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MNT inhibits the migration of human hepatocellular carcinoma SMMC7721 cells

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ABSTRACT

Max binding protein (MNT) is a member of the Myc/Max/Mad network that plays a role in cell proliferation, differentiation and apoptosis. We previously observed that MNT was differentially expressed in hepatocellular carcinoma (HCC) and interacted with Nck1 by 2-DE. Nck family adaptor proteins function to couple tyrosine phosphorylation signals, regulate actin cytoskeletal reorganization and lead to cell motility. In order to investigate the regulatory role of MNT in HCC migration, we used transient transfection with a MNT expressing vector to overexpress MNT protein in SMMC7721 cells, and MNT siRNA to knockdown MNT expression. Rho Family Small GTPase activation assay, Western blots and transwell assay were used to determine the migration potential of cells. We found that knockdown of MNT expression might promote SMMC7721 cell migration, while the overexpressed MNT could significantly inhibit cell migration. It further emphasized the role of MNT in inhibition of cell migration that might be a promising target for HCC chemotherapy.

1. Introduction

Worldwide, hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer deaths. Surgical resection is the only curative option for HCC, but only 10–30% of patients are amenable to surgical resection at the time of diagnosis. The 5-year overall survival rate is between 35% and 41% after resection of primary tumors and between 47% and 61% after liver transplantation [1,2]. Given this poor therapeutic efficacy, to develop newly diagnostic tool and therapeutic method is crucial for prescribing the most timely and effective treatment. Identification genes and biochemical pathways involved in hepatocarcinogenesis is important to develop novel preventive and therapeutic approaches against HCC.

Metastasis is a series of complicated steps in which cancer cells leave the original tumor site and migrate to other parts of the body [3]. The frequent metastasis is a major obstacle to further improve long-term survival rate of HCC patients [4]. Cell migration is a highly integrated multi-step cycle process including cell depolarization, extension of protrusions in the direction of migration, formation of stable adhesion near the leading edge of the protrusions, and detachment of the adhesion and retraction at the rear. This process plays a critical role in tumor metastasis and invasion, and governed by the remodeling of actin cytoskeleton [5,6].

The non-catalytic region of tyrosine kinase (Nck) adaptor protein is composed of three Src homology 3 (SH3) domains and a C-terminal SH2 domain. Nck can bind, via its SH2 domain, to a number of receptor tyrosine kinases, such as platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR) and ephrin receptor (Eph), as well as tyrosine phosphorylated docking proteins, such as p62Dok-1 and p130Cas [7–9]. On the other hand, the Nck SH3 domains are capable of binding to proline-rich sequences on a host of effecter proteins implicated in cytoskeleton regulation. For instance, Nck binding to Pak/PIX complex may stimulate Rho GTPases Rac1 and Cdc42 activation and regulate cell adhesion, migration as well as gene transcription [10,11].

In order to identify Nck SH2 domain binding proteins associated with HCC development, we employed the GST fused Nck1 SH2 domain to pull down associated proteins from either normal or HCC tumor samples. Then 2-DE gels were used to resolve the resulting complexes. The differentially expressed protein spots were analyzed by mass spectrometry. We identified the max binding protein (MNT), a novel Nck1 binding protein, and further investigated the regulatory role of MNT in HCC cell migration.

2. Material and methods

2.1. Cell lines and culture conditions

HCC cell line SMMC7721 was obtained from the Cell Bank, Chinese Academy of Medical Sciences (Shanghai, China). The cells were maintained in high-glucose Dulbecco's modified Eagle's media (DMEM) (Gibco, Invitrogen, Melbourne, Australia) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Melbourne,

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Australia), and incubated at 37 °C in a humidified chamber containing 5% CO_2.

2.2. Plasmid constructs and transfection

The full-length MNT cDNA was amplified and cloned into the pcDNA3.1 expression vector (Cyagen, Guangzhou, China). To increase MNT expression in vitro, when 70% cellular confluent occurred, the expression plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to manufacturer's instruction.

2.3. siRNA Transfection

Oligonucleotide siRNA duplex was synthesized by Shanghai Gene Pharma (Shanghai, China). The siRNA sequences for MNT were as follows: 5′- CACGCUUCAGUCAUCCAGA-3′ and 5′-CCUCGG AAAUCAGUGCGAU-3′. The siRNAs were transfected into SMMC-7721 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to manufacturer's instruction.

2.4. Western blot analysis

Harvested cultured cells were homogenized in Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Haimen, China). Protein quantification was determined by Bradford assay (Beyotime, Haimen, China). Equal amount of protein was separated on SDS–polyacrylamide gels first and then transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, USA). After blocking in 5% skim milk for 1 h at room temperature, the membranes were incubated at 4 °C with indicated primary antibody overnight. Then the membranes were treated by horseradish peroxidaseconjugated second antibody and detected by chemiluminescence (Amersham Biosciences, Piscataway, USA). The Western blot data were quantified by measuring the intensity of hybridized signals using Image analysis program of Fluor-ChemTM 8900 (Alpha Innotech, San Leandro, USA).

2.5. Rho family small GTPase activation assay

The intracellular activities of Rho family GTPases Rac1 and Cdc42 were respectively examined by Rac1 and Cdc42 activation kits (Upstate Biotechnology, Lake Placid, USA) according to manufacturer's protocols. In brief, cells were rinsed with ice-cold PBS and lysed with Mg^{2+} lysis/wash buffer. After clarifying the cell lysates with glutathione agarose and quantifying the protein concentrations, aliquots with equal amounts of proteins were incubated with Rac/Cdc42 assay reagent (PAK-1 PBD, agarose) at 4 °C for 1 h. GTPgS-pretreated lysates were used as positive controls. The precipitated GTP-bound Rac1 and Cdc42 were eluted in Laemmli reducing sample buffer and resolved in a 12% SDS–PAGE. Five percent of cell lysates were resolved in a 10% SDS–PAGE. Immunoblot was performed to detect Rac1 and Cdc42 with specific monoclonal antibodies.

2.6. Transwell assay

1×10^5 cells were suspended in 200 μl of serum-free DMEM medium and then seeded on the upper side of the invasion chamber (Millipore, Billerica, USA). The lower side of chamber was filled with DMEM supplemented with 10% fetal bovine serum. After incubation at 37 °C for 18 h, cells penetrated through the chamber were fixed with methanol for 15 min at room temperature and stained with 0.1% crystal violet for another 15 min. The upper surface of chamber was carefully wiped with a cotton-tipped applicator. Cells passing through the pores were counted in five non-overlapping 40× fields and photographed.

2.7. Statistical analysis

Statistical differences between two groups were determined by the Student's t test. A $P < 0.05$ was considered as statistical significance. The results were expressed as mean and ± standard deviation (SD) obtained from at least three experiments.

3. Results

3.1. MNT knockdown promotes the migration of human HCC SMMC7721 cells

To validate the potential role of MNT in HCC progression, we used MNT specific siRNA (MNT siRNA) to knockdown MNT expression in HCC SMMC7721 cells and then examined the effects of such manipulations on cell migration. The effectiveness of siRNA to suppress MNT expression was observed by Western blot. In contrast, the control siRNA treatment had little effect on MNT expression (Fig. 1A). It indicated that MNT siRNAs could effectively suppress MNT expression in HCC cells. Since MNT siRNA2 might cause more suppression, we employed MNT siRNA2 for subsequent experiments. We confirmed the role of MNT in HCC cell migration by specifically down-regulating its expression through RNA interference in vitro. After transfection with MNT siRNA, we examined the effects of MNT on migration of SMMC7721 cells by transwell migration assay. The results demonstrated that knockdown of MNT expression with siRNA significantly increased migratory potential of SMMC7721 cells (Fig. 1B, C).

3.2. MNT knockdown activates Rac1 and Cdc42 of human HCC SMMC7721 cells

We tested Rac1 and Cdc42 activities using GST-PBD fusion protein to capture the active forms of GTP-bound Rac1 and Cdc42, followed by immunoblot analysis with Rac1 and Cdc42 antibodies. As shown in Fig. 1D, down-regulation of MNT resulted in a dramatic increase in the activation for both Rac1 and Cdc42 compared to control cells.

3.3. Mnt-overexpression inhibits the migration of human HCC SMMC7721 cells

To further define the role of MNT, we applied transient transfection with a MNT expressing vector to overexpress MNT protein in SMMC7721 cells. Overexpressed MNT was observed by Western blot (Fig. 2A). Then the effects of MNT on SMMC7721 cells migration were determined by transwell migration assay. The results demonstrated that overexpression of MNT significantly decreased migratory potential of SMMC7721 cells (Fig. 2B, C).

3.4. Mnt-overexpression inactivates Rac1 and Cdc42 of Human HCC SMMC7721 cells

As shown in Fig. 2D, overexpressed MNT resulted in a dramatic decreased activation of both Rac1 and Cdc42 compared to control cells.

4. Discussion

MNT is a protein member of the Myc/Max/Mad network. This protein, vis a basic-Helix-Loop-Helix-zipper domain (bHLHzip), binds to the canonical DNA sequence CANNTG (the E box), and

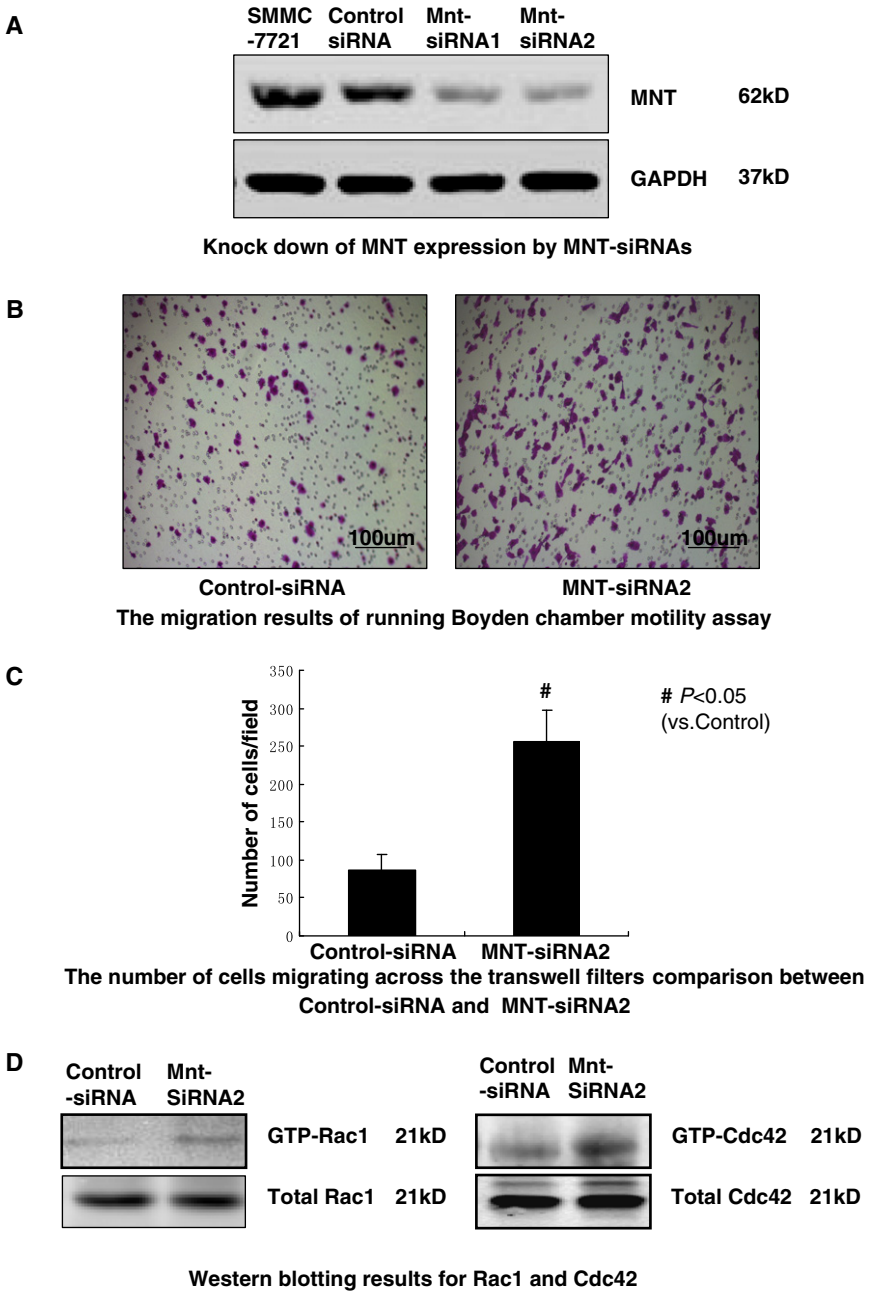

Fig. 1. MNT knockdown and activation of Rac1 and Cdc42 and the migration of human HCC SMMC7721 cells. (A) Knock down of MNT expression by MNT siRNAs. SMMC7721 cells were transfected with different MNT siRNAs or control siRNA for 48 h. Western blot demonstrated MNT protein levels were significantly inhibited by MNT siRNAs. (B) The migration results of running Boyden chamber motility assay. Cells (1×10^4) were re-suspended and seeded onto the filter in the upper compartment of the chamber and incubated for 18 h. Cells and cell extensions that migrated through the pores of transwell plates were counted and reported in (C). Photographs were taken using a Nikon microscope (phase contrast), magnification 100×, $^{\#}P<0.05$. (D) Western blotting results for Rac1 and Cdc42. The amount of active, GTP-loaded Rac1 and Cdc42 were determined by incubating cell lysates with purified GST-PBD fusion protein.

forms heterodimer with Max proteins. MNT functions as a transcriptional repressor or an antagonist for Myc-dependent transcriptional activation and cell growth. It represses transcription by connecting DNA binding protein at its N-terminal Sin3-interaction domain [12]. MNT is postulated as a tumor-suppressor by downregulating the growth of malignant cells and p53 mediated apoptosis in human HCC BEL-7404 cell line. Over-expression of MNT could greatly suppress p53-mediated apoptosis in the absence of serum. Therefore, MNT protein may function as a negative regulator to antagonize the activity of c-Myc in controlling cell growth and apoptosis in human HCC BEL-7404 cells [13].

RNA interference mediated by siRNA is a powerful tool for understanding gene function. We previously identified MNT as a novel binding protein for Nck1 SH2 domain in HCC tissues using 2-DE and MS analyses [14]. Nck SH2 domain belongs to the group I family and contains an aromatic amino acid (phenylalanine) at the betaD5 position. The protein tyrosine kinases and tyrosine phosphatases play pivotal role in a variety of important signaling pathways for multicellular organisms. The aberrant kinase activities have been associated with malignant transformation [15,16]. Thus, profiling the global state of tyrosine phoshporylation and identifying the differences between normal and tumor tissues,

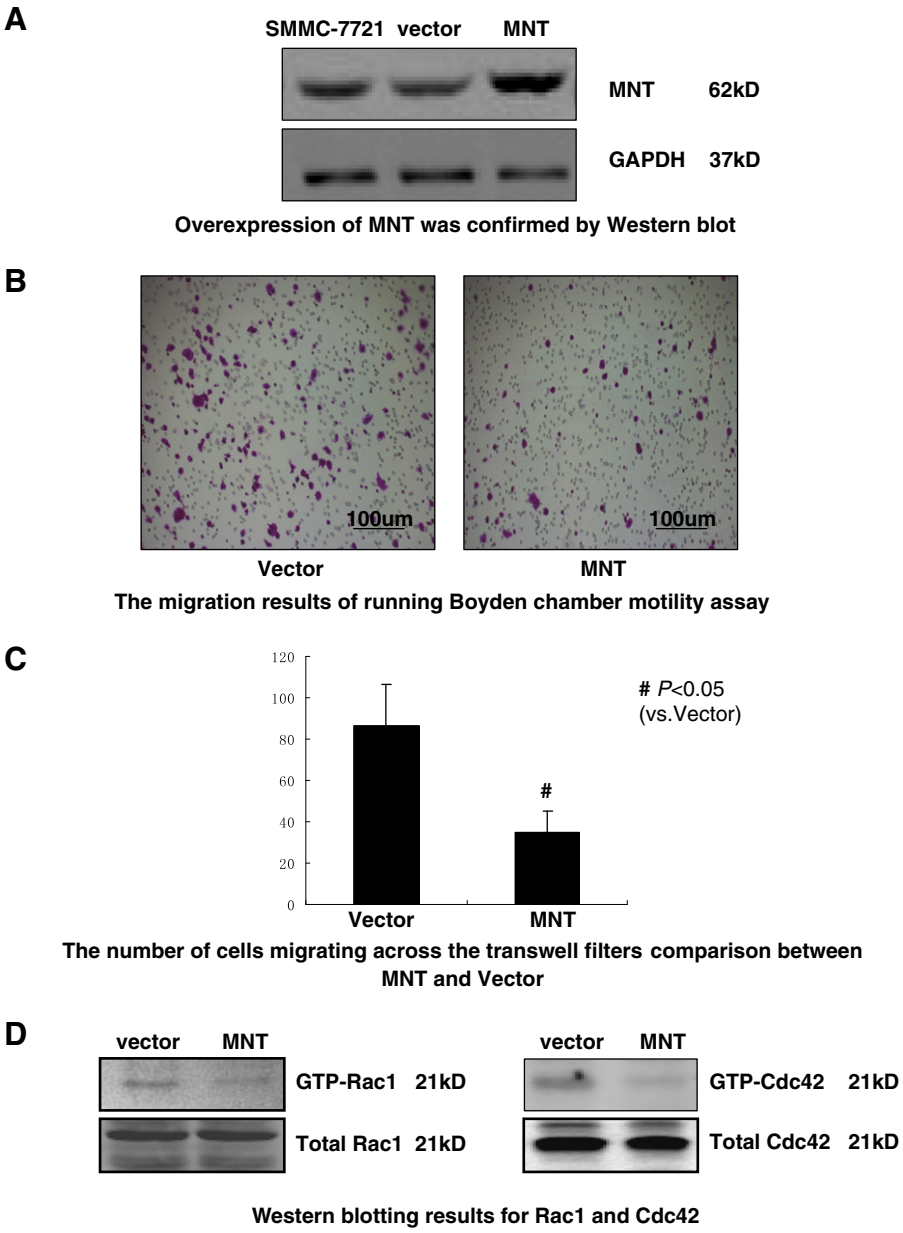

Fig. 2. Overexpression of MNT inactivated Rac1 and Cdc42 and inhibited the migratory ability of human HCC SMMC7721 cells. (A) SMMC7721 cells were transfected with empty vector, pcDNA3.1-MNT, respectively. Cell lysates were harvested 48 h after transfection. Overexpression of MNT was confirmed by Western blot. (B) The migration results bu running Boyden chamber motility assay, cells (1×104) were re-suspended and seeded onto the filter in the upper compartment of the chamber and incubated for 18 h. Cells and cell extensions that migrated through the pores of transwell plates were counted and reported in (C). Photographs were taken using a Nikon microscope (phase contrast), magnification 100×, $^{\#}P<0.05$. (D) Western blotting results for Rac1 and Cdc42. The amount of active, GTP-loaded Rac1 and Cdc42 were determined by incubating cell lysates with purified GST-PBD fusion protein.

may provide a useful tool for novel molecular diagnosis. Nck1 SH2 domain is a small modular protein domain specifically binding to tyrosine-phosphorylated peptide ligands. Therefore, characterizing the complex of SH2 domain binding protein may enrich low-abundance tyrosine phosphorylated proteins and efficiently capture information relevant to the activation of signaling pathways. Machita et al. developed a SH2 profiling method based on far-Western blotting by using GST-SH2 fusion proteins as probe. The SH2 binding profiles were found as useful molecular diagnostic tools to well classify tumor cell lines and predict clinical outcomes [17]. Combining affinity purification of Grb2-SH2 domain binding proteins with SILAC in cell lines, Blagoev et al. identified 28 proteins, including EGFR and Shc that were selectively enriched after EGF stimulation. This strategy was effective in identifying functional protein complexes [18]. However, these studies were performed in cells lines. We applied this strategy to investigate HCC associated proteins and observed differentially expressed MNT in HCC tissues. It indicated that MNT may be a novel Nck1-SH2 domain binding protein. Based on the role of Nck1 on actin polymerization, we speculated that MNT may be also involved in HCC metastasis and invasion pathways.

The Rho GTPases have been shown to be pivotal regulators of the actin filament system. In particular, activated Rac-1 has been implicated in facilitating lamellipodia formation, whereas, activated Cdc42 facilitates filopodia formation. It has been shown that the intracellular concentration and cross-talk between different rho proteins may drive the extensions and contractions and cause cellular locomotion [19]. This promoted us to investigate the

involvement of Rho GTPases in the migration of Human HCC SMMC7721 cells promoting by MNT konckdown. To elucidate the effect of MNT expression in HCC cells, overexpression and knockdown experiments were separately conducted in SMMC7721 cells. MNT cDNA was introduced into SMMC7721 cells, and MNT protein expression was successfully induced by overexpressed MNT in SMMC7721 cells (Fig. 2A). Mnt-overexpression also exhibited inhibition of migration and inactivation of Rac1 and Cdc42 in human HCC SMMC7721 cells (Fig. 2D). On the contrary, down-regulation of MNT might promote the migration of SMMC7721 cells and resulted in dramatic increases of Rac1 and Cdc42 activities compared to control cells.

In summary, our results demonstrated that MNT may inhibit the migration of SMMC7721 cells via activation of Rac1 and Cdc42. These observations were in agreement with recent reports suggesting MNT functions as tumor suppressor gene in HCC cell lines. Therefore, we concluded that MNT as a tumor suppressor can bind to Nck1 SH2 domain and suppress Nck1-mediated cellular migration. MNT and relevant regulatory signaling pathways may serve as promising targets for effective therapy of HCC.

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References

[1] D.M. Parkin, F. Bray, J. Ferlay, P. Pisani, Global cancer statistics, 2002, CA Cancer J. Clin. 55 (2005) 74–108.
[2] H.Y. Yoo, C.H. Patt, J.F. Geschwind, P.J. Thuluvath, The outcome of liver transplantation in patients with hepatocellular carcinoma in the United States between 1988 and 2001: 5-year survival has improved significantly with time, J. Clin. Oncol. 21 (2003) (1988) 4329–4335.
[3] G.P. Gupta, J. Massague, Cancer metastasis: building a framework, Cell 127 (2006) 679–695.
[4] X.D. Zhou, Recurrence and metastasis of hepatocellular carcinoma: progress and prospects, Hepatobiliary Pancreat. Dis. Int. 1 (2002) 35–41.
[5] A.J. Ridley, M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, A.R. Horwitz, Cell migration: integrating signals from front to back, Science 302 (2003) 1704–1709.
[6] H. Yamaguchi, J. Condeelis, Regulation of the actin cytoskeleton in cancer cell migration and invasion, Biochim. Biophys. Acta 1773 (2007) 642–652.
[7] F. Bladt, E. Aippersbach, S. Gelkop, G.A. Strasser, P. Nash, A. Tafuri, F.B. Gertler, T. Pawson, The murine Nck SH2/SH3 adaptors are important for the development of mesoderm-derived embryonic structures and for regulating the cellular actin network, Mol. Cell. Biol. 23 (2003) 4586–4597.
[8] Y. Miyamoto, J. Yamauchi, N. Mizuno, H. Itoh, The adaptor protein Nck1 mediates endothelin A receptor-regulated cell migration through the Cdc42-dependent c-Jun N-terminal kinase pathway, J. Biol. Chem. 279 (2004) 34336–34342.
[9] N. Jones, I.M. Blasutig, V. Eremina, J.M. Ruston, F. Bladt, H. Li, H. Huang, L. Larose, S.S. Li, T. Takano, S.E. Quaggin, T. Pawson, Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes, Nature 440 (2006) 818–823.
[10] Z. Li, M. Hannigan, Z. Mo, B. Liu, W. Lu, Y. Wu, A.V. Smrcka, G. Wu, L. Li, M. Liu, C.K. Huang, D. Wu, Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42, Cell 114 (2003) 215–227.
[11] Z.S. Zhao, E. Manser, L. Lim, Interaction between PAK and nck: a template for Nck targets and role of PAK autophosphorylation, Mol. Cell. Biol. 20 (2000) 3906–3917.
[12] A.S. Zervos, J. Gyuris, R. Brent, Mxi1, A protein that specifically interacts with Max to bind Myc-Max recognition sites, Cell 72 (1993) 223–232.
[13] H. Zhao, Y.H. Xu, Mad-overexpression down regulates the malignant growth and p53 mediated apoptosis in human hepatocellular carcinoma BEL-7404 cells, Cell Res. 9 (1999) 51–59.
[14] S.L. Shen, F.H. Qiu, T.K. Dayarathna, J. Wu, M. Kuang, S.S. Li, B.G. Peng, J. Nie, Identification of Dermcidin as a novel binding protein of Nck1 and characterization of its role in promoting cell migration, Biochim. Biophys. Acta 1812 (2011) 703–710.
[15] M.A. Lemmon, J. Schlessinger, Cell signaling by receptor tyrosine kinases, Cell 141 (2010) 1117–1134.
[16] B.A. Liu, K. Jablonowski, M. Raina, M. Arce, T. Pawson, P.D. Nash, The human and mouse complement of SH2 domain proteins-establishing the boundaries of phosphotyrosine signaling, Mol. Cell 22 (2006) 851–868.
[17] K. Machida, C.M. Thompson, K. Dierck, K. Jablonowski, S. Karkkainen, B. Liu, H. Zhang, P.D. Nash, D.K. Newman, P. Nollau, T. Pawson, G.H. Renkema, K. Saksela, M.R. Schiller, D.G. Shin, and B.J. Mayer, High-throughput phosphotyrosine profiling using SH2 domains, Mol Cell 26 (2007) 899–915.
[18] B. Blagoev, I. Kratchmarova, S.E. Ong, M. Nielsen, L.J. Foster, M. Mann, A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling, Nat. Biotechnol. 21 (2003) 315–318.
[19] M. Raftopoulou, A. Hall, Cell migration: Rho GTPases lead the way, Dev. Biol. 265 (2004) 23–32.