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Comparative gene expression analysis in melanocytes driven by tumor cell-derived exosomes

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Abstract

Abundant with organelle-like membranous structures, the tumor microenvironment is composed of cancer cells that secrete exosomes. Studies have shown that these secreted exosomes transport RNA and active molecules to other cells to reshape the tumor microenvironment and promote tumor growth. In fact, we found that exosomes derived from melanoma cells drive pre-malignant transition in primary melanocytes. However, there is little available in the scientific literature on how exosomes modulate melanocytes in the microenvironment to optimize conditions for tumor progression and metastasis. We therefore focused this current study on identifying these conditions genetically. Through RNA sequencing, we analyzed gene expression levels of melanocytes driven by exosomes derived from melanoma and lung cancer cells compared with those without exosome controls. Significant differences were found in gene expression patterns of melanocytes driven by exosomes derived from melanoma and lung cancer cells. In the melanocytes responding to exosomes derived from melanoma cells, genes of lipopolysaccharide and regulation of leukocyte chemotaxis were predominant. In the melanocytes responding to exosomes derived from lung cancer cells, genes of DNA replication and mitotic nuclear division played an important role. These results provide further mechanistic understanding of tumor progression promoted by tumor-

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Conflicts of Interest

The authors disclose no potential conflicts of interest.

derived exosomes. This will also help identify potential therapeutic targets for melanoma progression.

Keywords

RNA sequencing; melanoma; exosome; melanocyte; tumor progression

1. Introduction

Exosomes (40–100 nm) are organelle-like membranous structures shed into interstitial spaces and body fluids under diverse pathophysiologic conditions, including tumor development.^{1,2} The tumor microenvironment is abundant with exosomes secreted by the cancer cells themselves. Studies have shown that tumor-derived exosomes can transport RNA and active molecules to other cells to promote tumor growth.² Exosomes are increasingly being recognized as major contributors to the progression of malignant neoplasms. We have found that normal melanocytes can acquire invasiveness through the internalization of exosomes derived from melanoma cells.³ However, little is known about how exosomes modulate the melanocytes in the microenvironment to optimize conditions for tumor progression and metastasis. Defining the specific genes driven by tumor cell-derived exosomes may delineate our understanding of the mechanisms behind tumor progression.

The purpose of this study was to identify differentially expressed genes in melanocytes driven by tumor cell-derived exosomes through RNA sequencing and to translate those genes as therapeutic targets for melanoma metastasis. Our data indicate that there are significant differences in gene expression patterns of melanocytes driven by exosomes derived from melanoma and lung cancer cells. In the melanocytes responding to exosomes derived from melanoma cells, the genes response to lipopolysaccharide and regulation of leukocyte chemotaxis were predominate. Cytokine-cytokine receptor interaction was a major pathway that interacted with NHEM-c cells driven by A375 melanoma cell-derived exosomes. Genes of DNA replication and mitotic nuclear division played important roles in melanocytes reacting to exosomes derived from lung cancer cells. In melanocytes driven by lung cancer cell-derived exosomes, the cell cycle pathway may have contributed to tumor progression more than any other pathway. We also observed that there were 181 shared dysregulated genes (119 of upregulated and 62 of downregulated) in NHEM-c melanocytes driven by both *A375 melanoma exosomes* (melanocytes treated by exosomes derived from A375 melanoma cells) and by *A549 lung cancer exosomes* (melanocytes treated by exosomes derived from A549 lung cancer cells). These results provide further mechanistic delineation of tumor progression promoted by tumor-derived exosomes as well as potential therapeutic targets for melanoma progression.

2. Materials and methods

2.1. Cell lines and culture reagents

Primary human epidermal melanocytes, NHEM-c cells, were purchased from PromoCell (Heidelberg, Germany). The human malignant melanoma cell line (A375) and the human lung cancer cell line (A549) were purchased from American Type Culture Collection (Rockville, MD). A375 cells and A549 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% exosome-depleted fetal bovine serum (FBS) and penicillin (100 U/mL)/streptomycin (100 µg/mL). FBS was depleted of exosomes by ultracentrifugation at $100,000 \times g$ for 16 h at 4°C. NHEM-c cells were cultured in Melanocyte Growth Medium M2 supplemented with Supplement Mix (PromoCell, Heidelberg, Germany) in a 5% CO₂ incubator at 37°C. All other cell culture reagents were obtained from Life Technologies.

2.2. Isolation of exosomes

Exosomes were purified from cell culture supernatants by differential centrifugation as previously described.^{4,5} Initially, culture medium was collected and centrifuged at $400 \times g$ for 10 min to remove whole cells. The supernatant was then centrifuged at $15,000 \times g$ for 20 min to remove debris. The resulting cell-free medium was concentrated by ultrafiltration using Amicon stirred cell Model 8200 with a molecular weight cutoff membrane of 500,000 Daltons (Millipore, Billerica, MA). This concentrated material was then ultracentrifuged at $100,000 \times g$ for 90 min at 4°C to generate an exosome pellet. The pellet was resuspended and washed twice with PBS. Exosome quantity was determined using Nanodrop ND-1000 spectrophotometer at 420nm (Thermo Fisher Scientific, Pittsburgh, PA). Exosome purity was confirmed by Western blot analysis using exosome specific protein CD81 as we described before.⁵ An image from transmission electron microscopy (TEM) showed that the isolated vesicles ranged from 50–100nm in diameter (Supplementary Figure 1), consistent with the size of exosomes.

2.3. RNA isolation from melanocytes treated with cancer cell-derived exosomes

Total RNAs were isolated from NHEM-c melanocytes with three different treatments: NHEM-c melanocytes without exosomes (*control* 0_{exo}), NHEM-c melanocytes co-cultured with A375 melanoma cell-derived exosomes (*melanoma A375_{exo}*), and NHEM-c melanocytes co-cultured with A549 lung cancer cell derived exosomes (*melanoma A549_{exo}*). Briefly, NHEM-c melanocytes (at passage 12) were seeded in 6-well plates at a confluence of 70%. A total of 500 µL of either A375 cell-derived exosomes or A549 cell-derived exosomes (OD₄₂₀= 0.035) were added twice in 6-well plates at the following day, once in the morning and once in the afternoon. NHEM-c cells without exosome treatment were used as control cells. Each treatment group has three biological replicate samples. Total RNAs were isolated after 12 hrs of adding the exosomes at the second time. mirVana RNA isolation kit (Life Technologies) was used according to the manufacturer's guidelines. Total RNA was quantified using Nanodrop ND-1000 (Thermo Fisher Scientific). The integrity of these total RNAs was assessed using Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

2.4. RNA sequencing

Libraries were prepared using the TruSeq Stranded mRNA LT Sample Preparation Kit-Set A (catalog number: RS-122–2101) with poly-A enrichment (Life Technologies). The reference library of Homo sapiens hg38 was used. The quality and quantity library were validated according to the protocol. Sequencing run was performed at the University of Louisville Center for Genetics and Molecular Medicine's (CGeMM) Illumina NextSeq 500 using the NextSeq 500/550 75 cycle High Output Kit v2 (FC-404-2005).

2.5. Software packages for detecting differentially expressed genes (DEGs) in RNA-seq data

The RNA-seq data were analyzed using the Tuxedo Suite tools.^{6, 7} The pipeline is shown in Supplementary Figure 2. Quality control (QC) of the raw sequence data was performed using FastQC (version 0.10.1).⁸ The concatenated sequences were directly aligned to the Homo Sapiens hg38 reference genome assembly (hg38.fa) using tophat2 (version 2.0.13),⁹ generating alignment files in bam format. The genes in RNA-seq data were normalized using the Fragments Per Kilobase per Million mapped fragments (FPKM) normalization method implemented in the Cufflinks-CuffDiff2 package.⁷ DEGs were identified for the four pairwise comparisons: NHEM-c melanocytes treated with *melanoma A375_{exo}* vs. NHEM-c melanocytes without exosomes (*control 0_{exo}*), NHEM-c melanocytes treated with *lung cancer A549_{exo}* vs. NHEM-c melanocytes without exosomes (*control 0_{exo}*), NHEM-c melanocytes treated with *melanoma A375_{exo}* vs. NHEM-c melanocytes treated with *lung cancer A549_{exo}*, NHEM-c melanocytes treated with *melanoma A375_{exo}* + NHEM-c melanocytes treated with *lung cancer A549_{exo}* vs. NHEM-c melanocytes without exosomes (*control 0_{exo}*) using cufflinks-cuffdiff2 package (version 2.2.1) (Figure 1).^{6, 7} A combination of a q-value cutoff = 0.05 with different fold changes was used to determine DEGs in each treatment group.

2.6. Pathway analysis for biological functions

Pathway and network analysis of DEGs was performed with the aid of MetaCore version 6.27 (GenGO, Thomson Reuters, New York, N.Y.).

2.7. Detecting DEGs in human skin cutaneous melanoma (SKCM) dataset from The Cancer Genomics Atlas (TCGA) database

We selected the largest SKCM dataset from the TCGA database. This dataset contains one control and 472 primary melanomas. The dataset was downloaded from <http://www.gdac.broadinstitute.org>. The TCGA-SKCM data was analyzed using DESeq2 package to identify DEGs between a normal control and tumors.¹⁰

2.8. Accession number

The RNA sequencing data have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) and are accessible through GEO series accession number GSE 84683. (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cxojkcmfrovbk&acc=GSE84683>)

3. Results

3.1. Differentially expressed genes in NHEM-c melanocytes driven by exosomes derived from A375 melanoma cells (*melanoma A375_{exo}*) compared with those in NHEM-c melanocytes without exosome treatment (*control 0_{exo}*).

Our previous research showed that exosomes derived from either melanoma or lung cancer cells can promote the migration and invasion of the primary melanocytes through paracrine/autocrine signaling.³ We were interested in identifying which specific genes or pathways were involved in the process of facilitating malignant transition by exosomes derived from melanoma cells. We were also interested in clarifying whether there were any shared genes or pathways that contributed to tumor progression regardless of the specific tumor type. In this study, our goal was to provide a mechanism basis to translate those genes as therapeutic targets for melanoma metastasis.

We incubated exosomes derived from A375 melanoma cells with NHEM-c primary melanocytes and compared them with NHEM-c primary melanocytes without addition of exosomes (controls). Once incubated for 12 hours, total RNA from treated NHEM-c cells (*melanoma A375_{exo}*) and controls (*control 0_{exo}*) were isolated and proceeded to RNA sequencing. A combination of p-value and a q-value cutoff at 0.05 with a fold change (FC) at 2 or -2 determined the differentially expressed genes in melanocytes treated with *melanoma A375_{exo}* versus *control 0_{exo}*. The results showed that there were 30 upregulated genes and 17 downregulated genes in this comparison (Supplementary Table 1). Among those genes, MAGEA 4 (melanoma antigen family A4), GTSF1 (gametocyte specific factor 1), ID3 (inhibitor of DNA binding 3), and ID1 (inhibitor of DNA binding 1) had the highest fold changes (FC>5.0). Genes with the lowest fold changes (FC<-13) were RNY 4 (RNA, Ro-associated Y4), SFTA1P (surfactant associated 1), RNY 1 (RNA, Ro-associated Y1), and RNU 4-1 (RNA, U4 small nuclear 1).

Differentially expressed genes (DEGs) with a corrected p-value (q-value) cutoff at 0.05 for the pairwise comparisons were used for further analysis of enriched Gene Ontology: Biological Processes (GO:BP)^{11,12} and KEGG pathways¹³ using CATEGORY COMPARE¹⁴. The top highest enriched GO: BP terms and KEGG pathways of NHEM-c cells treated with *melanoma A375_{exo}* were compared with *control 0_{exo}*. Genes response to lipopolysaccharide and regulation of leukocyte chemotaxis were the dominate groups involved in NHEM-c cells treated with *melanoma A375_{exo}* (Table 1). Cytokine-cytokine receptor interaction was the major pathway that interacted with NHEM-c cells driven by *melanoma A375_{exo}* (Table 2).

To confirm gene expression with RNA sequencing analysis, we selected ID3 (fold change 5.1 by RNA sequencing) and CXCL2 proteins (fold change 1.9 by RNA sequencing) for Western blot. After 3 days of treatment with *melanoma A375_{exo}* and *melanocyte NHEM-c exo*, proteins from NHEM-c cells were extracted and subjected to Western blot analysis. CXCL2 protein expression increased in NHEM-c cells treated with *melanoma A375_{exo}* compared with cells treated with *melanocyte NHEM-c exo* (Figure. 2). The ID3 protein didn't show up in either treatment (data not shown). The elevation of CXCL2 protein expression is consistent with the upregulation of CXCL2 gene by RNA sequencing analysis

in NHEM-c cells treated with *melanoma A375_{exo}*, when compared to cells treated with *melanocyte NHEM-c_{exo}*.

3.2. Differentially expressed genes in NHEM-c melanocytes driven by exosomes derived from A549 lung cancer cells (*lung cancer A549_{exo}*) versus those in NHEM-c melanocytes without exosomes (*control 0_{exo}*).

Even though exosomes derived from different types of cancer cells can promote cancer progression, we postulated that they may have different signaling pathways during this process. We therefore compared the differentially expressed genes in NHEM-c cells driven by exosomes derived from A549 lung cancer cells (*lung cancer A549_{exo}*) with those in NHEM-c melanocytes without exosomes (*control 0_{exo}*). A q-value cutoff at 0.05 with a fold change (FC) of 2 was used to determine the differentially expressed genes in response to both treatment (*lung cancer A549_{exo}*) vs *control 0_{exo}*. A total of 1092 genes were upregulated, whereas 772 genes were downregulated. A list of the 34 upregulated genes with a fold change of 5 and 26 downregulated genes with a fold change of 3 is provided in Supplementary Table 2. The genes with the highest fold changes (FC 17) were VNN3 (vanin 3), HSD11B1 (hydroxysteroid (11-beta) dehydrogenase 1), CRHR1 (corticotropin releasing hormone receptor 1), PI3 (peptidase inhibitor 3, skin-derived), CRIP3 (cysteine-rich protein 3), LCN2 (lipocalin 2), LENEP (lens epithelial protein), and CSF2 (colony stimulating factor 2, granulocyte-macrophage). Those with the lowest fold changes (FC -4) were RPL35AP4, GSTA7P, PADI1 (peptidyl arginine deiminase, type I), and HMSD [histocompatibility (minor) serpin domain containing].

In the shared dysregulated genes in melanocytes by exosomes derived from both types of cells (*melanoma A375_{exo}* and *lung cancer A549_{exo}*), 119 genes were upregulated and 62 were downregulated. Figure 3 shows the Venn diagram of the dysregulated genes that overlapped in both comparisons. The dysregulated gene list with a 1.5 fold change -1.5 is shown in Supplementary Table 3. The following genes were among those with the highest fold changes: ID3 (inhibitor of DNA binding 3, dominant negative helix-loop-helix protein), ADGRL2 (adhesion G protein-coupled receptor L2), SERPINA3, PPEF1 (protein phosphatase, EF-hand calcium binding domain 1), ID2 (inhibitor of DNA binding 2, dominant negative helix-loop-helix protein), PMEPA1 (prostate transmembrane protein, androgen induced 1), and CXCL5 [chemokine (C-X-C motif) ligand 5]. The following genes were among those shared genes with lowest fold changes: IL24 (interleukin 24), CPA2 (carboxypeptidase A2), and TENM4 (teneurin transmembrane protein 4).

Differentially expressed genes (DEGs) in melanocytes at a q-value cutoff of 0.05 for the pairwise comparisons were used for further analysis of enriched Gene Ontology Biological Processes (GO:BP)^{8,9} and KEGG Pathways¹¹ using CATEGORY COMPARE.¹² The top 5 groups of enriched GO: BP terms (Table 3) and KEGG pathways (Table 4) in NHEM-c cells treated with *lung cancer A549_{exo}* versus those without exosomes (*control 0_{exo}*) are shown.

There were significant differences in the GO term and the KEGG pathway between the melanocytes driven by melanoma exosomes (*melanoma A375_{exo}*) and lung cancer exosomes (*lung cancer A549_{exo}*). The majority of genes in melanocytes driven by *melanoma A375_{exo}* were genes response to lipopolysaccharide and regulation of leukocyte chemotaxis. The

dominate genes in melanocytes driven by *lung cancer A549_{exo}* were genes for DNA repair and mitotic nuclear division. Pathways involved in cytokine-cytokine receptor interaction and RIG-I-like receptor signaling play a crucial role in melanocytes driven by *melanoma A375_{exo}*. In melanocytes driven by *lung cancer A549_{exo}*, cell cycle may contribute more than any other pathways.

3.3. Differences of gene expression patterns in NHEM-c melanocytes driven by A375 melanoma cell-derived exosomes (*melanoma A375_{exo}*) compared with those treated by A549 lung cancer cell-derived exosomes (*lung cancer A549_{exo}*).

We analyzed the changes of the fluctuations in gene expression of NHEM-c melanocytes treated with *melanoma A375_{exo}* versus those treated with *lung cancer A549_{exo}*. The results showed that there are 885 upregulated genes and 579 downregulated genes in melanocytes treated by *melanoma A375_{exo}* versus those by *lung cancer A549_{exo}*. Supplementary Table 4 lists dysregulated genes with 3 fold change ≥ 3 in this comparison. Among the ones with highest fold changes were VNN3 (vanin 3), HSD11B1 (hydroxysteroid [11-beta] dehydrogenase 1), PI3 (peptidase inhibitor 3, skin-derived), LCN2 (lipocalin 2), CSF2 (colony stimulating factor 2 [granulocyte-macrophage]), and CCL4L2 (chemokine (C-C motif) ligand 4-like 2). Genes with the lowest fold changes were PAGE5 (P antigen family, member 5), GTSF1 (gametocyte specific factor 1), RNU4-42P, LINC01549 (long intergenic non-protein coding RNA1549) and RNU4-42P.

CATEGORY COMPARE was also used to analyze the top rated gene categories and pathways in this comparison. Tables 5 and 6 show the top enriched GO: BP terms and KEGG pathways in melanocytes driven by *melanoma A375_{exo}* versus *lung cancer A549_{exo}*. Genes of mitotic nuclear division and DNA replication are the major categories of DEGs in this comparison. Cell cycle was the main pathway of the DEGs that was involved in NHEM-c cells driven by *melanoma A375_{exo}* as compared with those by *lung cancer A549_{exo}*.

3.4. Comparison of gene expression profiles in NHEM-c melanocytes treated with *melanoma A375_{exo}* and *lung cancer A549_{exo}* with those of without exosomes (*control 0_{exo}*).

After we compared the gene expression in NHEM-c cells by *melanoma A375_{exo}* versus *control 0_{exo}* and in NHEM-c cells by *lung cancer A549_{exo}* versus *control 0_{exo}* separately, we wanted to define the genes contributed to tumor progression, regardless of the specific tumor type. Therefore, we combined the gene signature in NHEM-c cells by *melanoma A375_{exo}* and *lung cancer A549_{exo}* into one group. We then compared this combined group with *control 0_{exo}*. The results showed that there were 44 of upregulated genes with a fold change of ≥ 3 and 7 of downregulated genes with a fold change of ≤ -2 , respectively (Supplementary Table 5). Among those with the highest fold changes were: HSD11B1 (hydroxysteroid [11-beta] dehydrogenase 1), PI3 (peptidase inhibitor 3), CSF2 (colony stimulating factor 2 [granulocyte-macrophage]), CCL20 (chemokine [C-C motif] ligand 20), ID3 (inhibitor of DNA binding 3), and ID1 (inhibitor of DNA binding 1). Among those with the lowest fold changes were: RPL35AP4 and RNY4 (RNA, Ro-associated Y4).

CATEGORY COMPARE showed that genes response to bacterium and to lipopolysaccharide were the major categories of DEGs that were involved in NHEM-c cells driven by *melanoma A375exo* and by *lung cancer A549exo* (Table 7). Cytokine-cytokine receptor interaction and cell cycle were the main pathways of the DEGs that were involved in NHEM-c cells driven by *melanoma A375exo* and by *lung cancer A549exo* (Table 8).

3.5. Comparison of the genes in NHEM-c melanocytes driven by *melanoma A375exo* with a prognostic 28-gene signature for high- or low-risk of melanoma patients.

The most developed molecular-based prognostic test is a 28-gene signature that was identified from melanoma tissue.¹⁵ This gene expression profile in primary cutaneous melanoma tumor tissue was reported to be able to distinguish patients with low risk of metastatic recurrence from patients with high risk of recurrence.⁸ Since melanoma exosomes have important roles in tumor progression, we wondered whether there were any genes that were dysregulated in NHEM-c melanocytes driven by *melanoma A375exo* that would appear in the 28-gene signature list. We found that ID2 was upregulated in NHEM-c melanocytes driven by *melanoma A375exo*. ID3 was also upregulated in NHEM-c melanocytes driven by *lung cancer A549exo*. But ID2 was downregulated in cutaneous melanoma with higher risk.¹⁵ A couple of CXCL family members had different expression levels in NHEM-c melanocytes driven by *melanoma A375exo*. CXCL2, CXCL3, and CXCL5 were upregulated in NHEM-c melanocytes driven by *melanoma A375exo*, but CXCL11 was downregulated in NHEM-c melanocytes driven by *melanoma A375exo*. CXCL2 was also upregulated in NHEM-c melanocytes driven by *lung cancer A549exo*. In melanoma with higher risk, CXCL14 was downregulated.¹⁵ These results confirm our previous hypothesis that exosomes may play important roles in tumor progression. These identified genes participating in exosome-promoted tumor progression may serve as markers for predicting the outcome of melanoma.

3.6. Comparison of the genes in NHEM-c melanocytes driven by *melanoma A375exo* with DEGs between primary melanoma and normal control in The Cancer Genomics Atlas (TCGA) database.

We also wondered whether the DEGs driven by *melanoma A375exo* will have any overlap with the DEGs between melanoma and non-melanoma. We took advantage of the TCGA database. There are 472 tumors and 1 normal control from the TCGA Skin Cutaneous Melanoma (SKCM) database.¹⁶ We detected 73 DEGs with q-value cutoff of 0.05 (Supplementary Table 6). However, none of the DEGs in this TCGA database overlapped with the DEGs in NHEM-c cells driven by *melanoma A375exo*. One major reason is that there is only one normal control in this database. A database with more controls may be required for finding a reliable conclusion.

4. Discussion

Exosomes can be derived from any cell type, but those derived from tumor cells may act as the fundamental intercellular messengers and contribute to tumorigenesis and metastasis. Several mechanisms may be involved in tumor progression driven by tumor exosomes, including metabolic reprogramming of stromal fibroblast,¹⁷ reprogramming of primary

melanocytes,³ immunosuppression,¹⁸ and modulation of microenvironment.¹⁹ Our results show that genes response to lipopolysaccharide and regulation of leukocyte chemotaxis are the dominate groups involved in NHEM-c cells treated with *melanoma A375exo*. Cytokine-cytokine receptor interaction is the major pathway that participated in changes of NHEM-c cells driven by *melanoma A375exo*. The major categories of genes and pathways in NHEM-c cells driven by *lung cancer A549exo* are different from those driven by *melanoma A375exo*. However, there were some shared genes in NHEM-c cells driven by exosomes derived from the two different types of cancer cells.

MAGEA 4 was one of the genes that was upregulated only in NHEM-c cells driven by *melanoma A375exo*. Interestingly, MAGEA 4 is a cancer/testis antigen that is overexpressed in various types of malignant tumors and is an immunotherapy target.²⁰ Highly expressed MAGEA 4 is associated with a poor prognosis with a lower than five-year survival rate for osteosarcoma.²¹ ID3 and ID1 are the other two genes that were upregulated in NHEM-c cells driven by *melanoma A375exo*. Interestingly, both ID3 and ID2 were upregulated in melanocytes treated with exosomes from both cancer types (*melanoma A375exo* and *lung cancer A549exo*). ID3 is critical for TGF- β 1 signaling in T cells. It was reported that ID3 can regulate IL-9 production in CD4 (+) T cells through the TAK1-ID3-E2A-GATA-3 pathway.²² ID3-deficient CD4⁺ T cells can differentiate into anti-tumor T_H9 cells instead of Treg cells in response to tumor antigens in the TGF- β -rich environment in tumor, resulting in the suppression of melanoma tumor growth in mice.²² Other ID members, ID2, ID3, and ID4 are direct downstream targets of the transforming growth factor (TGF)- β pathway. They contribute to cell proliferation, migration, and angiogenesis.²³

Our results confirm the previous hypothesis that there may be several different mechanisms involved in promoting tumor progression driven by tumor exosomes. One of the mechanisms we would like to highlight here is the cytokine-cytokine receptor pathway in driving melanoma progression by melanoma exosomes. Recent studies have found that tumor exosomes can convert normal mesenchymal stromal cells (MSCs) into tumor-associated MSCs by producing a large amount of CCR2 ligands, CCL2, and CCL7 in the tumor microenvironment and then promote tumor growth.²⁴ Another study also found that A375 and SK-MEL-28 melanoma cell-derived exosomes have increased protein levels of pro-angiogenic chemokine ligand CXCL1, CXCL2, and CXCL8 compared with the primary melanocyte-derived exosomes.²⁵ Further clarification of how the cytokines in exosomes derived from melanoma cells are participating in tumor progression will help us understand more of the mechanisms.

An unexpected finding was that genes in response to lipopolysaccharide were the dominate category of genes driven by *melanoma A375exo*. Lipopolysaccharides (LPS) are glycolipids found in the outer membrane of some bacteria. They are capable of acting as immunostimulators or immunomodulators.²⁶ Tumor cell-derived exosomes contain membrane structure. One may argue that changes of the genes in response to lipopolysaccharides may be one of the innate responses of the cells to the exogenous membrane-like exosomes. However, those genes in response to lipopolysaccharides were only observed in NHEM-c cells treated by *melanoma A375exo* but not by *lung cancer A549exo*. It is possible that *melanoma A375exo* contains more tumor antigens that can

trigger stronger immune responses than those by *lung cancer A549exo*. We acknowledge that using exosomes derived from melanocytes should be a better control than using no exosomes as a control. However, it is hard to get enough exosomes from primary melanocytes since this cell line has very limited passage numbers and secret far few exosomes compared with tumor cells.

When assessing the changes of NHEM-c cells driven by *lung cancer A549exo*, the number of the dysregulated genes was more than the number of the genes in NHEM-c cells driven by *melanoma A375exo*. One of the possible reasons is that different types of cancer may have different genotypes. When comparing the categories of the dysregulated genes, genes for DNA repair and mitotic nuclear division are the dominate genes in melanocytes driven by *lung cancer A549exo*, while gene responses to lipopolysaccharide and regulation of leukocyte chemotaxis are the majority of genes in melanocytes driven by *melanoma A375exo*. These data suggested that exosomes originating from different types of cancer may promote the changes of the cells through different mechanism/pathways.

5. Conclusion

In summary, our findings provide further mechanistic understanding of tumor progression promoted by tumor-derived exosomes. Those shared genes or pathways that were found prominent in NHEM-c cells by both *melanoma A375exo* and *lung cancer A549exo* treatment may be worth of further investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Control 0exo

Without exosome treatment

DEGs

Differentially expressed genes

GO:BP

Gene Ontology Biological Processes

Melanoma A375exo

A375 melanoma cell-derived exosomes

Lung cancer A549exo

A549 lung cancer cell-derived exosomes

Melanocyte NHEM-c exo

NHEM-c melanocyte-derived exosomes

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Figure 1.
Study design. A schematic diagram of our research design. w/: with, w/o: without.

Figure 2.

Western blot to confirm CXCL2 protein expression. Protein extracts (50ug) from NHEM-c cells treated with melanocyte NHEM-c exo or melanoma A375exo were assessed using the antibody against CXCL2. HSC70 was used as a loading control.

Figure 3.

Venn diagrams. (A) Shows a Venn diagram of the numbers of upregulated genes between NHEM-c treated with *A549exo* vs NHEM-c without exosomes (*control 0exo*) and NHEM-c treated with *A375exo* vs NHEM-c without exosomes (*control 0exo*). (B) Shows a Venn diagram of the numbers of the downregulated genes between NHEM-c treated with *A549exo* vs NHEM-c without exosomes (*control 0exo*) and NHEM-c treated with *A375exo* vs NHEM-c without exosomes (*control 0exo*).

Table 1.

The top 5 highest represented GO terms of differentially expressed genes (DEGs) in NHEM-c melanocytes treated with *melanoma A375_{exo}* versus those with *control O_{exo}* using CATEGORY COMPARE.

GO Term ID	Description	Number of Genes
GO:0032496	response to lipopolysaccharide	21
GO:0002688	Regulation of leukocyte chemotaxis	14
GO:0006695	Cholesterol biosynthetic process	11
GO:1902653	Secondary alcohol biosynthetic process	11
GO:0016126	Sterol biosynthetic process	11

Table 2.

The top 5 highest represented KEGG pathways in NHEM-c melanocytes treated with *melanoma A375_{exo}* versus those with *control 0_{exo}* using CATEGORY COMPARE.

KEGG Term ID	Pathway description	Number of Genes
4060	Cytokine-cytokine receptor interaction	17
4622	RIG-I-like receptor signaling pathway	10
4621	NOD-like receptor signaling pathway	8
0100	Steroid biosynthesis	7
4623	Cytosolic DNA-sensing pathway	7

Table 3.

Top 5 enriched GO terms of DEGs from NHEM-c cells treated with A549 lung cancer cell-derived exosome (*lung cancer A549_{exo}*) vs. those without exosomes (*control 0_{exo}*) using CATEGORY COMPARE.

GO Term ID	Description	Number of Genes
GO:0006281	DNA repair	182
GO:0007067	Mitotic nuclear division	176
GO:0006260	DNA replication	142
GO:0006261	DNA-dependent DNA replication	72
GO:0006271	DNA strand elongation involved in DNA replication	32

Table 4.

Top 5 enriched KEGG pathways from NHEM-c cells treated with A549 lung cancer cell-derived exosome (*lung cancer A549_{exo}*) vs. those without exosomes (*control 0_{exo}*) using CATEGORY COMPARE.

KEGG Term ID	Pathway description	Number of Genes
4110	Cell cycle	66
5323	Rheumatoid arthritis	39
4612	Antigen processing and presentation	31
3030	DNA replication	30
0100	Steroid biosynthesis	11

Table 5.

Top 5 enriched GO terms of DEGs in NHEM-c cells driven by *melanoma A375exo* versus those by *lung cancer A549exo* using CATEGORY COMPARE.

GO Term ID	Description	Number of Genes
GO:0007067	mitotic nuclear division	140
GO:0006260	DNA replication	115
GO:0007059	chromosome segregation	99
GO:0006261	DNA-dependent DNA replication	61
GO:0006271	DNA strand elongation involved in DNA replication	28

Table 6.

Top 5 enriched KEGG pathways of DEGs in NHEM-c cells driven by *melanoma A375exo* versus those by *lung cancer A549exo* using CATEGORY COMPARE.

KEGG Term ID	Pathway description	Number of Genes
4110	Cell cycle	53
5323	Rheumatoid arthritis	34
4612	Antigen processing and presentation	31
3030	DNA replication	24
5130	Pathogenic Escherichia coli infection	23

Table 7.

Top 5 enriched GO terms of DEGs in NHEM-c melanocytes by *melanoma A375exo* and *lung cancer A549exo* versus *Control 0_{exo}* using CATEGORY COMPARE.

GO Term ID	Description	Number of Genes
GO:0009617	Response to bacterium	51
GO:0032496	Response to lipopolysaccharide	44
GO:0002237	Response to molecule of bacterial origin	44
GO:0006695	Cholesterol biosynthetic process	16
GO:1902653	Secondary alcohol biosynthetic process	16

Table 8.

Top 5 enriched KEGG pathways of DEGs in NHEM-c melanocytes by *melanoma A375exo* and *lung cancer A549exo* versus *Control 0_{exo}* using CATEGORY COMPARE.

KEGG Term ID	Description	Number of Genes
4060	Cytokine-cytokine receptor interaction	29
4110	Cell cycle	20
0100	Steroid biosynthesis	9
3030	DNA replication	8
0900	Terpenoid backbone biosynthesis	6