RESEARCH

Transforming Growth Factor-Beta (TGF-β) Employs Epithelial-Mesenchymal Transition (EMT)-Inducible Cancer Cells to Secrete the Exosomal miRNAs Regulating the Immune Response

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Abstract: Epithelial–mesenchymal transition (EMT) is a vital program required for cancer invasion and metastasis. EMT inducer transforming growth factor-beta (TGF-β) is implicated in carcinogenesis by contributing metastasis, immune evasion, and immunosuppression. Cancer cells employ different mechanisms to suppress the differentiation and the activation of immune system cells. Recent studies have reported that exosomes derived from cancer cells induced by several internal and external factors may suppress the immune system. We aimed to illustrate whether TGF-β recruits cancer cells to secrete several miRNAs that could suppress the immune system. We performed all experiments with TGF-β-responsive (A549) non-small cell lung cancer and (PANC-1) pancreatic ductal adenocarcinoma cell lines used as *in vitro* models. We isolated exosomes secreted by TGF-β-induced cells. We analyzed miRNAs in exosomes by qPCR. We showed that miR-1246 was significantly increased in exosomes secreted by TGF-β-induced A549 cells, indicating that miR-1246 upregulation could contribute to exosome-mediated immunosuppression in lung cancer patients. Also, TGF-β upregulated expression levels of miR-17, miR-23a, miR-31, miR-145 and miR-181a as cellular and exosomal in A549 cells. Interestingly, TGF-β selectively induced exosomal miR-150 expression in A549 cells, suggesting that lung cancer cells might secrete exosomal miR-150 to suppress immune cells. Conversely, we observed that PANC-1 cells display reticent behaviour to secrete exosomal miRNAs in response to TGF-β treatment compared to A549 cells. Our study reveals that TGF-β selectively might regulate the expression of exosomal miRNAs in especially lung cancer cells. So, this study will contribute to further studies with lung cancer patients

INTRODUCTION

Transforming growth factor beta (TGF-β) is abundantly found in the tumor microenvironment at both primer and secondary tumor sites, and it is a bidirectional cytokine that can also act as an oncogenic and tumor suppressor on both cancer cells and stromal cells in tumor microenvironment [1]. In the early stages of carcinogenesis, TGF-β has a tumor suppressive action on cancer cells by inducing cell arrest and apoptosis, leading to antiproliferative response and the repression of tumor growth. However, it acts as an oncogenic factor in advanced stages and could induce stemness, epithelial–mesenchymal transition (EMT) and invasion [2]. Furthermore, TGF-β is a pleiotropic cytokine that suppresses the activation and differentiation of immune system cells and contributes immune evasion during metastasis [3-5].

Exosomes are membrane-surrounded vesicles with sizes ranging from 40-100 nm. They are secreted into the extracellular matrix by many different cell types and are also found in the peripheral circulation to function at distant tissue [6]. In addition, the exosomes have a complex content with multiple biomolecules such as messenger RNAs (mRNA), micro RNAs (miRNA), long non-coding RNAs (lnRNAs) and DNA fragments, heat-shock proteins, and enzymes [7-11]. Exosomes are increasingly considered as critical factors to maintain homeostasis with their ability to have paracrine and autocrine effects. However, it is also involved in the progression of many diseases, including cancer, osteoporosis, and diabetes [12-14]. Moreover, exosomes have been widely investigated for different processes in cancer progression as well as their role in suppression of the immune system. Tumor-derived exosomes (TEXs) released by cancer cells contain biomolecules contributed to invasion, angiogenesis, immune evasion and immunosuppression, suggesting that they reorganize the microenvironment for growth, dissemination and colonization of cancer cells [15].

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miRNAs are small non-coding RNAs, consisting of 22-25 nucleotides and play crucial roles in gene regulation by binding complementary to the 3' non-coding regions of mRNAs (3'-UTR) and preventing translation of mRNA. It has been considered that miRNAs are required for the post-transcriptional regulation of homeostasis at different processes throughout the life cycle of the cell [16, 17]. miRNAs function as crucial mediator of multiple oncogenic signal to promote the processes of proliferation, invasion, angiogenesis, immunosuppression and resistance to apoptosis in the tumor microenvironment [18]. Recent studies have shown that the tumor microenvironment modified by diverse intrinsic (mutations) or extrinsic (hypoxia, oxidative stress etc.) factors allows cancer cells, stromal cells, and immune system cells to release TEXs containing miRNAs that regulate immune response. However, it is unclear that whether the TGF-β could specifically manipulate the exosomal profiles of miRNAs derived from EMT inducible-cancer cells to repress immune response in the tumor microenvironment. Here, we hypothesized that TGF-β could play an active role in immunosuppression process through cancer cell-derived exosomal miRNAs.

Therefore, we selected the members of several miRNA families for the present study since it has the potential to broadly effect on signaling pathways that regulate the activation, differentiation and function of innate and adaptive immune response against tumor cells (Table 1) and found that miR-150 was selectively upregulated in the exosomes secreted by lung cancer cells. Moreover, TGF-β induced expressions of miR-17, miR-23a, miR-31, miR-145 and miR-181a at levels of cellular and exosomal in A549 cells, suggesting that these miRNAs play crucial roles in both TGF-β-induced aggressiveness phonotype and modulation of microenvironment in lung cancers. Eventually, this study suggests that exosomal miRNAs derived from TGF-β-induced tumor cells could be a potent vehicle for defence mechanism against immune response [3, 19-29].

MATERIALS and METHODS

Cell Culture, Reagents and Chemicals

In our study, cell culture studies were performed with TGF-β-responsive cancer cell lines used as *in vitro* models. A549 (non-small cell lung carcinoma) and PANC-1 (pancreatic ductal adenocarcinoma) were obtained from ATCC. The cells were grown in T-75 flasks containing complete Dulbecco's Modified Eagle Medium (DMEM) composed of 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin via incubation at 37°C, under 5% CO_2, and 95% relative humidity. All chemicals were of reagent grade and solvents were of HPLC grade. Human recombinant TGF-β1 were purchased from BioLegend (San Diego, CA).

Total Exosome RNA isolation

A549 and PANC-1 cells incubated with TGF-β (10 ng/ml) or not for 48 hours under serum-free and antibiotic-free conditions. We defined these cells that were not incubated with TGF-β as control group. At the end of incubation with TGF-β, the media were filtered using a 0.80 µm pore filter (Millipore), followed by centrifugation step of 5,000 × g for 5 min to discard cellular debris. The filtered and centrifuged media were subsequently used to isolate total exosomal RNA with the commercially available Qiagen exoRNeasy Serum / Plasma Maxi kit. The quality and amount of total RNA samples obtained from TEXs were spectrophotometrically measured with the Take 3 plate (Biotek Synergy H1 Hybrid Multi-Mode Reader).

Quantitative Real-Time PCR

cDNA reactions were performed using the miScript II RT Kit (Cat. No: 218161, Qiagen) RT^2 First Strand Kit (Cat. No: 330404, Qiagen) from miRNAs and mRNAs, respectively. Our study miR-17, miR-21, miR-23a, miR-25, miR-31, miR-145 miR-146a, miR-150, miR-155, miR-181a, miR-1246 and Snord68 miRNA as normalizers (Qiagen). We investigated miRNAs within the scope of this study because they play a role in the maturation, differentiation and activation of immune system elements (Table 1). qPCR analyses are performed with QuantiTect SYBR Green PCR Kit (Cat. No: 204143, Qiagen) and RT^2 SYBR® Green qPCR Mastermix (Cat. No: 330501, Qiagen) for miRNAs and mRNA using Roche Lightcycler 96 instrument, resprectively. All results were analyzed using the $2^{-\Delta\Delta Cq}$ method. Actin Beta (ACTB) was used as the reference gene. Primer sequences were designed for qPCR. They were given for E-cadherin, N-cadherin, Snail Family Transcriptional Repressor 2 (SNAI2), Matrix Metallopeptidase 2 (MMP-2), and ACTB in Table 2.

Statistical Analysis

Student's t-test was used to statistically determine the significant difference among different groups using an SPSS 23.0 version. p values of <0.05 were considered as statistically significant. All data are presented as mean values ± standard deviation (mean ± SD). p values of <0.05 and <0.01 are indicated with one and two asterisks, respectively.

Table 1. miRNAs target genes, cell type, and mechanism of action

miRNA	Target cell	Target gene	Effect	Reference
miR-17	Treg lymphocyte	Foxb3	Inhibition of the suppressive effect of Treg cells	[20]
miR-21	T lymphocyte ($CD4^+$)	GNAQ, PLEKHA1 and CXCR4	Inhibition of the TCR pathway	[21]
miR-23a	T lymphocyte ($CD8^+$)	Prdm1 and BLIMP-1	Inhibition of cytotoxic effect	[22]
miR-25	T lymphocyte ($CD8^+$)	cGAS and NCOA3	Inducing the escape of hypoxic tumors from the DAMP-induced immune response	[23]
miR-31	T lymphocyte ($CD8^+$)	Ppp6c	Inhibition of T cell due to PD-1 overexpression	[24]
miR-145	T lymphocyte ($CD4^+$)	CD28	CD28 inhibition required for T lymphocyte activation	[25]
miR-146a	T lymphocyte ($CD4^+$)	PRKCε	Inhibition of $CD4^+$ T lymphocyte differentiation into Th1 cells	[26]
miR-150	T lymphocyte ($CD4^+$)	AKT3/Bim	Inhibition of maturation and proliferation of $CD4^+$ T lymphocytes, induction of apoptosis	[27]
miR-155	Myeloid-derived suppressive cells (MDSC)	SOCS1	Inhibition of T lymphocyte activation by activating MDSC cells	[28]
miR-181a	T lymphocyte cells	DUSP5, DUSP6, SHP2, and PtPn22	Preventing thymocytes from maturing into $CD4^+$ and $CD8^+$ lymphocytes	[29]
miR-1246	Macrophage	IL17A, IL7R, LEF1, S1PR1, BCL2, and CD96	Transfromation of Type-1 Macrophage to TAMs	[3]

Table 2. The primers used in this study.

Gene	5'– >3'
E-cadherin	5'-TCCATTTCTTGGTCTACGCC-3' (forward) 5'-CACCTTCAGCCAACCTGTTT-3' (reverse)
N-cadherin	5'-CGCCATCATCGCTATCCTTCTGTG-3' (forward) 5'-AGCCGCTGCCCTCGTAGTCAAA-3' (reverse)
SNAI2	5'-GAGCATTTGCAGACAGGTCA-3'(forward) 5'-TGAATTCCATGCTCTTGCAG-3'(reverse)
MMP-2	5'- AGATCTTCTTCTTCAAGGACCGGTT-3' (forward) 5'- GGCTGGTCAGTGGCTTGGGGTA-3' (reverse)
ACTB	5'- CCACTGGCATCGTGATGG-3' (forward) 5'- GCGGATGTCCACGTCACACT-3' (reverse)

RESULTS

Confirming the activation of TGF-β signaling

In order to confirm the successful activation of the TGF-β signaling and EMT process in A549 and PANC-1 cell lines stimulated with TGF-β, we determined the expression levels of some EMT markers, including E-cadherin, N-cadherin, SNAI2, and MMP-2. As expected, we observed that TGF-β successfully suppressed E-cadherin expression while it increased expression levels of mesenchymal expression such as N-cadherin, SNAI2 and MMP-2 in A549 and PANC-1 cells (Figure 1). These cells are widely used as model cells for TGF-β-mediated EMT process, so we have confirmed that it successfully activated its signaling and induced EMT process in A549 and PANC-1 cells.

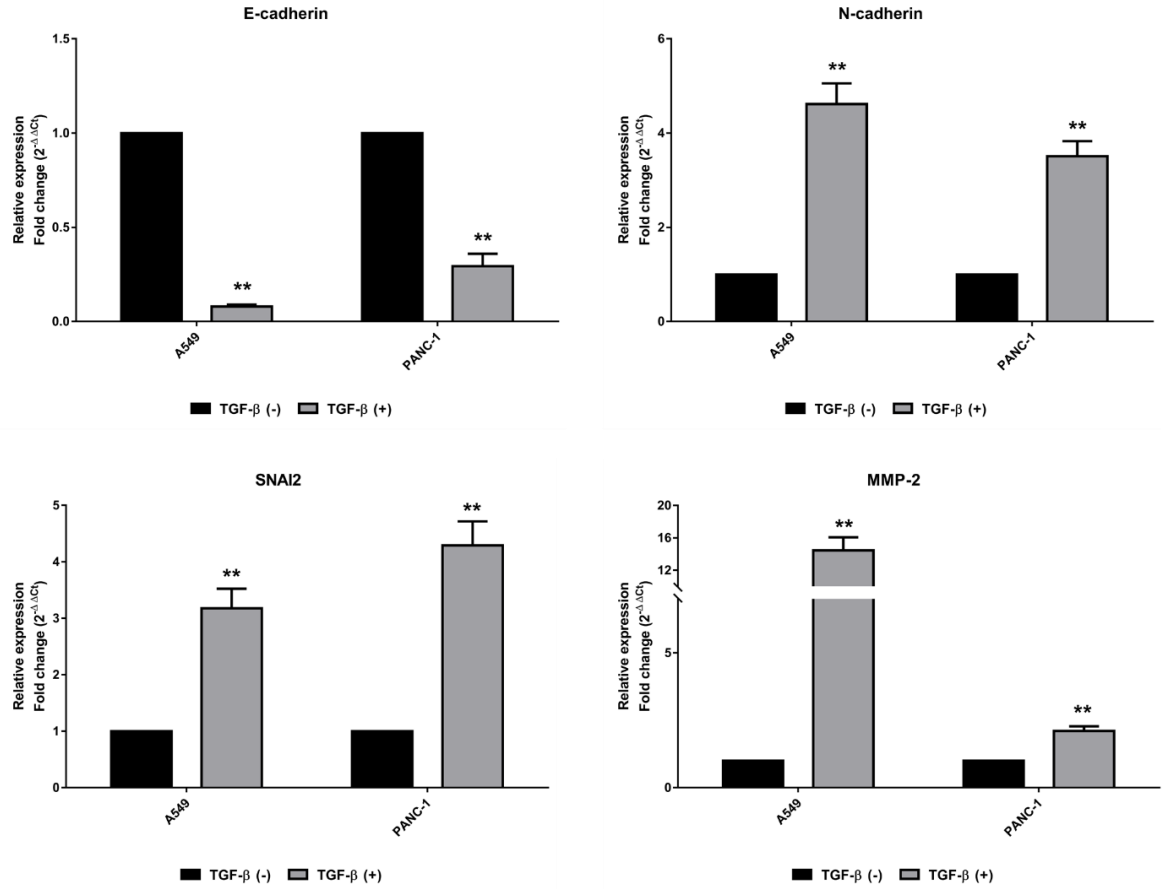

Figure 1. The cells strongly respond to TGF-β stimulation. Determination of changes in expression levels of E-cadherin, N-cadherin, SNAI2, and MMP-2 in serum starved-A549 and PANC-1 cell lines incubated with TGF-β for 48 hours by qPCR analysis.
Data are shown as mean ± SD.
** Values significantly different from the control groups (p <0.01).

Detection of miRNA expression profiles in TGF-β induced A549 and PANC-1 cells at the cellular and exosomal level

After we validated that TGF-β induced EMT in A549 and PANC-1 cells, we aimed to determine expression levels of miR-17, miR-21, miR-23a, miR-25, miR-31, miR-145 miR-146a, miR-150, miR-155, miR-181a, and miR-1246 in the parent cells as well as exosomes secreted by them. We observed no significant changes in the cellular and exosomal levels of miR-21, miR-25, and miR-146a genes in TGF-β-induced A549 cells (Figure 2). However, we found that TGF-β induced in cellular and exosomal levels of miR-17, miR-23a, miR-31, miR-145, miR-181a, and miR-1246 while it specifically increased miR-150 expression in only A549 cells-derived exosome (Figure 2). The results showed that TGF-β-responsive lung cancer cells have ability to secrete exosomes containing the miRNA genes that regulate immune response.

In the pancreatic cancer model, we observed that cellular and exosomal levels of the related miRNA genes had slightly regulated by TGF-β compared to A549 cells. We found that TGF-β increased the cellular expressions of miR-17 and miR-23a genes in PANC-1 cells

while there were no significant changes in the exosomal expressions of these miRNA genes derived from these cells (Figure 3). We also showed that TGF-β-induced PANC-1 cells produced less exosomal miR-150 than A549 cells, suggesting that pancreatic cancer cells could be uncommunicative to secrete exosome carrying immunosuppression related miRNAs in response to TGF-β stimulation (Figures 3). Moreover, we showed that TGF-β induces the cellular expressions of miR-21, miR-145, and miR-181a while it suppressed exosomal expressions of them in PANC-1 cells (Figure 4). All these results indicated that TGF-β could stimulate pancreatic cancer cells to regulate miRNA expressions at cellular and TEXs levels via distinct mechanisms, needed to be elucidated with further studies.

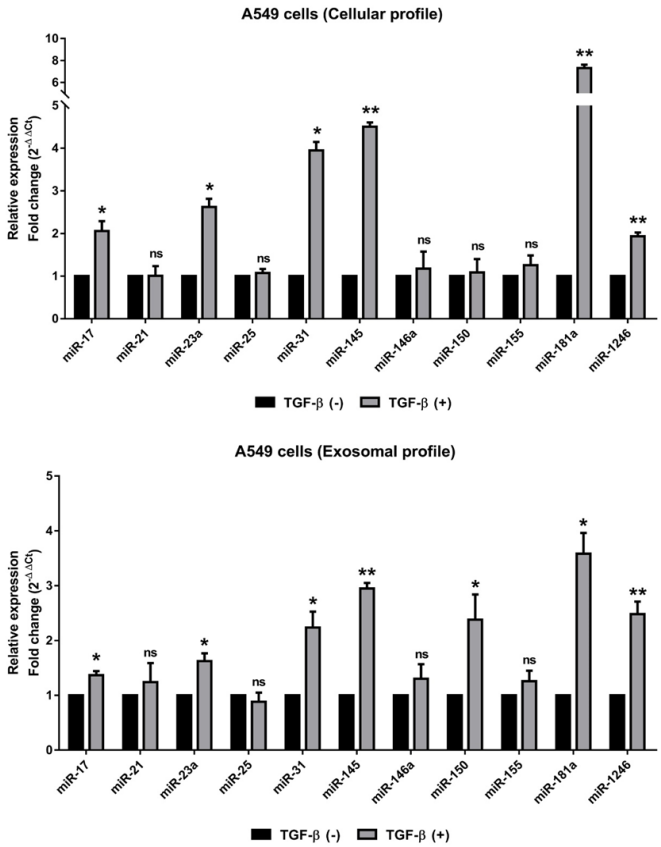

Figure 2. A549 cells specifically secrete the exosomes carrying upregulated miR-150 in response to TGF-β. After serum-starved A549 cells were stimulated with TGF-β for 48 h, cellular and exosomal levels of miR-17, miR-21, miR-23a, miR-25, miR-31, miR-145 miR-146a, miR-150, miR-155, miR-181a, and miR-1246 were determined by qPCR assay and normalized to Snord68, used as endogenous reference RNA.
Data are shown as mean ± SD.
* Values significantly different from the control groups (p <0.05).
** Values significantly different from the control groups (p <0.01).

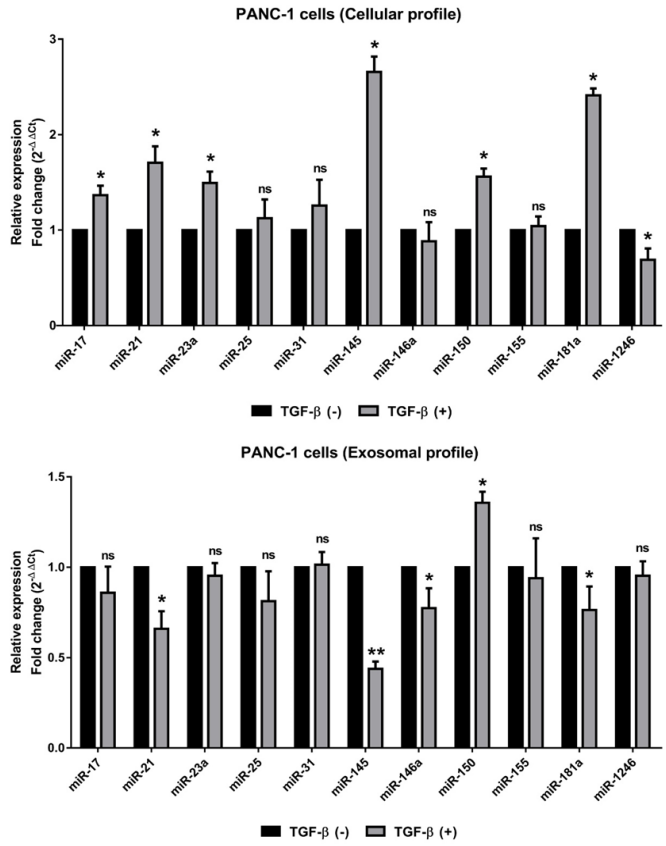

Figure 3. PANC-1 cells cannot be sufficiently responsive to TGF-β to produce exosomal miRNAs that regulate immune system. After serum-starved PANC-1 cells were stimulated with TGF-β for 48 h, cellular and exosomal levels of miR-17, miR-21, miR-23a, miR-25, miR-31, miR-145 miR-146a, miR-150, miR-155, miR-181a, and miR-1246 were determined by qPCR assay and normalized to Snord68, used as endogenous reference RNA.
Data are shown as mean ± SD.
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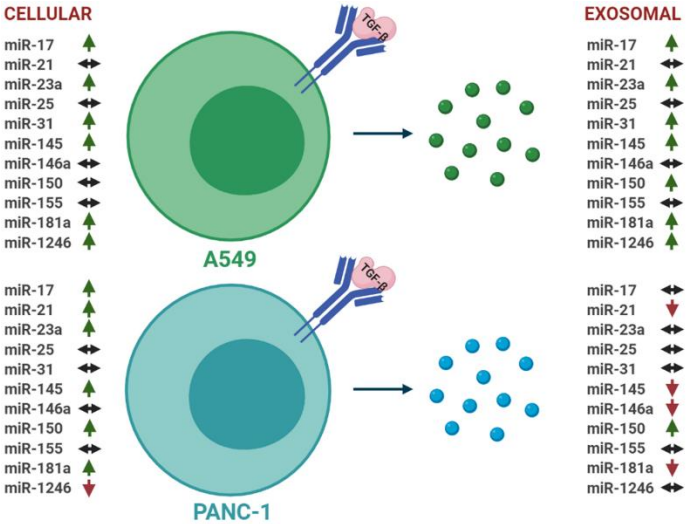

Figure 4. Schematic summary of the changes in the expression profiles of TGF-β-induced related miRNAs at the cellular and exosomal level.

DISCUSSION

It is important to understand all aspects of immunosuppression mechanisms to identify reliable biomarkers in prognosis and effective therapy. Cancer cells have an ability to inhibit immune system cells via several mechanisms. Among identified mechanisms, exosomes derived from cancer cells induced by external or intracellular factors play significant roles in the inhibition of immune system. A recent study showed that T lymphocyte cells can be suppressed by TEXs containing elevated programmed death-ligand 1 (PD-L1) that inhibits the activation of immune system cells by binding to programmed cell death protein 1 (PD-1) on their [30]. Exosomes could also influence on the adjacent and distant cells through the miRNAs in their structures [9]. However, it is yet unknown whether TGF-β induces cancer cells to release exosomal miRNA, which has an ability to dysregulate the function of immune system cells. Here, the present study revealed a novel and potential role of TGF-β in selectively suppressing immune system to promote cancer progression as well as its role in EMT and invasion.

TGF-β signaling regulates the transcription of genes involved in many biological and pathological processes such as EMT, apoptosis, angiogenesis, senescence, cell movement, invasion, and suppression of the immune system [31]. TGF-β is a strong actor that can directly inhibit the activation and differentiation on almost all immune system cells types [32]. It has been indicated that TGF-β-mediated exosomes serve as adjuvant factors by regulating bone marrow mesenchymal stem cells (BMSCs) to attenuate and repair cartilage injuries, favoring in the protection of homeostasis [33, 34]. A recent study also showed that TGF-β induces colorectal cancer cells to produce miR-200b enriched in exosome of colorectal cancer cells, and subsequently promotes the proliferation of other colorectal cancer cells [35]. All these studies clearly suggested that exosomal miRNAs derived from cancer cells induced TGF-β could also be potential vehicles to modulate immune cells in the tumor microenvironment.

Although there are no studies showing the role of exosomal miR-145 in lung cancer, it was reported that it is found in the exosomes of lung injury patients who undergoing sepsis and lung tissues from mice with sepsis-induced lung injury [36]. Therefore, we firstly showed that exosomal miR-145 could play role as an extracellular regulator in

TGF-β-mediated modification of tumor microenvironment for lung cancers (Figure 2). Furthermore, we reported that miR-181a level was elevated in the exosomes secreted from lung cancer cells (Figure 2). Shan et al. previously reported that there are not statistically a correlation for miR-181a expression between exosome samples of lung cancer patients and normal persons, however, the same study also referred these results is due to the small sample size [37]. Thus, further studies are needed to demonstrate the potential prognostic role of miR-181a for lung cancer. Besides, it has been stated that exosomal miR-1246 is implicated in cell proliferation and the resistance to radiotherapy for lung cancer [38]. In this study, we showed that miR-1246 was upregulated as cellular and exosomal in EMT-inducible lung cancer cells in response to TGF-β, indicating that it has regulator roles in TGF-β-mediated EMT and immunosuppression to promote lung carcinogenesis. Whereas TGF-β has been shown to upregulate the cellular level of miR-31, there is not a literature showing its effect on the exosomal level of miR-31 [39]. Here, we found that the level of miR-31 increased in TEXs from lung cancer cells with TGF-β stimulation in the present study (Figure 2).

In our study, we have shown that TGF-β cannot induce significant increases in the exosomal levels of the related miRNAs in pancreatic cancer cells. There is no study showing the role of miR-150 gene, it was showed to increase partially at exosomal level in PANC-1 cells induced by TGF-β (Figure 3). Moreover, TGF-β significantly promotes miR-150 expression both A549 and PANC-1 cells derived exosomes (Figure 2 and Figure 3). Recently Wu et al. showed that miR-150 may promote tumorigenesis by inhibiting the suppressor gene liver kinase B1 (LKB1) expression in lung cancer [40]. In addition, MacIver et al. reported that LKB1 plays a critical role for regulation of viability and activation of $CD4^+$ and $CD8^+$T cells [41]. Therefore, we hypnotized TGF-β could mediate an indirect mechanism to suppress immune response by inducing intracellular level of miR-150 via cancer cell derived exosomes. In this respect, our results are new information for the literature, and the effects of TGF-β on exosomal miRNAs in pancreatic cancer cells should be globally investigated to find novel miRNA genes. Finally, our results clearly demonstrated the different roles of TGF-β especially in resistance mechanisms of lung cancers against immunotherapy (All results are summarized in Figure 4).

Conclusion

Consequently, our results show the novel role of TGF-β as a crucial regulator in cancer progression, contribute to a further an understanding of the relationships between cancer cell and immune system cells and provide new insights on exosome-based non-invasive diagnostic approaches to predict whether immunosuppression would develop or not for cancer patients (Figure 5).

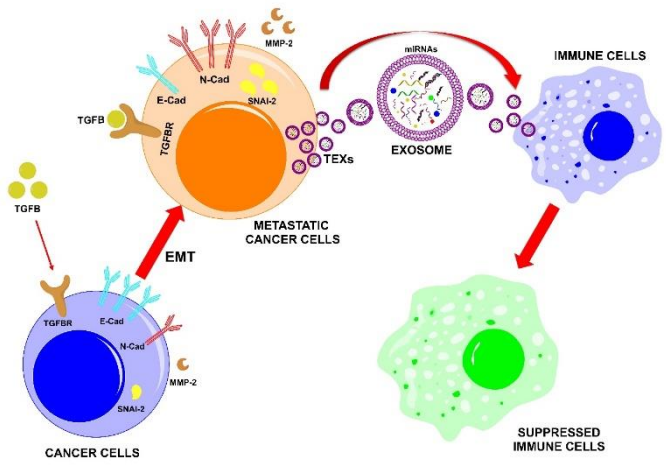

Figure 5. The potential effects of TGF-β on cancer cells and tumor microenvironment in immunosuppression.

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Author contributions

EK and HK designed the experiment, performed the experiments and drafted the manuscript. EK and HK contributed to the experimental studies, reviewed and approved the final manuscript.

Conflict of interest disclosure

The authors confirm that this article content has no conflicts of interest.

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