Role of the p55-gamma subunit of PI3K in ALK-induced cell migration: RNAi-based selection of cell migration regulators

Minchul Seoa,b, Jong-Heon Kimb, and Kyoungho Sukb

aDepartment of Agricultural Biology, National Institute of Agricultural Sciences, RDA, Wanju-gun, Republic of Korea; bDepartment of Pharmacology, Brain Science & Engineering Institute, BK21 Plus KNU Biomedical Convergence Program, Kyungpook National University School of Medicine, Daegu, Republic of Korea

ABSTRACT

Recently, unbiased functional genetic selection identified novel cell migration-regulating genes. This RNAi-based functional selection was performed using 63,996 pooled lentiviral shRNAs targeting 21,332 mouse genes. After five rounds of selection using cells with accelerated or impaired migration, shRNAs were retrieved and identified by half-hairpin barcode sequencing using cells with the selected phenotypes. This selection process led to the identification of 29 novel cell migration regulators. One of these candidates, anaplastic lymphoma kinase (ALK), was further investigated. Subsequent studies revealed that ALK promoted cell migration through the PI3K-AKT pathway via the p55γ regulatory subunit of PI3K, rather than more commonly used p85 subunit. Western blot and immunohistochemistry studies using mouse brain tissues revealed similar temporal expression patterns of ALK, phospho-p55γ, and phospho-AKT during different stages of development. These data support an important role for the p55γ subunit of PI3K in ALK-induced cell migration during brain development.

KEYWORDS

ALK; brain development; cell migration; PI3K; RNAi

Cell migration, an evolutionarily conserved mechanism that underlies embryogenesis, wound healing, immune responses, cancer metastasis, and embryogenesis is governed by chemokines and growth factors.1-3 The molecular mechanisms of cell migration have been extensively studied during the past several decades. Cell migration is thought to be controlled by complex regulatory mechanisms that are likely mediated by numerous genes. Here, we attempted to identify novel genes that regulate cell migration using an in vitro loss-of-functional selection with short hairpin RNA (shRNA). Lentiviral-delivered shRNAs were used to produce stable transcript knockdown in mouse fibroblast cells and to conduct loss-of-functional genetic selection.4 The genome-wide functional selection process is illustrated in Figure 1. Pooled recombinant lentiviruses expressing shRNAs were generated by transfecting HEK293T cells with pHAGE-mir30-RFP-shRNA (which targeted the mouse genome), pVSV-G, pTat, pPM2, and pRev. NIH3T3 fibroblast cells were infected with the 63,996 pooled lentiviral mouse shRNA library at an MOI of 1.5,6 Two days after infection, shRNA-infected cells were selected with puromycin, placed in the upper compartment of a transwell unit, and allowed to migrate through a perforated membrane to the lower compartment. Cells that exhibited accelerated or impaired migration were isolated from lower or upper compartments after 5 and 24 hr of incubation, respectively. Cells with the desired phenotypes were enriched by repeating this procedure 5 times. After enrichment, genomic DNA was isolated, and shRNAs that were integrated into chromosomes were retrieved by PCR amplification, cloned, and sequenced. Half-hairpin barcode sequences were used to identify the shRNAs.

From this genome-wide selection process, 29 novel cell migration-regulating shRNAs were identified (Table 1) and 10 were selected for further investigation: Mtmr1 (Myotubularin related protein 1), Lats2 (Large tumor suppressor 2), Dock3 (Dedicator of cyto-kinesis 3), Myo5a (Myosin VA), Ptpn14 (Protein tyrosine phosphatase, non-receptor type 14), Csnk2a2 (Casein kinase 2, α prime polypeptide), Arid4a (AT rich interactive domain 4A (RBP1-like)), Ppp3cc (Protein phosphatase 3, catalytic subunit, gamma isoform), Irf4 (Interferon regulatory factor 4), and Alk (Anaplastic lymphoma...
kinase (Ki-1)). The cell migration-regulating activity of these genes was individually tested by transient knockdown using a synthesized siRNA targeting sequence of each gene. The cell migration-regulating activities of these candidates were confirmed in NIH3T3 fibroblast and mouse embryonic fibroblast (MEF) cells using the transwell migration assay (Table 2). The PI3K/PTEN/AKT signaling pathway was identified as a converging point using network analysis of these cell migration regulators. To determine whether the PI3K/PTEN/AKT signaling pathway was involved in the accelerated or impaired migration induced by the selected shRNAs, we first assessed Akt phosphorylation after knocking down dock3, mtmr1, ptpn14, lats2, and myo5a, and overexpressing alk and irf4. A knockdown of mtmr1, dock3, myo5a, or ptpn14, but not of lats2, or the overexpression of alk or irf4 induced Akt phosphorylation. In addition, we used pharmacological inhibitors of PI3K or AKT to evaluate the role of PI3K/AKT signaling in the accelerated or impaired cell migration by these shRNAs. The accelerated cell migration observed after mtmr1, dock3, myo5a, or ptpn14 (but not lats2) knockdown was significantly attenuated by AKT or PI3K inhibitors in the cell migration assays. Similarly, the accelerated cell migration observed for alk or irf4 overexpression was also attenuated by these inhibitors. Taken together, these results support that the PI3K/AKT pathway is critical for the diverse cell migration regulators identified by an unbiased functional selection.

The cell migration-promoting gene Alk was subjected to further investigation. ALK was previously identified as an oncogene in human anaplastic large cell lymphoma and neuroblastoma, displaying the classical structural features of a receptor tyrosine kinase (RTK). ALK mediates several signal transduction pathways and modulates various cellular functions.7 Many receptor tyrosine kinases transduce their signals via specific interactions with proteins containing SH2 domains, such as the regulatory subunits of PI3K.8 PI3K plays an important role in neurite outgrowth during nerve growth factor-stimulated differentiation and in brain development.9,10 In addition, each PI3K regulatory subunit possesses specific roles in signal transduction, based on its association with different RTKs.11,12,13 Regulatory subunits of all class I PI3K have 2
SH2 domains and an inter-SH2 domain, but contain different NH2-terminal sequences. The p85 regulatory subunit contains SH3 and bcr homology domains in their N-terminal, while the p55 regulatory subunit contains a unique 34 amino acid sequence in their N-terminal.

The p55 subunit, one of the regulatory subunits of PI3K class I, is primarily expressed in prenatal (e.g., 13.5- and 17.5-day) and postnatal brains. Furthermore, the p55 subunit regulates DNA synthesis, cell cycle progression, and tumor angiogenesis. We recently demonstrated that the receptor tyrosine kinase Alk enhanced phosphorylation of the p55 regulatory subunits (Tyr199 residue) of PI3K through a physical interaction. The Alk-induced phosphorylation of the p55 regulatory subunit of PI3K was accompanied by Akt phosphorylation. T he critical role of p55γ and its phosphorylation in the ALK-induced Akt activation was confirmed by siRNA-mediated knockdown of p55γ. These data indicate that p55γ was critically involved in receptor tyrosine kinase Alk-promoted PI3K/AKT activation and cell migration.

Increasing evidence indicates that ALK modulates various cellular functions, such as proliferation, angiogenesis, metabolism, and migration. Furthermore, an important role for ALK in nervous system development and function has also been reported. Despite these data, the relationship between p55γ and ALK in brain development is poorly understood. Here, we assessed brain expression levels of ALK and p55 during mouse embryogenesis, postnatal development, and adulthood.

Table 1. The list of 29 novel cell migration-regulating genes identified in this study.

| Symbols      | Target genes                  | Target sequences                  | GenBank accession No. |
|--------------|-------------------------------|-----------------------------------|-----------------------|
| H2-Q10       | RIKEN cDNA A930006J02 gene     | TTAGAAATCAGGACCATATGCTTG          | BC042572              |
| A930006J02Rik| RIKEN cDNA A930006J02 gene     | AATGCATAAATGCTGCAGGA             | AK020818              |
| Atmin        | ATM interactor                | TTATACTACCTCACATTGCG              | NM_177700             |
| D63003301Rik | RIKEN cDNA D63003301 gene      | AAGTCCATAGGAACTGTCGCA             | XM_00101707           |
| Gm379        | Gm379 predicted gene 379      | ATGTCAATTGCTGTTCTCTCCT           | XM_142052             |
| Gm1971       | Gm1971 predicted gene 1971    | TTAATCCGCGGGAGAGGAAGGG            | XM_001472879          |
| Gm5615       | Predicted gene 5615           | TTGTCACCCGATTGCCTGTTGA           | XM_001333783          |
| Gm12273      | Gm12273 predicted gene 12273  | TAAAGGAAGTGCCAAATCCTTG           | XM_001479118          |
| Gpckow       | G patch domain and KOW motifs | TTCACTTCTGGATTATCCTCCT            | XM_173747             |
| Iprip2       | A 4.5-triphosphate receptor interacting protein-like 2 | TTCACTTCT GGATTATCCTCCT | XM_001033380 |
| LOC668961    | LOC668961 spindlin 2 family member | TACGTGTATAATATGGGATCCCTG        | XM_001006595          |
| Mpc1         | Mitochondrial precursor y 1   | TAAGTTGATCATGATAAGACTG            | XM_001818             |
| Otud6b       | OTU domain containing 6B      | ATTAGGGAAAGTAAGAATCCT             | XM_152812             |
| Ptx4         | Pentraxin 4                   | TAGTCCTGAAGCCTTGTGGCC            | XM_001163416          |
| Rfpl4        | Ret finger protein-like 4     | TATAGATGGGAGTGCC                 | XM_152825             |
| Trms9        | Tripartite motif-containing 59 | AAGTCCATAGGAACTGTCGCA     | XM_002586             |
| Usp45        | Ubiquitin specific peptide 45 | TTATAGCCCTAAAGATGCTG             | XM_152825             |
| Zbed3        | Zinc finger, BED domain containing 3 | TAGATGCGTGAAGCAGGGGACGG          | XM_028106             |
| Sep15        | Selenoprotein                 | TAAGTTAATAATGCTTGATCG             | XM_035102             |
| Adam2        | A disintegrin and metallopeptidase domain 2 | TAATGATCTCGTTCTCCTCCTG | XM_009618 |
| Lrgm1        | Immunity-related GTPass family member 1 | AAGAGATCTAAGGTAACCTGCC | XM_008326 |
| Atn4a        | AT rich interactive domain 4A (RBP1-like) | ATATTGCGCTGATACAGCGGCTG | XM_0108195 |
| Cdcd34       | Coiled-coil domain containing 34 | TTATAGGCTAAGCCTGGTACTG          | XM_026613             |
| Cmm2mb       | CKL-like MARVEL transmembrane domain containing 28 | TTCTCTGTCGGTTCAAGAGCA | XM_028524 |
| Defb20       | Defensin beta 20              | ATTTAATATCTAGAAAGATGCGTGC        | XM_176950             |
| Frm6d        | FERM domain containing 6      | TAATGATCTCGTTCTGCTGGTGC          | XM_028127             |
| Gpr143       | G protein-coupled receptor 143| ATTAGGCTAAGCCTGGTGACG            | XM_010951             |
| Hpd1         | 4-hydroxyphenylpyruvate dioxygenase-like | ATGGCTTCTTGGCTGCTGCTG | XM_146256 |
| Rp17         | Ribosomal protein L7          | TAGGGCTTCTGCGCGCAGGTG            | XM_011291             |

Table 2. The list of 29 novel cell migration-regulating genes identified in this study.

| Symbols      | Target genes                  | Target sequences                  | GenBank accession No. |
|--------------|-------------------------------|-----------------------------------|-----------------------|
| Mtrm1        | NM_016985                     | Myotubulin related protein 1      | 1.75 ± 0.14           |
| Lats2        | NM_015771                     | Large tumor suppressor 2          | 1.58 ± 0.09           |
| Dock3        | NM_153413                     | Dedicator of cytokinesis 3        | 1.50 ± 0.08           |
| Myo5a        | NM_010864                     | Myosin VA                         | 1.60 ± 0.13           |
| Pptm14       | NM_008976                     | Protein tyrosine phosphatase, non-receptor type 14 | 1.70 ± 0.14 |
| Csnk2a2      | NM_009974                     | Casein kinase 2, alpha prime polypeptide | 0.61 ± 0.09 |
| Arid4a       | NM_00108195                   | AT rich interactive domain 4A (RBP1-like) | 0.54 ± 0.06 |
| Ppp3cc        | NM_008915                     | Protein phosphatase 3, catalytic subunit, gamma isoform | 0.57 ± 0.10 |
| Irf4         | NM_013674                     | Interferon regulatory factor 4    | 0.53 ± 0.11           |
| Alk          | NM_007439                     | Anaplastic lymphoma kinase (Ki-1) | 0.55 ± 0.08   |
well postnatal days (PD) 3. However, their expression decreased in the adult brain (Fig. 2). Levels of Akt phosphorylation strongly correlated with Alk and phospho-p55γ levels. Immunohistochemical analysis of brain tissue confirmed Alk expression in early development and during the early postnatal period (Fig. 3). As shown in Figure 3, Alk-positive cells were primarily seen in the migrating zone (CP of ED14.5 brain; SVZ of ED18.5 and PD3), while no Alk-immunoreactive cells were observed in adult cortical layers (I–VI). These results indicate that Alk promotes cell migration in vitro as well as in vivo by specifically interacting with the p55γ subunit of PI3K.

In summary, the loss-of-function selection strategy was successfully utilized to identify a large number of genes that control cell migration. Furthermore, many of the identified cell migration-regulating genes have not been previously associated with cell migration. Cell migration occurs through a multistep process requiring the coordinated actions of many genes. Therefore, additional studies are necessary to clarify the precise regulatory mechanisms responsible for the effects of these cell migration regulators. Finally, these results advance our understanding of the cell migration process and ultimately provide new therapeutic targets for the treatment of diseases, which involve cell migration, such as cancer invasion/metastasis, inflammatory disease, angiogenesis, and regeneration of injured tissue.

**Figure 2.** A similar temporal expression pattern of ALK, p55γ, and AKT during mouse brain development. Levels of ALK, phospho-p55γ, and phospho-AKT were assessed by Western blot analysis of whole brain lysates at different time points: embryonic stages (e.g., ED14.5, ED18.5), postnatal day (PD) 3, and adult brain. Tubulin acted as a loading control. Results of densitometric analysis are presented as means ± SDs (n = 3); * p values of < 0.05 indicate significance between the indicated conditions.
Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the NRF grant funded by the Korean government (MSIP) (No. 2015R1A2A1A10051958, 2008-0062282).

References

[1] Vicente-Manzanares M, Webb DJ, Horwitz AR. Cell migration at a glance. J Cell Sci 2005; 118:4917-9; PMID:16254237; https://doi.org/10.1242/jcs.02662
[2] Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR. Cell migration: integrating signals from front to back. Science 2003; 302:1704-9; PMID:14657486; https://doi.org/10.1126/science.1092053
[3] Franz CM, Jones GE, Ridley AJ. Cell migration in development and disease. Dev Cell 2002; 2:153-8; PMID:11832241; https://doi.org/10.1016/S1534-5807(02)00120-X
[4] Seo M, Lee S, Kim JH, Lee WH, Hu G, Elledge SJ, Suk K. RNAi-based functional selection identifies novel cell migration determinants dependent on PI3K and AKT pathways. Nat Commun 2014; 5:5217; PMID:25347953; https://doi.org/10.1038/ncomms6217
[5] Schlabach MR, Luo J, Solimini NL, Hu G, Xu Q, Li MZ, Zhao Z, Smogorzewska A, Sowa ME, Ang XL, et al. Cancer proliferation gene discovery through functional genomics. Science 2008; 319:620-4; PMID:18239126; https://doi.org/10.1126/science.1149200
[6] Silva JM, Li MZ, Chang K, Ge W, Golding MC, Rickles RJ, Siolas D, Hu G, Paddison PJ, Schlabach MR, et al. Second-generation shRNA libraries covering the mouse and human genomes. Nat Genet 2005; 37:1281-8; PMID:16200065
[7] Roskoski R, Jr. Anaplastic lymphoma kinase (ALK): structure, oncogenic activation, and pharmacological inhibition. Pharmacol Res 2013; 68:68-94; https://doi.org/10.1016/j.phrs.2012.11.007
[8] Schlessinger J, Ullrich A. Growth factor signaling by receptor tyrosine kinases. Neuron 1992; 9:383-91; PMID:1326293; https://doi.org/10.1016/0896-6273(92)90177-F
[9] Waite K, Eickholt BJ. The neurodevelopmental implications of PI3K signaling. Curr Topics Microbiol Immunol 2010; 346:245-65; PMID:20582530
[10] Kimura K, Hattori S, Kabuya Y, Shizawa Y, Takayanagi J, Nakamura S, Toki S, Matsuda Y, Onodera K, Fukui Y. Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. J Biol Chem 1994; 269:18961-7; PMID:8034653

Figure 3. Spatiotemporal expression pattern of ALK during mouse brain development. Sagittal sections were immunostained with anti-ALK antibody and visualized with DAB in the mouse brain at embryonic days (ED) 14.5, 18.5, at postnatal days (PD) 3, and in the adult. Scale bar = 2 mm (upper). Laminar patterns of ALK-immunoreactive cells are shown. Alternatively, for immunofluorescence analysis, brain tissue sections were immunostained with an anti-ALK antibody and Cy3-conjugated secondary antibody (lower). The images represent the boxed region (upper panel) in the cerebral neocortical area. While ALK-positive cells were mainly seen in the migrating zone (CP of ED14.5 brain; SVZ of ED18.5 and PD3), no ALK-immunoreactive cells were observed in adult cortical layers (I – VI). P, pia mater; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter. Scale bar = 100 μm.
[11] Van Horn DJ, Myers MG, Jr., Backer JM. Direct activation of the phosphatidylinositol 3'-kinase by the insulin receptor. J Biol Chem 1994; 269:29-32; PMID:8276809

[12] Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Hager WG, King F, Roberts T, Ratnofsky S, Lechleider RJ. SH2 domains recognize specific phosphopeptide sequences. Cell 1993; 72:767-78; PMID:7680959; https://doi.org/10.1016/0092-8674(93)90404-E

[13] Inukai K, Funaki M, Anai M, Oghara T, Katagiri H, Fukushima Y, Sakoda H, Onishi Y, Ono H, Fujishiro M, et al. Five isoforms of the phosphatidylinositol 3-kinase regulatory subunit exhibit different associations with receptor tyrosine kinases and their tyrosine phosphorylations. FEBS Lett 2001; 490:32-8; PMID:11172806; https://doi.org/10.1016/S0014-5793(01)02132-9

[14] Pons S, Asano T, Glashieen E, Miralpeix M, Zhang Y, Fisher TL, Myers MG Jr, Sun Xi, White MF. The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. Mol Cell Biol 1995; 15:4453-65; PMID:7542745; https://doi.org/10.1128/MCB.15.8.4453

[15] Wang G, Cao X, Lai S, Luo X, Feng Y, Xia X, Yen PM, Gong J, Hu J. PI3K stimulates DNA synthesis and cell-cycle progression via its p55PIK regulatory subunit interaction with PCNA. Mol Cancer Ther 2013; 12:2100-9; PMID:23939377; https://doi.org/10.1158/1535-7163.MCT-12-0920

[16] Wang G, Deng Y, Cao X, Lai S, Tong Y, Luo X, Feng Y, Xia X, Gong J, Hu J. Blocking p55PIK signaling inhibits proliferation and induces differentiation of leukemia cells. Cell Death Differ 2012; 19:1870-9; PMID:22722333; https://doi.org/10.1038/cdd.2012.70

[17] Hu J, Liu S, Wang J, Luo X, Gao X, Xia X, Feng Y, Tao D, Wang G, Li X, et al. Overexpression of the N-terminal end of the p55gamma regulatory subunit of phosphatidylinositol 3-kinase blocks cell cycle progression in gastric carcinoma cells. Int J Oncol 2005; 26:1321-7; PMID:15809724

[18] Wang G, Chen C, Yang R, Cao X, Lai S, Luo X, Feng Y, Xia X, Gong J, Hu J. p55PIK-P13K stimulates angiogenesis in colorectal cancer cell by activating NF-kappaB pathway. Angiogenesis 2013; 16:561-73; PMID:23354733; https://doi.org/10.1007/s10456-013-9336-y

[19] Wasik MA, Zhang Q, Marzec M, Kasprzycka M, Wang HY, Liu X. Anaplastic lymphoma kinase (ALK)-induced malignancies: novel mechanisms of cell transformation and potential therapeutic approaches. Semin Oncol 2009; 36:S27-35; PMID:19393833; https://doi.org/10.1053/j.seminoncol.2009.02.007

[20] Polgar D, Leisser C, Maier S, Strasser S, Rüger B, Deutke M, Khorchide M, Simonitsch I, Cerni C, Krupitzka G. Truncated ALK derived from chromosomal translocation t(2;5)(p23;q35) binds to the SH3 domain of p85-PI3K. Mutation Res 2005; 570:9-15; PMID:15680399; https://doi.org/10.1016/j.mrfmmm.2004.09.011

[21] Słupianek A, Nieborowska-Skorska M, Hoser G, Morriione A, Majewski M, Xue L, Morris SW, Wasik MA, Skorski T. Role of phosphatidylinositol 3-kinase-Akt pathway in nucleophosmin/anaplastic lymphoma kinase-mediated lymphomagenesis. Cancer Res 2001; 61:2194-9; PMID:11280786

[22] Yao S, Cheng M, Zhang Q, Wasik M, Kelsh R, Winkler C. Anaplastic lymphoma kinase is required for neurogenesis in the developing central nervous system of zebrafish. PloS one 2013; 8:e63757; PMID:23667670; https://doi.org/10.1371/journal.pone.0063757

[23] Palmer RH, Vernersson E, Gräbbe C, Hallberg B. Anaplastic lymphoma kinase: signalling in development and disease. Biochem J 2009; 420:345-61; PMID:19459784; https://doi.org/10.1042/BJ20090387

[24] Vernersson E, Khoo NK, Henriksson ML, Roos G, Palmer RH, Hallberg B. Characterization of the expression of the ALK receptor tyrosine kinase in mice. Gene Exp Patterns 2006; 6:448-61; PMID:16458083; https://doi.org/10.1016/j.modgep.2005.11.006

[25] Iwahara T, Fujimoto J, Wen D, Cupples B, Bucay N, Arakawa T, Mori S, Ratzkin B, Yamamoto T. Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. Oncogene 1997; 14:439-49; PMID:9053841; https://doi.org/10.1038/sj.onc.1200849