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COMMENTARY

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

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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.

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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

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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

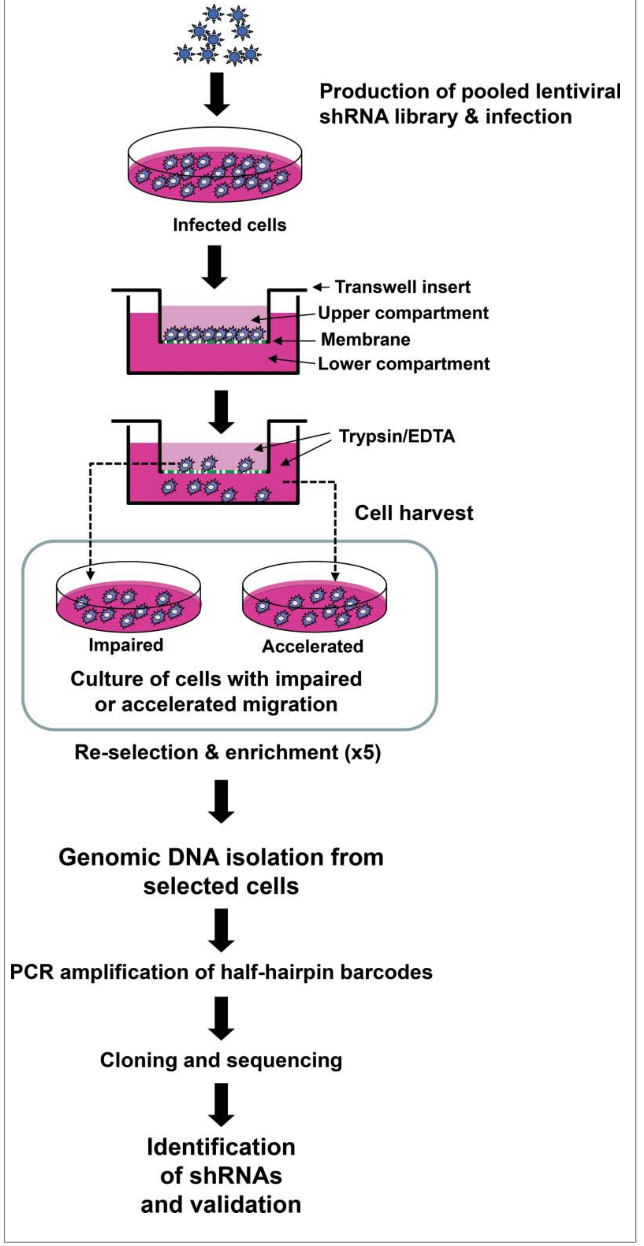

Figure 1. Schematic illustration of *in vitro* loss-of-function selection for cell migration-regulating genes. The production of a 63,996 pooled lentiviral shRNA library targeting 21,332 mouse genes was performed by the transient transfection of HEK293T cells with pHAGE-mir-30-RFP-shRNA, pVSV-G, pTat, pPM2, and pRev. For the introduction of the pooled lentiviral shRNA library, NIH3T3 fibroblast cells were seeded at a density of 1×10^6 cells/100-mm culture plate and infected with the lentiviral shRNA library at an MOI of 1 in the presence of polybrene (8 μg/ml). Two days after infection, cells were selected with puromycin (10 ng/ml) for 7 d and then detached and seeded onto transwell culture inserts. After seeding, cells were allowed to migrate across a porous membrane at 37°C for 5 or 24 hr to determine if they had an increased or decreased migration phenotype, respectively. Migrated and non-migrated cells were collected by trypsin-EDTA treatment from the lower and upper faces of the inserts, respectively, and re-seeded onto transwell culture inserts for a second round of selection. This process was repeated 5 times. After the final round, genomic DNA was isolated from migrating and non-migrating cells in order to identify the shRNAs integrated into the combined cells. DNA was subjected to PCR and sequencing. shRNA segments were amplified using the following primer set: forward, AGTGAAGCCACAGATGTA; reverse, CCTCCCCTACCCGGTAGA. All clones were sequenced. The sequences of 22-27 nt, corresponding to the half-hairpins of shRNAs, were used to identify shRNAs.

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	Histocompatibility 2, Q region locus 10	TTAGAAATCAGGACCATATGCTTG	BC042572
A930006J02Rik	RIKEN cDNA A930006J02 gene	AATGCAATAAACTGTGGAAGGA	AK020818
Atmin	ATM interactor	TTATAATACACTCACATTTGCATGCC	NM_177700
D630033O11Rik	RIKEN cDNA D630033O11 gene	AAGTTCCATATGGGACTGTGCA	XM_001001707
Gm379	Gm379 predicted gene 379	ATGTTCAATTCGTTCTTCTCCT	XM_142052
Gm1971	Gm1971 predicted gene 1971	TTATAATCCTGGTGGTGGAGGA	XM_001472879
Gm5615	Predicted gene 5615	TTGTCACCGGATTCATGTTGGA	NM_001033783
Gm12273	Gm12273 predicted gene 12273	TAAGGAAGTGGCCAAATTCGGG	XM_001479118
Gpkow	G patch domain and KOW motifs	TTCCACTTTGATTATCTCAGCTTG	NM_173747
Itpripl2	Inositol 1,4,5-triphosphate receptor interacting protein-like 2	TTCACATAAGAACCAAACACGA	NM_001033380
LOC668961	LOC668961 spindlin 2 family member	TACGTGTATAATATGGGATCCCTG	XM_001006595
Mpc1	Mitochondrial pyruvate carrier 1	TAAGGTTTAGCATTGATAAGGCTG	NM_018819
Otud6b	OTU domain containing 6B	ATTAGCGGGAAGAGTAACACCT	NM_152812
Ptx4	Pentraxin 4	TAGTGCCTGAACGCTTAGGGCC	NM_001163416
Rfpl4	Ret finger protein-like 4	TATAGATATGGGAGCCATCGCT	NM_138954
Trim59	Tripartite motif-containing 59	AAGTTCTTAGATAAACTCTGGTTGC	NM_025863
Usp45	Ubiquitin specific peptidase 45	TTAATTCGCCAATAAAGATGCGT	NM_152825
Zbed3	Zinc finger, BED domain containing 3	TAGATGCTGAAGGCAGGGAGCC	NM_028106
Sep15	Selenoprotein	TAAGTATTAAATTCGTACTGCATGCC	NM_053102
Adam2	A disintegrin and metallopeptidase domain 2	TAAATCGATATCCTTCTCGGCG	NM_009618
Irgm1	Immunity-related GTPase family M member 1	AAGAGATCTAAGGTAACCTGGC	NM_008326
Arid4a	AT rich interactive domain 4A (RBP1-like)	ATAATTCGTCATTGAGACGCCT	NM_001081195
Ccdc34	Coiled-coil domain containing 34	TAAATGTAAGCTCCGGGTAGCT	NM_026613
Cmtm2b	CKLF-like MARVEL transmembrane domain containing 2B	TTCTCGTCTTGCTTCAAGAGCA	NM_028524
Defb20	Defensin beta 20	ATTTATAATATCCAGACAAGGATGCCT	NM_176950
Frmd6	FERM domain containing 6	TAATAGTATCTTGCAATTCGGTTGC	NM_028127
Gpr143	G protein-coupled receptor 143	ATGGATTTCAACAGTACTGGCA	NM_010951
Hpdl	4-hydroxyphenylpyruvate dioxygenase-like	ATTTGTTCTTCTTTGCCGGGCT	NM_146256
Rp17	Ribosomal protein L7	TAAGGGTTCCTGGCACAGTGGC	NM_011291

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 p55γ 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.[14] Furthermore, the p55γ subunit regulates DNA synthesis,[15] differentiation, proliferation,[16] cell cycle progression,[17] and tumor angiogenesis.[18] We recently demonstrated that the receptor tyrosine kinase Alk enhanced phosphorylation of the p55γ regulatory subunits (Tyr199 residue) of PI3K through a physical interaction.[4] The Alk-induced phosphorylation of the p55γ regulatory subunit of PI3K was accompanied by Akt phosphorylation.[4] The critical role of p55γ and its phosphorylation in the ALK-induced Akt activation was confirmed by siRNA-mediated knockdown of p55γ.[4] 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.[19-21] Furthermore, an important role for ALK in nervous system development and function has also been reported.[22-25] Despite these data, the relationship between p55γ and ALK in brain development is poorly understood. Here, we assessed brain expression levels of ALK and p55γ during mouse embryogenesis, postnatal development, and adulthood. ALK and phospho-p55γ were highly expressed in the mouse brain at embryonic days (ED) 14.5 and 18.5, as

Table 2. Cell migration-regulating activity of 10 genes selected.

Symbols	GenBank accession No.	Remark	Fold change
Mtmr1	NM_016985	Myotubularin 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
Ptpn14	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_001081195	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

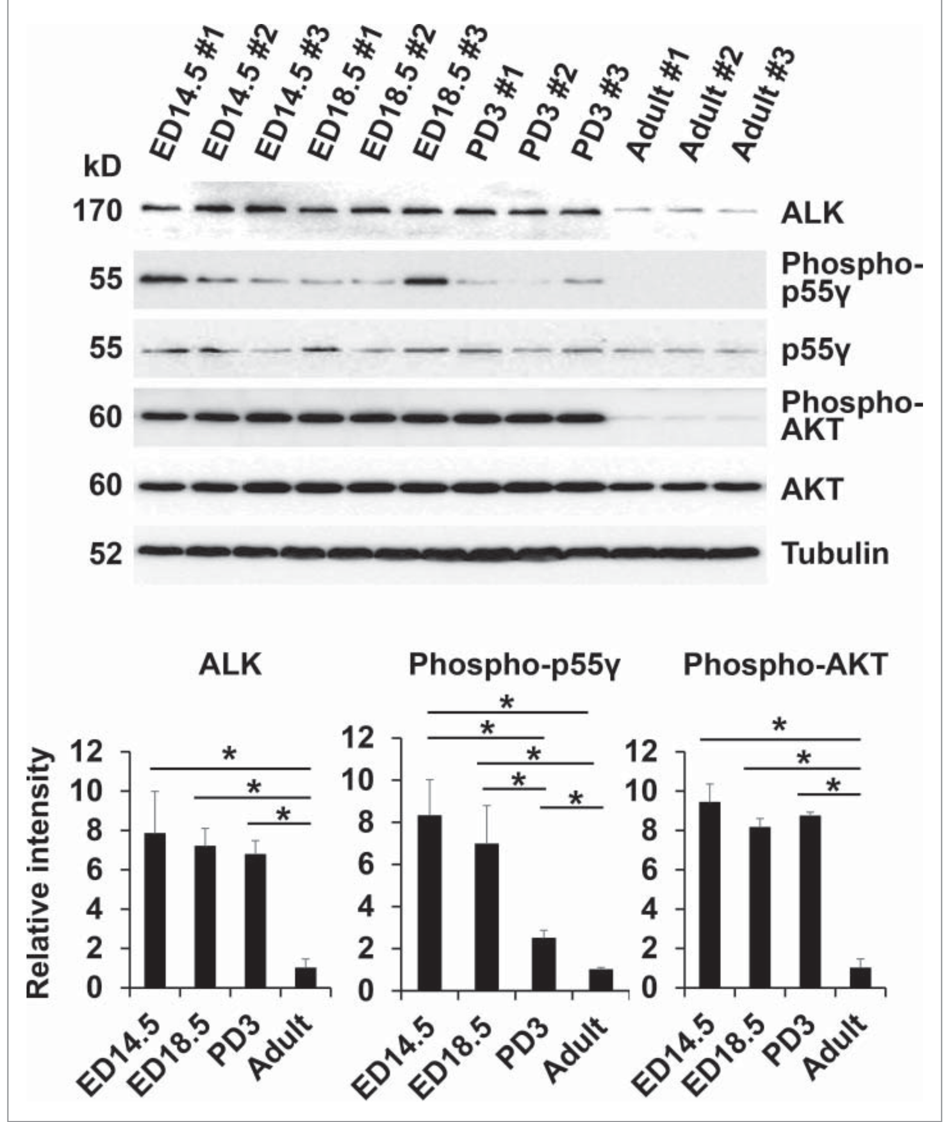

Figure 2. A similar temporal expression pattern of ALK, p55γ, and AKT during mouse brain development. Levels of ALK, phospho-p55γ, and phospho-AKT were assessed by Western blot analysis of whole brain lysates at different time points: embryonic stages (e.g., ED14.5, ED18.5), postnatal day (PD) 3, and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

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.

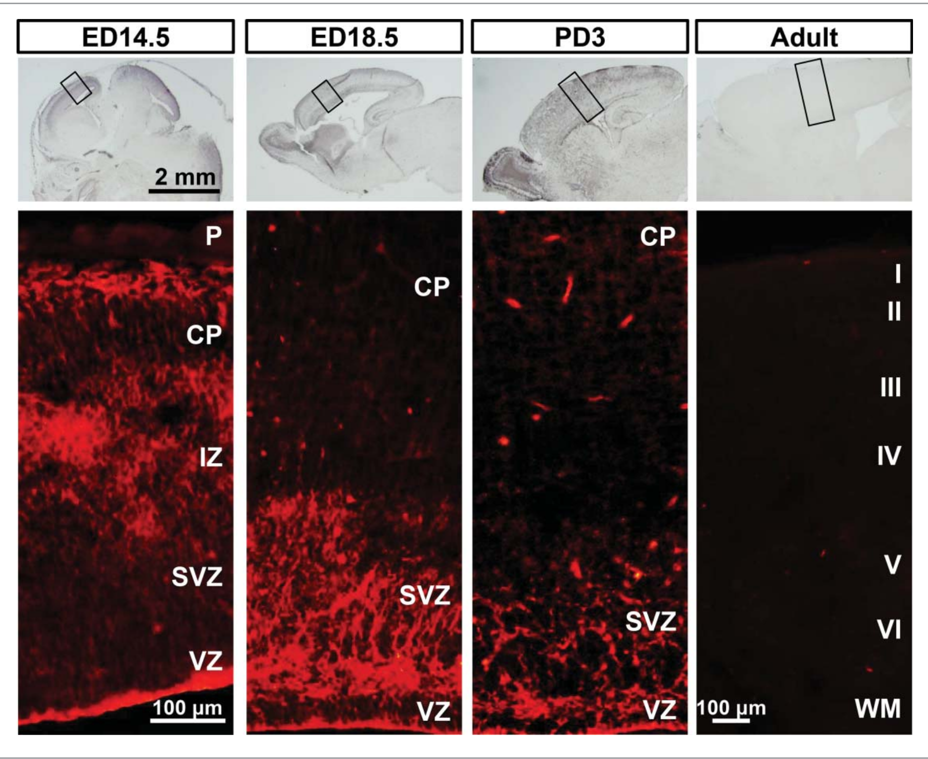

Figure 3. Spatiotemporal expression pattern of ALK during mouse brain development. Sagittal sections were immunostained with anti-ALK antibody and visualized with DAB in the mouse brain at embryonic days (ED) 14.5, 18.5, at postnatal days (PD) 3, and in the adult. Scale bar = 2 mm (*upper*). Laminar patterns of ALK-immunoreactive cells are shown. Alternatively, for immunofluorescence analysis, brain tissue sections were immunostained with an anti-ALK antibody and Cy^{TM}3-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 meter; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter. Scale bar = 100 μm.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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