raamsdonk, C. D., Bezrookove, V., Green, G., Bauer, J., Gaugler, L., O'Brien, J. M., Simpson, E. M., Barsh, G. S., and Bastian, B. C. *Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi.* Nature. 2008.
23. Chen, P. W., Murray, T. G., Salgaller, M. L., and Ksander, B. R. *Expression of MAGE genes in ocular melanoma cell lines.* J.Immunother. 1997. 20:265-275.
24. Waard-Siebinga, I., Blom, D. J., Griffioen, M., Schrier, P. I., Hoogendoorn, E., Beverstock, G., Danen, E. H., and Jager, M. J. *Establishment and characterization of an uveal-melanoma cell line.* Int.J.Cancer. 1995. 62:155-161.
25. Kan-Mitchell, J., Mitchell, M. S., Rao, N., and Liggett, P. E. *Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes.* Invest Ophthalmol.Vis.Sci. 1989. 30:829-834.
26. Luyten, G. P., Naus, N. C., Mooy, C. M., Hagemeijer, A., Kan-Mitchell, J., Van Drunen, E., Vuzevski, V., de Jong, P. T., and Luider, T. M. *Establishment and characterization of primary and metastatic uveal melanoma cell lines.* Int.J.Cancer. 1996. 66:380-387.
27. Smyth, G. K. *Linear models and empirical bayes methods for assessing differential expression in microarray experiments.* Stat.Appl.Genet.Mol.Biol. 2004. 3:Article3. Epub;%2004 Feb 12.:Article3.
28. Narayanan, R., Kenney, M. C., Kamjoo, S., Trinh, T. H., Seigel, G. M., Resende, G. P., and Kuppermann, B. D. *Trypan blue: effect on retinal pigment epithelial and neurosensory retinal cells.* Invest Ophthalmol.Vis.Sci. 2005. 46:304-309.
29. Lemeer, S., Jopling, C., Naji, F., Ruijtenbeek, R., Slijper, M., Heck, A. J., and den Hertog, J. *Protein-tyrosine kinase activity profiling in knock down zebrafish embryos.* PLoS.ONE. 2007. 2:e581.
30. Thomas, S. M. and Brugge, J. S. *Cellular functions regulated by Src family kinases.* Annu.Rev.Cell Dev. Biol. 1997. 13:513-609.
31. Cooper, J. A., Reiss, N. A., Schwartz, R. J., and Hunter, T. *Three glycolytic enzymes are phosphorylated at tyrosine in cells transformed by Rous sarcoma virus.* Nature. 1983. 302:218-223.

32. Diella, F., Cameron, S., Gemund, C., Linding, R., Via, A., Kuster, B., Sicheritz-Ponten, T., Blom, N., and Gibson, T. J. *Phospho.ELM: a database of experimentally verified phosphorylation sites in eukaryotic proteins.* BMC.Bioinformatics. 2004. 5:79.:79.
33. Koike, A., Kobayashi, Y., and Takagi, T. *Kinase pathway database: an integrated protein-kinase and NLP-based protein-interaction resource.* Genome Res. 2003. 13:1231-1243.
34. Milde-Langosch, K., Bamberger, A. M., Rieck, G., Grund, D., Hemminger, G., Muller, V., and Loning, T. *Expression and prognostic relevance of activated extracellular-regulated kinases (ERK1/2) in breast cancer.* Br.J.Cancer. 2005. %20;92:2206-2215.
35. Mizumoto, Y., Kyo, S., Mori, N., Sakaguchi, J., Ohno, S., Maida, Y., Hashimoto, M., Takakura, M., and Inoue, M. *Activation of ERK1/2 occurs independently of KRAS or BRAF status in endometrial cancer and is associated with favorable prognosis.* Cancer Sci. 2007. 98:652-658.
36. Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. *The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2.* Embo J 1998. 17:5001-14.
37. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. *Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.* Cell 1997. 88:593-602.
38. Summy, J. M. and Gallick, G. E. *Src family kinases in tumor progression and metastasis.* Cancer Metastasis Rev. 2003. 22:337-358.
39. Missotten, G. S., Journee-de Korver, J. G., Wolff-Rouendaal, D., Keunen, J. E., Schlingemann, R. O., and Jager, M. J. *Heat shock protein expression in the eye and in uveal melanoma.* Invest Ophthalmol. Vis.Sci. 2003. 44:3059-3065.
40. Faingold, D., Marshall, J. C., Antecka, E., Di Cesare, S., Odashiro, A. N., Bakalian, S., Fernandes, B. F., and Burnier, M. N., Jr. *Immune expression and inhibition of heat shock protein 90 in uveal melanoma.* Clin.Cancer Res. 2008. 14:847-855.
41. Babchia, N., Calipel, A., Mouriaux, F., Faussat, A. M., and Mascarelli, F. *HSP90 inhibitors 17-AAG and 17-DMAG target wild-type B-Raf signaling for the proliferation of human uveal melanoma cell lines.* Invest Ophthalmol.Vis.Sci. 2008. ..