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Integrated analysis of genomics and proteomics reveals that CKIP-1 is a novel macrophage migration regulator

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ABSTRACT

Casein kinase-2 interacting protein-1 (CKIP-1) has been identified to play an important role in cell morphology, differentiation and apoptosis. However, the role of CKIP-1 in other cellular processes is still unknown. Here we investigated transcriptome profiles of WT and CKIP-1-deficient mouse embryonic fibroblasts (MEFs), and found that innate immunity and cell migration related pathways were significantly correlated with CKIP-1 expression. As macrophage is a key cell type in innate immunity, we then used murine macrophage RAW264.7 cells to discover CKIP-1 interacting proteins by immunoprecipitation/mass spectrometry (IP/MS). Analysis of these proteins revealed migration related pathways were enriched. Further experiments indicated that knockdown of CKIP-1 in RAW264.7 cells resulted in impaired cell migration. Our study suggests that CKIP-1 is a novel regulator of macrophage migration.

1. Introduction

The pleckstrin homology (PH) domain-containing protein casein kinase 2 interacting protein-1 (CKIP-1) was originally identified as an interacting protein of casein kinase 2 (CK2) α subunit [1]. Previous studies based on cultured cells showed that CKIP-1 might participate in regulation of muscle cell differentiation [2], cell mophorlogy [3,4] and cell apoptosis [5]. The physiological role of CKIP-1 *in vivo* was discovered by the genetic study. We previously showed that CKIP-1 deficiency in mice results in increased osteoblast activity and accelerated bone formation, indicating CKIP-1 physiologically suppressed bone formation [6,7]. Recently, we reported that CKIP-1-deficient mice exhibited spontaneous cardiac hypertrophy with aging and sensitivity to pressure overload-induced cardiac hypertrophy [8]. However, the role of CKIP-1 in other physiological processes needs further investigation.

In recent years, knowledge based methods such as Gene Set Enrichment Analysis (GSEA) have been developed to aid in extracting biologically interesting information using the genome-wide transcriptome data. And GSEA has been successfully used in identifying important pathways in oncogenesis and diabetes [9,10] Here we performed an unbiased screen for transcriptomes of CKIP-$1^{+/+}$, CKIP-$1^{+/-}$ and CKIP-$1^{-/-}$ mouse embryonic fibroblasts (MEFs). Then GSEA was used to identify all the pathways significantly correlated with CKIP-1 expression. Except for gene expression data, interacting proteins will also provide clues for the protein's function. So we used IP/MS to identify proteins interacting with CKIP-1 and applied DAVID to identify over-represented pathways. Migration related pathways were enriched in both data sets. Further experimental results confirmed that knockdown of CKIP-1 in RAW264.7 macrophage cells resulted in impaired cell migration. Thus, CKIP-1 may be a novel regulator of macrophage migration.

2. Materials and methods

2.1. Cells and reagents

RAW264.7 cells were cultured in RPMI 1640 medium with standard formulations whereas 293FT and mouse embryonic fibroblasts (MEF) were cultured in DMEM. N-formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma.

2.2. RNA Isolation and cDNA preparation

Total cellular RNA was extracted from each sample using Trizol reagent according to the mannual provided by manufactor (Invitrogen). DNase-treated total RNA was prepared, and cDNA were got through a T7 based linear RNA amplification method and

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subsequent enzymatic reaction. The amplified RNA (aRNA) was purified with NucleoSpin Extract II (MN). The cDNA labeling approach with Klenow enzyme after reverse transcription was used. Test samples were labeled with Cy5-dCTP and the reference samples were labeled with Cy3-dCTP, then the labeling was reversed in the other array. Arrays were scanned with a confocal LuxScan 10 KA scanner (CapitalBio), and the data of obtained images were extracted with LuxScan 3.0 software (CapitalBio).

2.3. Microarray study design and data analysis

CKIP-1 knockout (CKIP-$1^{-/-}$), heterozygous mutant (CKIP-$1^{+/-}$) and wildtype (CKIP-$1^{+/+}$) mouse embryonic fibroblasts (MEFs) were used as test samples and 3 distinct wildtype MEFs were used as reference samples. Data was normalized by LOWESS algorithm [11]. Then probesets with the signal intensity (Cy3 or Cy5) $\leqslant 400$ in any array were removed. After these steps, 8,272 probesets representing 5,877 genes survived. The intensity ratios for the probesets were calculated as intensity of the test sample divided by intensity of the reference sample. Intensity ratios of 6 microarrays were log transformed, and then hierarchical clustering analysis was performed with the Pearson correlation distance measure and average linkage with Cluster (version 3.0) and graph was displayed with TreeView.

Relative expression value for each probeset of CKIP-$1^{-/-}$, CKIP-$1^{+/-}$ or CKIP-$1^{+/+}$ MEFs was obtained by geometric mean of intensity ratios of two reversed labeling arrays (Ratio = $(\text{Ratio1} * \text{Ratio2})^{1/2}$). Then Gene Set Enrichment Analysis (GSEA) was used to determine whether a priori defined set of genes (KEGG gene sets) was statistically significant correlated with CKIP-1 expression. In this study, the javaGSEA implementation was used for GSEA analysis. Pearson correlation coefficient values between all 5,877 genes and CKIP-1 were pre-ordered ranked, and 1,000 permutations were applied to sample labels to test if each priori defined KEGG gene set was randomly distributed along the gene list.

2.4. Immunoprecipitation (IP)

Immunoprecipitations were carried out essentially as described [6]. Briefly, RAW264.7 cell in the TNE lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and a cocktail of protease and phosphatase inhibitors. To remove debris, lysates were centrifuged at 10,000*g* for 10 min. Then a two-step IP protocol with 3 h primary antibody incubation and subsequent overnight incubation with Protein A/G Sepharose beads. Beads were then washed with TNE buffer for 3 times.

2.5. SDS/PAGE and mass spectrometry (MS)

IPs were resolved on 4–20% precast Novex Tris–Glycine gels to half-length. Gels were minimally stained with Coomassie brilliant blue to differentiate IgG bands. Each lane was then cut into 10 molecular weight regions and a heavy chain band. These bands were digested with 100 ng of trypsin overnight, extracted twice with 100% acetonitrile, and dried in a Savant Speed-Vac. Peptides were then resuspended in 5% methanol and loaded onto a BioBasic C18 column. LTQ Orbitrap Velos (Thermo) was run in a data-dependent mode, where each sample was eluted in a 1 h 5–30% acetonitrile gradient with a Easy-nLC II liquid chromatography system (Thermo).

2.6. IP/MS Data analysis

Mass spectrometry data were searched against mouse protein RefSeq database using Mascot software in Proteome Discoverer 1.3 Suites. The false positive rates were set to 1% and 5% as restrict and relax cutoff respectively. The mass tolerance was set to 10 ppm and 0.5 Da for precursor and fragment ion. Oxidation (M), Acetyl (Protein-N term), DeStreak(C) and Phospho (S/T/Y) were chosen as variable modifications; and one missed cleavage on trypsin was allowed. IP/MS results were transferred into an in-house built FileMaker-based relational database where protein identification numbers (protein GIs) were converted to the GeneID identifiers according to the NCBI "gene2accession" table. Data filtering and deconvolution were performed as described in the text. Pathway enrichment analysis for potential interacting proteins of CKIP-1 was performed by DAVID (http://david.abcc.ncifcrf.gov/).

2.7. Viral infection

CKIP-1 shRNA were inserted to U6-Puro-GFP vector for knock down assay. CKIP-1-lentivivral shRNA-1 (5′-CCTGAGTGACTATGAGAAGTT-3′), CKIP-1-lentivivral shRNA-2 (5′-AGTGCGAAGAGCTCCGGAAAT-3′), control shRNA (5′-TTCTCCGAACGTGTCACGT-3′) were transfected with packing plasmids into 293FT cells for 2 days, and virus particles containing CKIP-1 or control shRNAs were used to infect RAW264.7 cells. All the infected cells were cultured in medium with 5 μg/ml puromycin for 2 days.

2.8. Immunoblotting

For western experiment, cells were lysed in the TNE lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1%

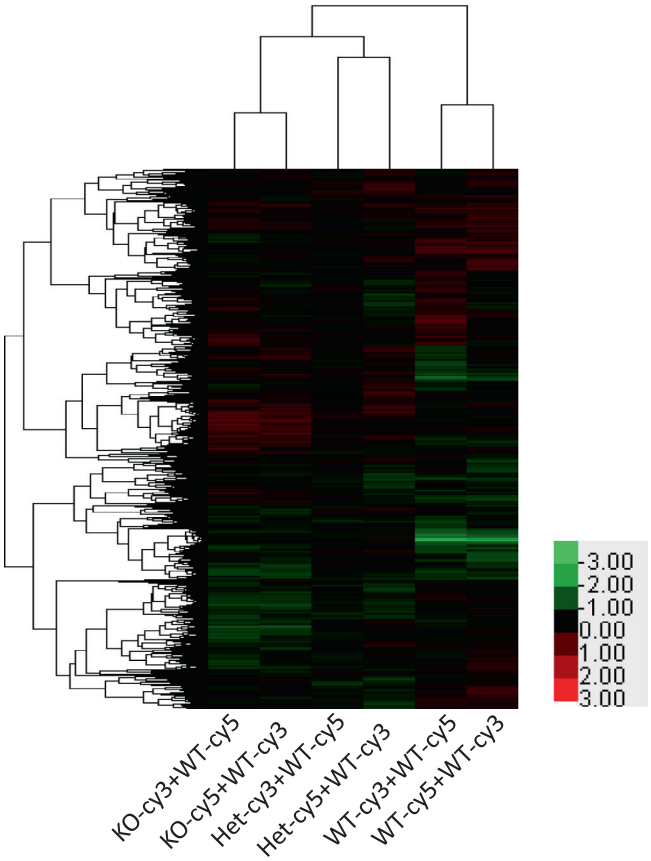

Fig. 1. Gene expression differences in CKIP-$1^{-/-}$, CKIP-$1^{+/-}$ and CKIP-$1^{+/+}$ MEFs. Expression patterns of 8,272 probesets representing 5,877 genes of 6 microarrays were shown in a heatmap. Data was log transformed and hierarchical clustering analysis was performed with the pearson correlation distance measure and average linkage. The data was colored red for high expression while green for low expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NP-40, and a cocktail of protease and phosphatase inhibitors. Primary antibodies used were: Goat polyclonal CKIP-1 antibody (Santa Cruz), mouse monoclonal GAPDH antibody (Santa Cruz). Standard immunoblot protocol was used.

2.9. Transwell migration assay

For migration experiments, 6.5 mm-diameter Transwell™ chambers (Corning, NY) with 8 μm pore size were used. 1×10^5 cells were added in the upper well in 100 ml RPMI 1640 medium and allowed to migrate to the bottom compartment containing 1 nM N-formyl-methionyl-leucyl-phenylalanine (fMLP) used as chemoattractant (F3506, Sigma–Aldrich) for 24 h, followed by wiping off the non-migrated cells with a cottons wab. For quantification of migration, Transwell filters were fixed in methanol for 10 min and stained with 0.1% Crystal Violetin 20% (v/v) methanol for 5 min and mounted on a glass slide. The evaluation of the completed transmigration was performed under the microscope, and

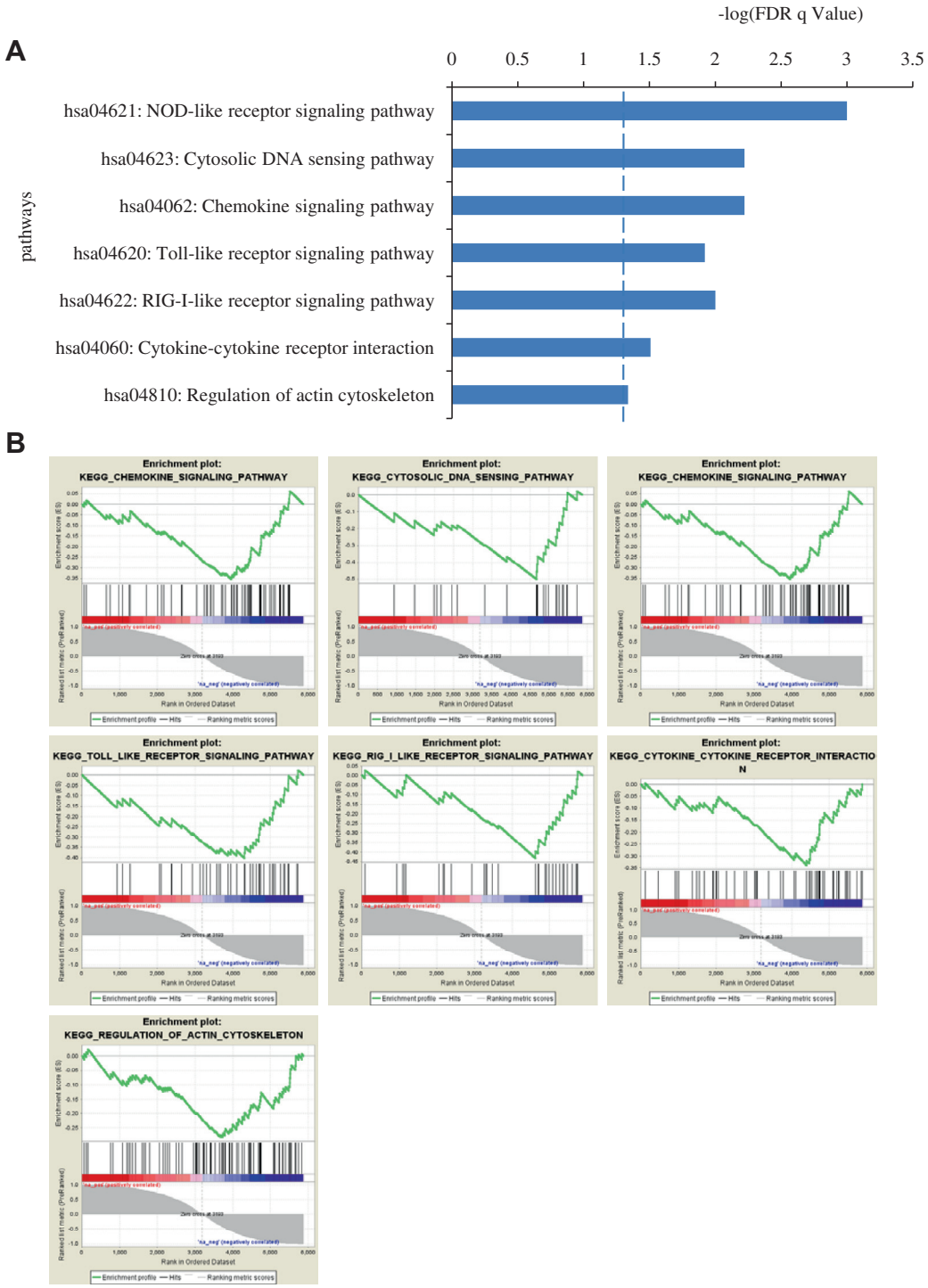

Fig. 2. KEGG Pathways significantly correlated with expression of CKIP-1 in MEFs. (FDR q value < 0.05). (A) pathways identified by GSEA to be significantly correlated with expression of CKIP-1 in MEFs with a threshold of FDR q value < 0.05 (dashed line: FDR q value equal to 0.05); (B) Enrichment plot for each pathway, 7 pathways were all negatively correlated with CKIP-1 expression.

the cell number on each filter was counted in five randomly selected fields under a microscope (400×).

3. Result

3.1. Signaling pathways correlated with CKIP-1 expression in MEFs

To identify relevant pathways with CKIP-1 expression, we obtained unbiased transcription data from CKIP-1-deficient (CKIP-$1^{-/-}$), heterozygous mutant (CKIP-$1^{+/-}$) and wildtype (CKIP-$1^{+/+}$) MEFs. Hierarchical clustering analysis of 6 microarrays correctly clustered to 3 clusters (Fig. 1). Then we used GSEA to discover pathways which were correlated with CKIP-1 expression. In the end, 7 pathways were found negatively correlated with CKIP-1 expression, including several classical innate immunity-related pathways and cell migration-related pathways (Fig. 2). The most significantly altered pathway was NOD-like receptors signaling pathway. NOD-like receptors (NLRs) are intracellular sensors of pathogen-associated molecular patterns (PAMPs) that enter the cell via phagocytosis or pores and dassociated molecular patterns (DAMPs) that are associated with cell stress. They are part of pattern recognition receptors and play key roles in regulation of innate immune response [12]. In addition, some involved pathways such as cytosolic DNA-sensing pathway, Toll-like receptors

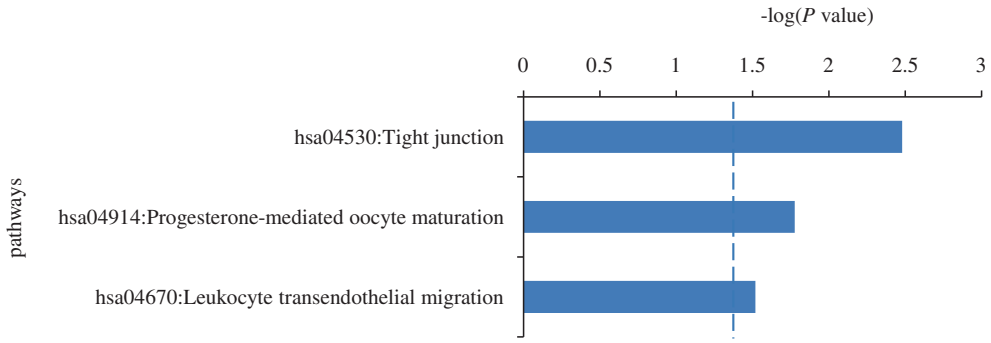

Fig. 3. KEGG Pathways significantly enriched in CKIP-1 interacting proteins analyzed by DAVID (dashed line: *P* value equal to 0.05).

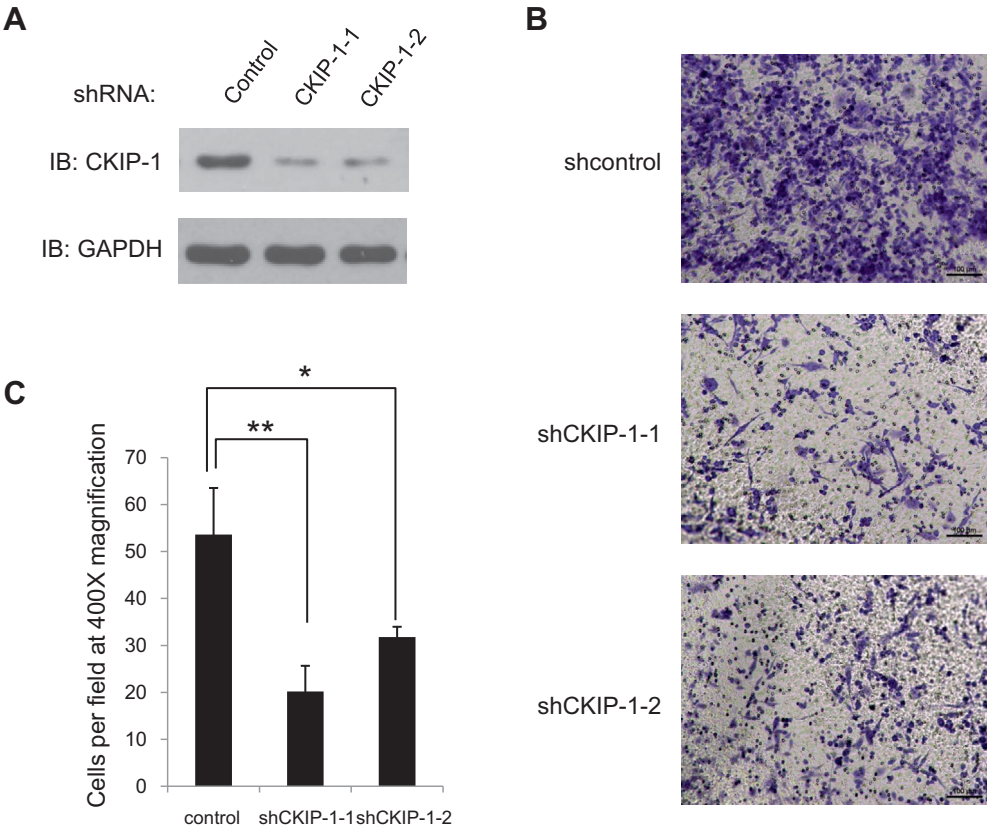

Fig. 4. CKIP-1 KD in macrophages leads to defective migration. (A) Western blot analysis of CKIP-1 protein expression in control and CKIP-1 KD RAW264.7 cells. (B-C) Transwell assay were performed in transwell cell culture chambers, control and CKIP-1 KD RAW264.7 cells in 100 μl 1640 media was added to the upper reservoir. 1640 medium with 100 nM fMLP was added to the lower reservoir. After 24 h, cells remaining on the upper membrane were scraped off, and cells on the lower membrane surface were stained with Crystal Violetin. (B) Pictures were taken from a representative field at 100×magnification and (C) counted at random under a microscope (400×). The experiments were performed three times in triplicate with similar results. $^{*}p < 0.01$, $^{**}p < 0.001$ (unpaired two-tail *t* test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

signaling pathway as well as RIG-I-like receptors signaling pathway are all well established innate immunity pathways [13–15]. Other signaling pathways enriched were chemokine signaling pathway and cytokine-cytokine receptor interaction. Chemokines are a family of small cytokines, or signaling proteins secreted by cells. Their names are derived from their ability to induce directed chemotaxis in nearby responsive cells; they are chemotactic cytokines. Members of the chemokine superfamily are crucially involved in both innate and adaptive responses. By binding to their receptors, chemokines conduct the migration of cells into infectious foci and subsequent activation of these cells, enabling the host to achieve effective and efficient removal of microbes [16,17]. Taken together, analysis of transcriptome of WT and CKIP-1-deficient MEFs revealed that CKIP-1 expression affected innate immunity pathways.

3.2. IP-MS identified CKIP-1 interacting proteins

Given that pathways significantly affected by CKIP-1 expression were enriched in innate immunity, we were very interested in proteins interacting with CKIP-1 and decided to identify them in macropahges, a key cell type in innate immunity. Murine macrophage cell line RAW264.7 was used to perform CKIP-1-IP/MS. We carried out insolution trypsin digestion of the CKIP-1-IP and the IgG-IP products, as well as in-gel digestion of individual bands. After MS analysis, we identified a total of 14 unique peptides that corresponded to the CKIP-1 protein, and no peptide that corresponded to the CKIP-1 protein was identified in IgG-IP products, suggesting that our CKIP-1-IP product contains the CKIP-1 protein. To be selected as candidate CKIP-1 interacting proteins, a minimum of two unique peptides per protein was required in the CKIP-1-IP. Then, to remove as much as potential contamination and/or nonspecific binding of proteins from the IgG portion of the antibody, we removed those proteins that were identified in the CKIP-1-IP with unique peptides number 2 times less than that of IgG-IP. In the end, a total of 27 proteins were identified as putative CKIP-1 interacting proteins in RAW264.7 cells (Supplementary Table 1).

3.3. Pathway enrichment analysis of the CKIP-1 interacting proteins

We next used DAVID to identify pathways enriched with 27 potential CKIP-1 interacting proteins. We found that these proteins were enriched in "hsa04530: Tight junction", "hsa04914: Progesterone-mediated oocyte maturation" and "hsa04670: Leukocyte transendothelial migration" pathways (Fig. 3). Among these pathways, we found that two pathways are related to cell migration. Leukocyte transendothelial migration pathway controls leukocytes crossing blood vessels. This process occurs through diapedesis, in which the cells moves in an ameboid fashion through tightly apposed endothelial borders and, in some cases, through the endothelial cell itself [18]. Tight junctions are the closely associated areas of two cells whose membranes join together forming an impermeable barrier to fluid. They also help to maintain the polarity of cells and regulate cell migration [19,20]. Thus, these results strongly suggest a role of CKIP-1 in regulation of macrophage migration.

3.4. KD of CKIP-1 result in defective macrophage migration

To confirm our findings that CKIP-1 regulates macrophage migration, we established two stable CKIP-1 knockdown (KD) RAW264.7 cell lines. These CKIP-1 KD cells showed a marked reduction of CKIP-1 protein expression (Fig. 4A). We then analyzed the CKIP-1 KD effect on cell migration. The migration of CKIP-1 KD cells in the presence of the chemoattractant fMLP was significantly impaired compared with control cells. (Fig. 4B and C). These results indicate that CKIP-1 is required for chemoattractant-induced macrophage migration.

4. Discussion

In summary, through integrating gene expression microarray data and CKIP-1-IP/MS data, unexpected CKIP-1 function in macrophages was revealed. Our study provided a successful case for integrating high-throughput analysis and traditional functional assays. Gene expression microarray data found chemokine signaling pathway and innate immunity related pathways were significantly correlated with CKIP-1 expression, meanwhile, interacting proteins of CKIP-1 in macrophage was enriched for migration related pathway. Both datasets suggest that CKIP-1 affect macrophage migration. Furthermore, functional data suggest that CKIP-1 is a crucial regulator of macrophage migration.

CKIP-1 has been reported to be involved in regulation of cell morphology and actin cytoskeleton, so it is not unexpected that CKIP-1 plays a role in cell migration. However, it's very interesting that CKIP-1 could be a regulator in innate immunity. CKIP-1 is expressed at substantially higher levels in macrophage (www.biogps.gnf.org), which plays a pivotal role in antimicrobial immune defence and tissue healing. Therefore, further studies employing a CKIP-1 deficient mouse model will be necessary to understand in detail the physiologic functions of this protein in the signaling pathways that regulate inflammatory mediator production and subset macrophage development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.109.

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