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Whole genome expression microarray reveals novel roles for Kif4 in monocyte/macrophage cells

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Abstract. – OBJECTIVE: Kinesin superfamily member 4 (Kif4), a conventional kinesin, is a microtubule-dependent molecular motor. The active movement of Kif4 participates in several cellular functions, including DNA repair, mitosis, the transport of macromolecules, survival of neurons and even tumorigenesis and progression. However, the role of Kif4 in monocyte/macrophage cells has not been reported. Our work aimed to increase understanding and further investigations of Kif4 in monocyte/macrophage cells.

MATERIALS AND METHODS: RAW264.7 cells were transfected with Kif4 small interfering RNA (siRNA), and whole genome expression microarray analysis was employed to analyze gene expression after cells treatment with or without Kif4 siRNA.

RESULTS: Our data found multiple differentially expressed genes which were enriched in the top 5 biological processes about innate immune response, immune response, response to interferon-beta, immune system process and cellular response to interferon-beta. 23 most significant pathways had been identified and enriched pathways indicated enrichment in peroxisome, lysosome, fatty acid metabolism, cell adhesion molecules and so on.

CONCLUSIONS: Our work may help understand the roles of Kif4 in monocyte/macrophage cells and would give useful information on basic research and the function of monocyte/macrophage cells.

Key Words:
Kinesin superfamily member 4, Whole genome, Microarray, Monocyte/macrophage cell.

Introduction

The kinesin superfamily (KIFs) is a major component of the intracellular transport system and Kinesin superfamily proteins are important molecular motors involved in the transportation of a variety of cytoplasmic cargos. Kinesins play vital roles in the maintenance of cell morphology and the realization of physiological functions[1]. In recent years, researchers have shown an increased interest in kinesin superfamily protein 4 (Kif4). Kif4 is a highly conserved protein in different species[2] and is an essential factor involved in a variety of cellular processes, such as DNA repair and replication[3], the stability of gene[4-6], regulating mitosis, meiosis[6,7] and division of the cytoplasm[8]. Kif4 also plays critical roles in the development and survival of neurons[9,10], virus infection[11], nervous system disease and tumor progression[12-17].

Monocyte/macrophage is one of the important immune cells. They are highly versatile and multifunctional immune effector cells and they equipped with pathogen recognition to engulf invading microbes from injured sites. Furthermore, they display a high degree of plasticity and can alter their phenotype to suit the particular microenvironments[18]. Monocyte/macrophage cells also can secrete a wide array of cytokines, present antigens to T cells and lymphocytes and contribute to the pathogenesis of inflammatory and tumor progression. So, monocyte/macrophage cells are an increasingly attractive therapeutic target in many pathologic states[19].

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So far, however, little is known about the functions of Kif4 in immune cells, especially in monocyte/macrophage cells. To identify the expression profiles and the roles of Kif4 in monocyte/macrophage cells, we delivered Kif4 small interfering RNA into RAW264.7 cells and used microarray analysis to identify differentially expressed genes in cells. We obtained the microarray data from the experiments to offer a resource for investigators to study Kif4 function in immune cells and will provide a novel perspective for understanding the role of both individual gene products and entire pathways in RAW264.7 monocyte/macrophage cells.

Materials and Methods

Cell Culture

RAW264.7 cells (American Type Culture Collection; ATCC, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (all from Gibco, Grand Island, NY, USA). Cells were cultured at 37°C in a humidified air with 5% CO_2. The RAW264.7 cells were seeded at a density of 5×10^5 cells/well into 6-well plates and treated with 2 mL of medium.

Small Interfering RNA (siRNA) Transfection

The following siRNA sequences were used: Kif4 sense, 5'-GCUGAAGUUUAGGCAAUTT-3' and antisense, 5'-AUUGCCUAAACUUCUCAGCTT-3'; Negative control (NC) sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3' synthesized by Gene Chem, Co., Ltd. (Shanghai, China). The siRNA transfections were performed in Opti-MEM serum-free medium (Invitrogen, Carlsbad, CA, USA). To form complexes, the Kif4 siRNA (SiRNA group), NC siRNA (NC group; 6 μL) and Lipofectamine™ 2000 (4.5 μL; Invitrogen, Carlsbad, CA, USA) were each diluted in Opti-MEM and incubated at room temperature for 5 min; then Kif4 siRNA or NC siRNA were mixed gently with Lipofectamine™ 2000 respectively and incubated for 20 min at room temperature. The complexes were added to the RAW264.7 cells in 6-well plates. After 6 h, the cells were placed in fresh complete medium. After incubating either 24 h, total RNA was extracted. The experiment was repeated three times and samples labeled as NC1 *vs.* SiRNA1, NC3 *vs.* SiRNA3 and NC5 *vs.* SiRNA5.

Preparation of RNA and Quality Analysis

Total cellular RNA was isolated by TRIzol RNA Isolation Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer. NanoDrop ND-1000 spectrophotometer was used to detect RNA quantity and quality. The quality of gene data was shown in the box plot.

RNA Labeling and Array Hybridization

Prepared total RNA samples were labeled and hybridized to the Mouse Gene Expression 4x44K Microarray V2 (Agilent Technologies, Santa Clara, CA, USA) according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol. Total RNA from each sample was amplified, labeled with Cy3-UTP and purified by RNeasy Mini Kit (Qiagen, Hilden, Germany). After the labeling efficiency quality check measured by NanoDrop ND-1000, 1 μg of each labeled cRNA was fragmented by adding 11 μL 10×Blocking Agent and 2.2 μL of 25×Fragmentation Buffer, then heated at 60°C for 30 min, and finally diluted by 55 μL 2×GE Hybridization buffer. 100 μL of hybridization solution was dispensed into the gasket slide and assembled to the microarray slide. The Agilent Hybridization Oven was used to incubate the slides for 17 hours at 65°C. The hybridized microarrays were then washed, fixed and scanned using the Agilent G2505C DNA Microarray Scanner (Agilent Technologies, Santa Clara, CA, USA).

Statistical Analysis

Acquired array images were analyzed by the Agilent Feature Extraction software (version 11.0.1.1, Agilent Technologies, Santa Clara, CA, USA). Quantile normalization and subsequent data processing were then performed with the GeneSpring GX v12.1 software (Agilent Technologies, Santa Clara, CA, USA). After quantile normalization of the raw data, further data analysis were used the R script. Scatter plot and Volcano Plot were performed to identify differentially expressed genes. Differentially expressed genes were identified through Fold Change filtering (fold change ≥ 1.5) and the p-value was <0.05. Gene function was described by Gene Ontology (GO). Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway analysis was used to determine

Table I. RNA quantification and quality assurance by NanoDrop ND-1000.

Sample ID	OD260/280 Ratio	OD260/230 Ratio	Conc. (ng/μL)	Volume (μL)	Quantity (ng)
NC1	1.94	2.47	219.78	70	15384.60
SiRNA1	1.92	2.20	225.29	70	15770.30
NC3	1.89	2.47	321.16	80	25692.80
SiRNA3	1.88	2.49	312.56	70	21879.20
NC5	1.93	2.40	427.47	80	34197.60
SiRNA5	1.92	2.38	400.25	80	32020.00

the genes in different biological pathways. When the *p*-value is lower, the GO term and KEGG pathway are more significant ($p<0.05$).

Results

RNA Quality Analysis

For NanoDrop ND-1000 spectrophotometer, the OD A260/A280 ratios of RNA samples were between 1.8 and 2.0 and the ODA260/A230 ratios were all more than 1.8 (Table I). This indicated that the quality of the samples was superior and suitable for the microarray analysis. The intensity distributions of all samples were shown in the box plot. The distributions of $\log_2$ ratios were similar among the samples (Figure 1A).

Determination of Differentially Expressed Genes

The data from three independent samples demonstrated that 1216 genes were differentially expressed between the NC group and SiRNA group. Scatter plot showed differentially expressed genes in the two groups (Figure 1B). Volcano Plot was used to identify differential expression with statistical significance. The vertical lines correspond to 1.5-fold decrease or increase, and the horizontal line represents a *p*-value of 0.05. Each red dot represented a differentially expressed gene with statistical significance in the plot (Figure 1C).

GO Category and KEGG Pathway Analysis

The affected genes were categorized on the basis of their function under GO terms. The genes were grouped into the major categories of biological process (BP), molecular function (MF) and cellular component (CC). The categorized genes were shown graphically and the top 20 terms in each category were listed according to the size of Enrichment Score (Figure 2). In the

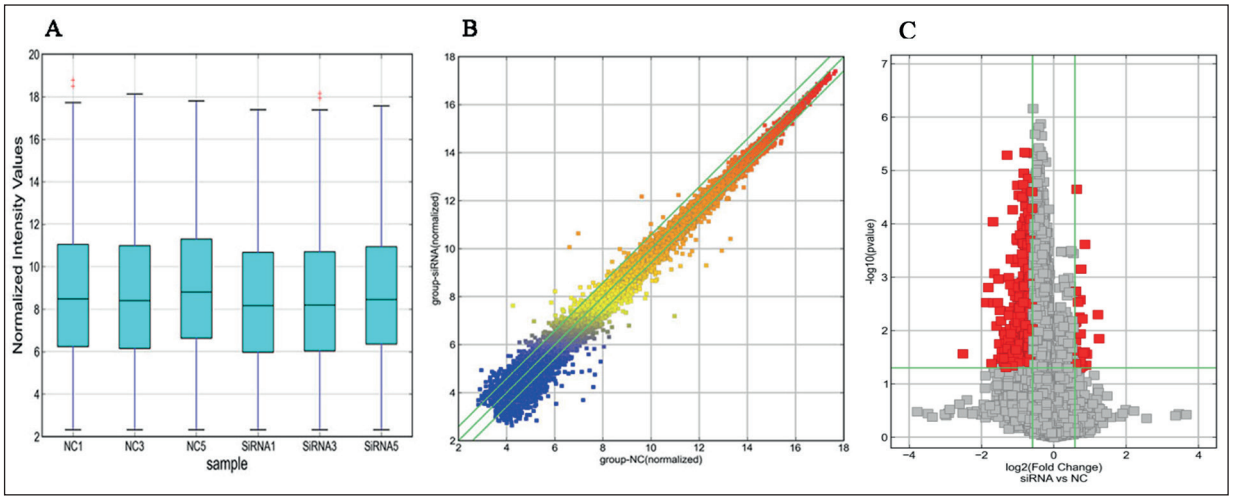

Figure 1. Quality evaluation of gene data and differential gene expression screening. ***A,*** The box plot was used to compare the distributions of the intensities from all samples. ***B,*** A scatter plot indicated gene expression variation between the two groups. ***C,*** Volcano plot was performed to identify differential gene expression between the two groups. The vertical lines on the left and right correspond to 1.5-fold decrease and increase, and the horizontal line represents a *p*-value of 0.05.

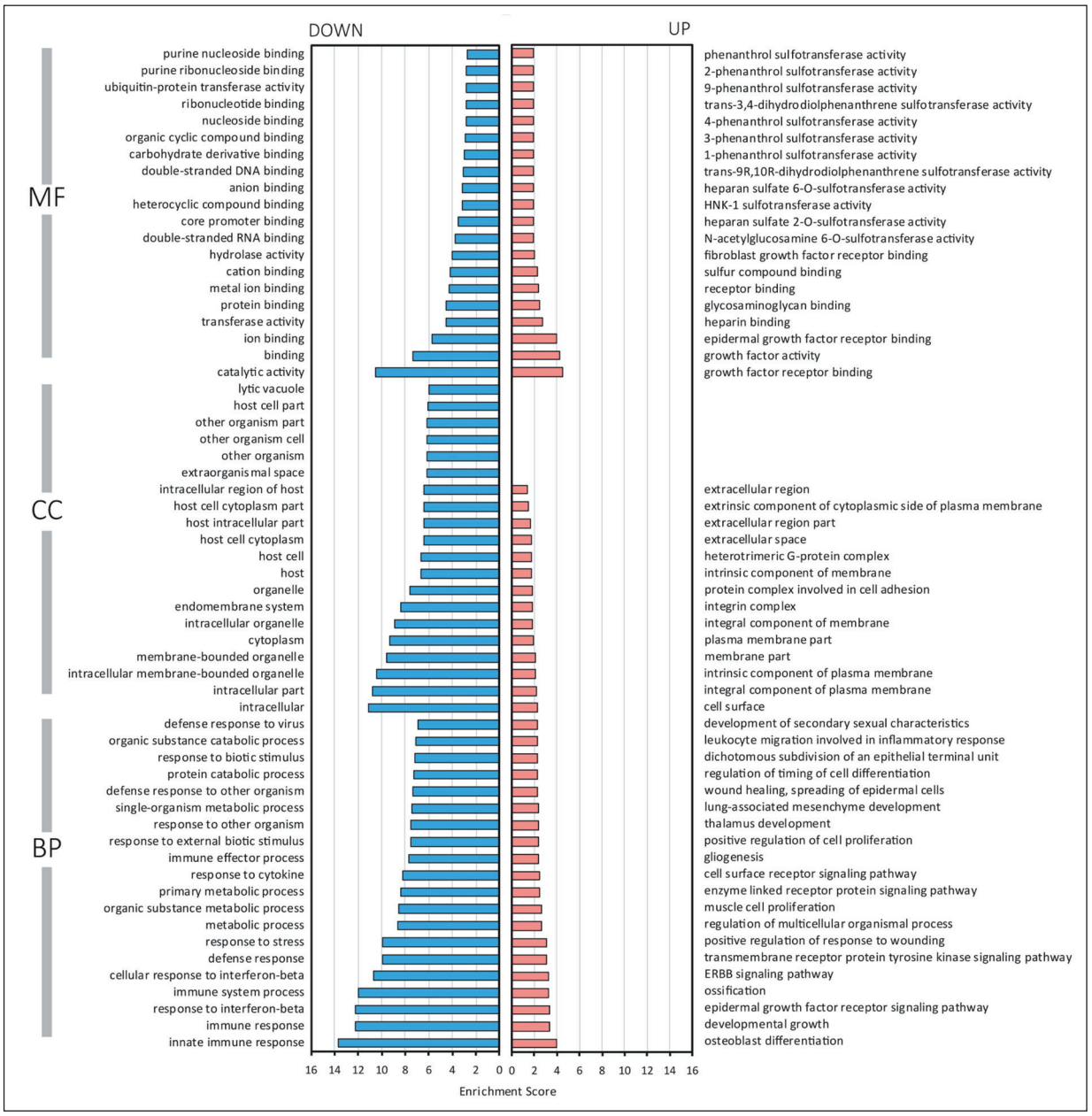

Figure 2. Gene Ontology (GO) functional categorization of the differentially expressed genes. The genes were grouped into the major categories of molecular function (MF), cellular component (CC) and biological process (BP). Numbers on the x-axis represent Enrichment Score for each GO terms. Enrichment Score represents the enrichment score value of the GO terms, which equals -log10 (*p*-value).

BP analysis, "innate immune response", "immune response", "response to interferon-beta", "immune system process", "cellular response to interferon-beta" were enriched significantly. In the MF analysis, the significantly enriched terms were "catalytic activity", "binding", "ion binding", "transferase activity", "protein binding". In the CC category, "intracellular", "intracellular part", "intracellular membrane-bounded organelle", "membrane-bounded organelle", "cytoplasm" were the significant terms.

Furthermore, KEGG pathway analysis was employed to identify the roles of differentially expressed genes in biological pathways. 20 KEGG pathways displayed a significant enrichment ($p<0.05$) in downregulated genes (Table II), and 3 KEGG pathways in upregulated genes (Table III).

Table II. Downregulated expressed genes associated with KEGG pathways with Fisher-*p*-value.

Pathway ID	Pathway name	Fisher-*p*-value	Genes
mmu04146	Peroxisome	0.00063225	ACSL1//ACSL5//CAT//CROT//HACL1//IDH1//NUDT12//PEX13//PMVK//PRDX1
mmu04142	Lysosome	0.001583387	ABCB9//AGA//AP3M1//ASAH1//CTSE//GBA//LAPTM4A// NAGLU//PLA2G15//PPT1//SCARB2//SLC11A1
mmu05166	HTLV-I infection	0.005411997	ADCY9//AKT3//ATM//CHEK2//CREM//H2-Q10//H2-Q8//H2-T10//H2-T24//H2-T9//ITGB2L//MAP2K4//NRP1//PIK3CD//PRKX//PTTG1//SLC25A31//WNT10A//XPO1
mmu00520	Amino sugar and nucleotide sugar metabolism	0.007263766	AMDHD2//CYB5R3//HK2//NANP//UGP2//UXS1
mmu05164	Influenza A	0.008530607	AKT3//CXCL10//DNAJC3//IFIH1//MAP2K4//MX2//OAS1G//PIK3CD//PML//RSAD2//STAT2//TLR3//XPO1
mmu01212	Fatty acid metabolism	0.008858538	ACADSB//ACSL1//ACSL5//CPT2//PPT1//SCD1
mmu03450	Non-homologous end-joining	0.01022955	DCLRE1C//PRKDC//RAD50
mmu04514	Cell adhesion molecules (CAMs)	0.01303647	CD274//H2-Q10//H2-Q8//H2-T10//H2-T24//H2-T9//ICOSL//ITGB2L//PVRL2//SELPLG//SIGLEC1//SPN
mmu04110	Cell cycle	0.01568791	ATM//CDC14A//CDC25A//CDK7//CDKN2D//CHEK2//PRKDC//PTTG1//RBL1//SMC1B
mmu05168	Herpes simplex infection	0.01647455	H2-Q10//H2-Q8//H2-T10//H2-T24//H2-T9//HCFC2//IFIH1//IFIT1//OAS1G//PML//PVRL2//STAT2//TAP2//TLR3
mmu04650	Natural killer cell mediated cytotoxicity	0.01656133	CD48//H2-Q10//H2-Q8//H2-T10//H2-T24//H2-T9//ITGB2L//PIK3CD//RAET1A//RAET1C//SOS1
mmu04668	TNF signaling pathway	0.01691003	AKT3//BIRC3//CSF1//CXCL10//GM5431//IFI47//MAP2K4//PIK3CD//RPS6KA5
mmu05169	Epstein-Barr virus infection	0.02219575	AKT3//H2-Q10//H2-Q8//H2-T10//H2-T24//H2-T9//MAP2K4//PIK3CD//POLR3GL//PRKX//RBPJ//SPN//STAT3//XPO1
mmu00511	Other glycan degradation	0.02554953	AGA//FUCA2//GBA
mmu04612	Antigen processing and presentation	0.02676803	H2-Q10//H2-Q8//H2-T10//H2-T24//H2-T9//PSME2//TAP2
mmu00071	Fatty acid degradation	0.02877699	ACADSB//ACSL1//ACSL5//ADH7//CPT2
mmu00600	Sphingolipid metabolism	0.02877699	ASAH1//CERK//GBA//SPTLC1//SPTLC2
mmu05212	Pancreatic cancer	0.03145713	AKT3//BRCA2//CDC42//PIK3CD//STAT3//VEGFA
mmu05150	Staphylococcus aureus infection	0.03620351	C1RA//C3AR1//CFB//ITGB2L//SELPLG
mmu05330	Allograft rejection	0.04471008	H2-Q10//H2-Q8//H2-T10//H2-T24//H2-T9

Discussion

Kif4 protein is widely expressed in various tissues, especially strong in juvenile tissues[20]. Kif4 is known as one of the chromosome binding kinesins and plays an important role in the cell cycle. Kif4 can interact with DNA and DNA related protein to maintain the stability of chromatin and chromosome structure[4]. Midorikawa et al[9] had shown that Kif4 is involved in cell proliferation and neuronal survival in brain development. Kif4 was also found abnormal expression in a variety of cancers[21]. However, no report has clarified the clinical values and roles of Kif4 in monocyte/macrophage cells.

Table III. Upregulated expressed genes associated with KEGG pathways with Fisher-*p*-value.

Pathway ID	Pathway name	Fisher-*p*-value	Genes
mmu04012	ErbB signaling pathway	0.001284773	AREG//HBEGF
mmu04810	Regulation of actin cytoskeleton	0.007773268	FGF9//ITGAX
mmu05205	Proteoglycans in cancer	0.008412588	FGF9//HBEGF

Genome-wide RNA sequencing microarrays are widely used in various biological and medical research. Over the past decade, they have become an important method to analyze the function and regulation of individual genes from many organisms[22]. So, these data were likely to be valuable for those wishing to study the roles Kif4 gene played in RAW264.7 monocyte/macrophage cells, including cell cycle regulation, survival, apoptosis, innate immune response, metabolic process and so on.

Of note, the microarray data revealed that differentially expressed genes were enriched in cell growth and survival, such as Kif26A 5.73-fold downregulation (p=0.027), IFIT3 2.81-fold downregulation (p=0.0042), and Hoxb8 2.61-fold downregulation (p=0.010). Kif 26A belongs to the Kinesin 11 subfamily and is involved in microtubule stabilization, cell growth, cell division and motility[23,24]. The IFN-induced protein with tetratricopeptide repeat 3 (IFIT3) is predominantly induced by type I interferons and is regulated by the pattern recognition. IFIT3 is involved in many processes in macrophage including cell growth and macrophage phagocytosis of apoptotic cells[25]. Homeobox B8 (Hoxb8) was a key regulating cell differentiation gene[26] and could alter survival, differentiation and growth in myeloid cells[27]. These suggested that Kif4 siRNA may inhibit the growth and survival of monocyte/macrophage cells.

Based on the gene expression results, we also observed significant enrichment about immune process in the downregulated genes, such as innate immune response (GO: 0045087; p=2.02344E-14), immune response (GO: 0006955; p=6.08478E-13), immune system process (GO: 0002376; p=9.60597E-13), immune effector process (GO: 0002252; p=1.88822E-08) and antigen processing and presentation (GO: 0019882; p=6.60378E-06). The genes of H2-Q10, H2-T10, H2-T24, IFIT1, IFIT2, IFIT3, TLR3, CSF1, CXCL10, IL7, ISG20, PSME2 and TNFSF13B were all downregulated by Kif4 siRNA. Meanwhile, some KEGG pathways exhibited a statistically significant enrichment in immune process, such as Cell adhesion molecules (CAMs) (ID: mmu04514; p=0.013), Antigen processing and presentation (ID: mmu04612; p=0.027) signaling pathways. These results indicated that Kif4 possibly involved in regulation of immune response in monocyte/macrophage cells.

Monocyte/macrophage cells play a central role in the initial stages and further progression of atherosclerosis. Especially, macrophage foam cells can carry out processes that promote atherosclerosis progression. The generation of these cells is relevant to an imbalance of lipid metabolism[28]. Lipid metabolism disorder influences macrophage phenotype, inflammatory status and the progression of atherosclerotic plaque[29]. Peroxisomes are essential organelles that play a key role in redox signaling and lipid homeostasis[30]. By the bioinformatics analysis, the data detected that GO functional category lipid metabolic process (GO: 0006629; p=2.52751E-05) was significantly reduced and several differentially expressed genes enriched in Peroxisome pathway (ID: mmu04146; p=0.00063), Fatty acid metabolism pathway (ID: mmu01212; p=0.0089) and Fatty acid degradation pathway (ID: mmu00071; p=0.029). The data of the present study demonstrated that Kif4 may be a potential factor to participate in lipid metabolism and then affect the biological function of macrophages.

Phagocytosis and autophagy are important functions of macrophages and essential to both innate and adaptive immunity. Lysosomes are the final steps in phagocytosis and autophagy. Lysosomes are the membrane-delimited organelles responsible for degradation and recycling of both extracellular and intracellular macromolecules[31]. Lysosomal dysfunction results in many diseases, including infectious diseases, neurodegeneration and aging[32]. When comparing the SiRNA group with the NC group, we found lysosome-related genes with decreased expression, PPT1, TMEM165, NAGLU and ZKSCAN3. Meanwhile, the KEGG pathways term Lysosome pathway (ID: mmu04142; p=0.0016) was markedly inhibited. Hence, Kif4 may affect the lysosome-related genes expression and participate in regulating monocyte/macrophage metabolism of the lysosome.

This study might provide a whole genome exploration on the roles of Kif4 in monocyte/macrophage cells and would give useful information on basic research and function of monocyte/macrophage cells. To the best of our knowledge, this is the first time to demonstrate that Kif4 may be a potentially important factor for regulating the various function of monocyte/macrophage cells. Further investigation should be performed to find out the exact roles of the identified genes in this work.

Conclusions

We hope that this comparative analysis of the whole genome from this study will drive future investigation into the dysregulation of individual genes and biological pathways in monocyte/macrophage cells that will improve our understanding of the novel function of Kif4.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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