Inflammatory Cytokines Regulate Exosomal MicroRNA and Protein Expressions in Osteoblast-like Saos2 Cells and Human Gingival Fibroblasts

（炎症性サイトカインは骨芽細胞様 Saos2 細胞およびヒト歯肉線維芽細胞のエクソソーム中のマイクロ RNA およびタンパク質発現を調節する）

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Preface

This article is based on a main manuscript, “Inflammatory Cytokines Stimulate Exosomal MicroRNA and Protein Expressions in Osteoblast-like Saos2 Cells” in the International Journal of Oral-Medical Sciences, and a reference manuscript, “Tumor Necrosis Factor-α Regulates Exosomal miRNA and Protein Expressions in Human Gingival Fibroblasts” in the International Journal of Oral-Medical Sciences.

Abstract

Exosomes are extracellular vesicles secreted by cells that contain microRNAs (miRNAs) and proteins and are thought to be involved in host cell function and cell-cell communication. In this study, osteoblast-like Saos2 cells were stimulated by tumor necrosis factor α (TNF-α) or interleukin 1β (IL-1β) and human gingival fibroblasts (HGF) were stimulated by TNF-α to investigate the relative amount of exosome miRNAs and proteins in the inflammatory state. Relative amounts of miRNAs in the exosomes secreted by Saos2 cells with and without stimulation by TNF-α (10 ng/ml) or IL-1β (1 ng/ml) were measured by real-time polymerase chain reaction (PCR). Relative amount of miRNAs in

the exosomes from HGF with or without stimulation by TNF-α (10 ng/ml) were analyzed by real-time PCR. Marker proteins in the exosomes such as programmed cell death 6-interacting protein (PDCD6IP), heat shock protein 70 (HSP70), tumor susceptibility gene 101 protein (TSG101), cluster of differentiation 63 (CD63), CD9 and CD81 in Saos2 and HGF were analyzed by Western blot. Relative amounts of miR-150-5p, miR-200b-3p, miR-223-3p and miR-144-5p in the exosomes from conditioned media of Saos2 cells were much higher than that in the Saos2 cells. These four kinds of miRNA levels in the exosomes were significantly increased by TNF-α, and IL-1β increased only the relative amounts of miR-150-5p and miR-200b-3p in the exosomes from Saos2 conditioned media. Relative amounts of miR-150-5p, miR-200b-3p, miR-223-3p and miR-144-5p in the exosomes from conditioned media of HGF were much higher than the expression levels of these miRNAs in the HGF. MiR-150-5p, miR-200b-3p and miR-144-3p expressions in the exosomes were significantly decreased by TNF-α. TNF-α and IL-1β increased expression levels of PDCD6IP, HSP70 and TSG101 proteins in the exosomes from Saos2 cells. TNF-α increased the amounts of PDCD6IP and CD81 in the exosomes from HGF. Thus, inflammatory cytokine stimulation resulted in differential responses to relative amounts of exosomal miRNAs from Saos2 cells and HGF. These data suggest that distinct roles of osteoblasts and gingival fibroblasts in the progression of periodontitis.

1. Introduction

Periodontitis is a chronic inflammatory disease caused by periodontopathic bacteria and viruses that results in the progressive destruction of periodontal ligaments and alveolar bone (1). Periodontopathic bacteria are the risk factor of periodontal disease, and inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are produced as a result of host immune response might cause gingival inflammation and alveolar bone resorption (2, 3).

Exosomes are a type of extracellular vesicles of about 40~100 nm diameter secreted by cells and contain proteins and RNA reflecting the functions of host cells. Therefore, the contents of exosomes derived from diseased cells may serve as biomarkers of the diseases (4).

Exosomes have several specific marker proteins which distinguish them from other extracellular vesicles such as programmed cell death 6-interacting protein (PDCD6IP) also known as apoptotic linked-gene-product 2 interacting protein X (ALIX), heat shock protein 70 (HSP70), tumor susceptibility gene 101 protein (TSG101), cluster of differentiation 63 (CD63), CD9, CD81 and complement component 6 (C6) (5-7). Tobón-

Arroyave *et al.* reported salivary concentrations of CD9 and CD81 exosome-related tetraspanins were decreased in patients with periodontitis (8). In addition to proteins, exosomes contain biochemical substances such as cytokines, mRNA and miRNA, and play an essential role in cell-cell communication through the transfer of genetic substances (9).

Exosomal miRNAs have been reported to exhibit physiological and pathological functions such as immunomodulation and cancer progression (10). Mesenchymal cell-derived exosomal miR-150-5P has therapeutic potential for rheumatoid arthritis (RA) mediated by suppression of matrix metalloproteinase 14 (MMP14) and vascular endothelial growth factor (VEGF) (11). MiR-200b-3p and miR-206 in urinary exosomes might serve as non-invasive biomarkers for steroid-induced osteonecrosis of femoral head (12). MiR-223-3p in salivary exosomes regulates gasdermin D-mediated pyroptosis by targeting nucleotide-binding oligomerization (NOD) domain-like receptor (NLR) pyrin domain-containing 3 (NLRP3) in periodontitis (13). Exosomal miR-369-3p, miR-379-5p, miR-493-3p and miR-1296-5p were promising biomarkers for the diagnosis of large-artery atherosclerosis stroke, and their diagnostic efficiency is superior to that of their counterparts in plasma (14). Exosomal miR-144-5p from bone marrow-derived macrophage in type 2 diabetes impairs bone fracture healing via targeting Smad1 (15).

However, there was few study whether inflammatory cytokines affect the expression of miRNAs in the exosomes from osteoblasts and gingival fibroblasts. In this study, the effects of inflammatory cytokines on the relative amounts of miRNAs and proteins in the exosomes isolated from osteoblasts or gingival fibroblasts conditioned media were studied.

2. Materials and Methods

Materials

Alpha minimum essential medium (α-MEM), fetal calf serum (FCS), penicillin and streptomycin and TrypLE™ Express were obtained from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified eagle medium (DMEM) was procured from FUJIFILM Wako Pure Chemical Corp. (Osaka Japan). ExoQuick-TC were purchased from System Biosciences Inc. (Mountain View, CA, USA). TRIzol® RNA isolation reagents were from Thermo Fisher Scientific (Waltham, MA, USA). Mir-X™ miRNA First-Strand Synthesis Kit, TB Green® Advantage® qPCR Premix, PrimeScript™ RT reagent Kit and TB Green® Fast qPCR Mix were obtained from Takara Bio (Shiga, Japan). Radioimmunoprecipitation (RIPA) lysis buffer was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Hybond 0.2 μm polyvinylidene difluoride (PVDF) membrane was obtained from GE Healthcare Japan (Tokyo, Japan). Clarity Western ECL Substrate was from Bio Rad (Hercules, CA, USA). All chemicals used were of analytical grade.

Cell culture

Osteoblast-like Saos2 cells were cultured at 37℃ in 5% CO_2 and 95% air in α-MEM containing 10% FCS and 1% penicillin and streptomycin. Primary human gingival fibroblasts (HGF) were prepared from patient gingival connective tissue explants, as previously described, and cultured at 37°C in 5% CO_2 and 95% air in DMEM containing 10% FCS and 1% penicillin and streptomycin (16). Saos2 cells and HGF were grown to confluence in 75cm² cell culture flasks. After washed with α-MEM or DMEM supplemented with 1% penicillin and streptomycin without FCS to remove FCS-derived exosomes, cells incubated in these medium for 12 h. After stimulation of Saos2 cells with TNF-α (10 ng/ml) or IL-1β (1 ng/ml) and HGF with TNF-α (10 ng/ml), the cells and conditioned media were collected as following analyses.

Exosome isolation

Collected conditioned medium was centrifuged at 3,000 x g for 15 min to removed cell debris. ExoQuick-TC was added to the supernatant and incubated at 4℃ for 12 h. Then, the incubated medium was centrifuged at 1,500 x g for 30 min in order to form exosome pellets. The exosomal pellet was used for following analyses.

Real-time PCR

Total RNAs from the cells and exosomes were isolated using TRIzol® RNA isolation reagents. cDNA was synthesized with a Mir-X™ miRNA First-Strand Synthesis Kit. Then, real-time PCR was performed using a TB Green® Advantage® qPCR Premix. Real-time PCR was performed using the following primers: hsa-miR-150-5p forward; 5'-TCTCCCAACCCTTGTACCAGTG-3', hsa-miR-200b-3p forward; 5'-TAATACTGCCTGGTAATGATGA-3', hsa-miR-223-3p forward; 5'-TGTCAGTTTGTCAAATACCCCA-3', hsa-miR-379-5p forward, 5'-TGGTAGACTATGGAACGTAGG-3', hsa-miR-144-5p forward; 5'-GGATATCATCATATACTGTAAG-3', mRQ 3' reverse primer, U6 forward primer, and U6 reverse primer including a Mir-X™ miRNA First-Strand Synthesis Kit using a SYBR® Advantage® qPCR Premix (Clontech) in a TP950 thermal cycler dice real time system (TaKaRa). U6 were used as an internal standard. The amplification reactions were

performed in 25 μl of the final reaction mixture containing: 2×SYBR Advantage qPCR Premix (12.5 μl); 10 μM forward and reverse primers (final concentration was 0.2 μM), and 25 ng (2.0 μl) cDNA. To reduce variability between replicates, PCR premixes which contained all reagents except for cDNA, were prepared and aliquoted into 0.2 ml Hi-8-tubes. The thermal cycling condition was 10 s at 95ºC with 40 cycles of 5 s at 95ºC and 20 s at 60ºC. Post-PCR melting curves confirmed the specificity of single-target amplification. The initial amount of RNA was quantified using a standard curve, and fold expressions of miRNA relative to U6 were determined in quadruplicate.

Western blotting

Western blotting was performed using exosomal proteins purified from conditioned media of Saos2 cells stimulated with TNF-α (10 ng/ml) or IL-1β (1 ng/ml) and HGF stimulated with TNF-α (10 ng/ml). Total protein lysates were extracted using RIPA lysis buffer, applied same amounts (105 μg) of total proteins for each lane and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Hybond 0.2 μm PVDF membrane. The membranes were blocked with 3% skim milk for 30 min at room temperature, and incubated overnight with anti-PDCD6IP polyclonal antibody (12422-1-AP; Proteintech, Rosemont, IL, USA), anti-

HSP70 polyclonal antibody (10995-1-AP; Proteintech), anti-CD63 monoclonal antibody (556019; BD Pharmingen, Franklin, NJ, USA), anti-CD9 monoclonal antibody (SHI-EXO-M01; Cosmo Bio, Tokyo, Japan), anti-CD81 monoclonal antibody (SHI-EXO-M03; Cosmo Bio), and anti-TSG101 polyclonal antibody (14497-1-AP; Proteintech). Anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase (HRP) were used as the secondary antibodies (Sigma Aldrich, St. Louis, MO, USA). Immune response was detected by Clarity Western ECL Substrate.

Statistical Analysis

Triplicate samples were analyzed for each experiment. All statistical analyses were performed using one-way analysis of variance (ANOVA) or Student's *t*-test.

3. Results

Relative amounts of miR-150-5p, miR-200b-3p, miR-223-3p and miR-144-5p in the exosomes from conditioned media of Saos2 cells were much higher than the relative amounts of these miRNAs in the cells (Fig. 1A-D). These four kinds of miRNA levels in

the exosomes from Saos2 cells were significantly increased by TNF-α (Fig. 2A-D). IL-1β significantly increased only the relative amounts of miR-150-5p and miR-200b-3p in the exosomes (Fig. 2A, B). Relative amounts of miR-223-3p and miR-144-5p tended to increase with IL-1β stimulation, but not significantly (Fig. 2C, D). Results of Western blotting, PDCD6IP, HSP70 and TSG101 in the exosomes from Saos2 cells were increased by TNF-α and IL-1β stimulations (Fig. 3).

Relative amounts of miR-150-5p, miR-200b-3p, miR-223-3p and miR-144-5p in the exosomes from conditioned media of HGF were much higher than the relative amounts of these miRNAs in the cells (Fig. 4A-D). TNF-α significantly decreased the relative amounts of miR-150-5p, miR-200b-3p and miR-144-5p in the exosomes from HGF (Fig. 5A, B, D). Relative amount of miR-223-3p tended to decrease with TNF-α stimulation, but not significantly (Fig. 5C). Results of Western blotting, PDCD6IP and CD81 in the exosomes from HGF were increased by TNF-α stimulations. α-tubulin was used as an internal control for Western blot, however it could not be detectable in the exosomes (Fig. 6). CD63 and CD9 could not be detected in the cells and exosomes. Therefore, those data were not shown (Fig 6).

Discussion

Periodontitis is widely known as a chronic infectious disease caused by bacterial, environmental and host factors, however the pathological processes of progression of periodontitis has not yet been elucidated in detail. Recently, several miRNAs (hsa-miR-146, hsa-miR-19a, hsa-miR-20a, hsa-miR-142-3p, hsa-miR-155 and hsa-miR-203) are involved in the signal transductions of inflammatory responses (17). MiRNAs can control gene expression at the post transcriptional level by binding to the target mRNAs and initiating either their cleavage or reduction in the translational efficiency. In this study, we have demonstrated that miRNAs were enriched in the exosomes compared to in the cells. These results supported by previous study that miRNAs are selectively packed into the exosomes (18). Profiles of miRNAs and mRNAs in the exosomes have been found to differ from those of parental cells (19). MiRNAs are not randomly integrated into exosomes, but have a mechanism by which the parent cells induce specific intracellular miRNAs into the exosomes (20). It is also suggested that the lipid membrane of exosomes protects miRNAs from degradation by RNases, thus ensuring high miRNA concentrations (21). It is very interesting to investigate the target cells of exosomes secreted by osteoblasts and fibroblasts. As target cells, it may act on gingival epithelial cells, gingival fibroblasts, periodontal ligament-derived cells and osteoblasts, so further research should be needed.

This study showed that different sets of miRNAs increased in response to TNF-α or IL-1β in osteoblast-like Saos2 cells. Relative amounts of miR-150-5p, miR-200b-3p, miR-223-3p and miR-144-5p in the exosomes were significantly increased by TNF-α. In addition, the relative amounts of miR-150-5p and miR-200b-3p in the exosomes were increased significantly by IL-1β. Since the signaling pathways after stimulation of Saos2 cells with TNF-α or IL-1β are different, it is considered that the set of miRNAs increased after stimulation were different. These results were supported by previous report which described miR-150-5p, miR-200b-3p, miR-223-3p and miR-144-5p were highly expressed in the inflamed gingiva (16). TNF-α and IL-1β are soluble proteins that bind to the specific receptors and induce intracellular signaling pathways. These inflammatory cytokines are also involved in the inflammation and tissue damage in periodontitis (22, 23). MiR-150 alleviates neuropathic pain development and suppresses the cyclooxygenase-2 (COX-2), IL-6 and TNF-α expressions in the chronic sciatic nerve injury rats (24). MiR-150 can inhibit the production of inflammatory cytokines by targeting arrestin beta-2 (ARRB2). ARRB2 forms a dimer with phosphodiesterase 4 (PDE4), a cAMP-specific PDE which involved in the hydrolysis of the intracellular signaling molecule. MiR-150 inhibits the nuclear factor-kappa B (NF-κB) pathway and TNF production by reducing ARRB2/PDE4 dimers and increasing cAMP levels (25).

MiR-200b may suppress cellular senescence and inflammatory response via zinc finger E-box binding homeobox 2 (ZEB2) in pulmonary emphysema (26). MiR-223 decreases inhibitor of kappa-B kinase α (IKKα) protein and mitogen activated protein kinase phosphatase-5 (MKP-5) mRNA expressions in HGF, and might control inflammation in the periodontium (27). COX2 is an enzyme involved in the production of prostaglandins from arachidonic acid. Prostaglandin E2 increases vascular dilation and capillary permeability, stimulates osteoclast bone resorption (28). COX2 is an inducible enzyme expressed in the periodontium involved in the inflammatory process, is negatively regulated by miR-144-5p (29).

Results of Western blotting showed that the exosomal PDCD6IP, HSP70 and TSG101 from Saos2 cells were increased by TNF-α and IL-1β. Although the biosynthesis of exosomes remains not fully understood, it has been reported that the syndecan heparan sulphate proteoglycans and their cytoplasmic adaptor syntenin control the formation of exosomes. PDCD6IP supports exosome budding by interacting directly with syntenin (42, 43). Exosomes derived from TNF-α preconditioned gingival mesenchymal stem cells could regulate inflammation and osteoclastogenesis (44). TSG101 ameliorated endotoxin induced cardiac dysfunction by enhancing Parkin-mediated mitophagy (45). HSP70

levels in the exosomes were elevated in mycobacteria infected RAW 264.7 macrophages (46). HSP70 alleviated spinal cord injury through inhibiting NF-κB pathway (47).

Whereas miRNA in the exosomes from HGF behaved differently from that in exosomes from Saos2 cells. MiR-150-5p, miR-200b-3p and miR-144-5p levels in the exosomes from HGF were significantly decreased by TNF-α (Fig. 5). MiRNA analysis of plasma samples from RA patients showed that miR-150-5p expression was lower in RA patients than in healthy controls (30). MiR-150-5p directly targets and downregulates MMP-14 and VEGF (11). Expression levels of MMP-8, MMP-13 and MMP-14 in the gingival crevicular fluid (GCF) were increased in the patients with periodontitis (31). MMPs (including MMP-14) stimulate alveolar bone resorption and periodontal tissue destruction, playing a central role of pathogenesis of periodontitis (32), suggesting altered miR-150 expression may contribute to the progression of periodontitis. MiR-200b is involved in the multiple biological events such as angiogenesis, cell migration and secretion of inflammatory cytokines (33). MiR-200b significantly decreased in mucosa with inflammatory bowel disease (IBD) compared to the normal tissues (34). Gene expressions of E-cadherin and cyclin D1 were significantly decreased in IBD and positively correlated with miR-200b (34, 35). E-cadherin is a calcium dependent cell adhesion molecule which establishes cell-cell adhesion and also plays a critical role in

maintaining a barrier function of gingival epithelium (36). The decrease in expression level of E-cadherin caused by *Prophyromonas gingivalis*-lipopolysaccharide leasd to destruction of the epithelial barrier function in gingival epithelial cells, and antioxidant can restore the impaired function by scavenging reactive oxygen species (37). MiR-144-3p ameliorated the progression of osteoarthritis by targeting IL-1β (38). IL-1β expression was significantly increased in saliva and GCF of the patients with periodontitis (39, 40). IL-1β is expressed mainly by macrophages and dendritic cells, but gingival fibroblasts, periodontal ligament cells and osteoblasts can also secrete it (41). Negative correlation between IL-1β and miR-144-3p suggested that miR-144-3p might be an effective therapeutic agent for periodontitis.

Results of Western blotting, PDCD6IP and CD81 in the exosomes from HGF were increased by TNF-α stimulations. Aberrant upregulation of CD81 is accompanied by the decrease in regulatory T cells that limit inflammation and increase in Th17 cells that mediate inflammation (48). The amounts of TSG101 in the exosomes from the saliva and CD9 in the exosomes from the GCF of periodontitis patients were higher than healthy subjects (49). The amounts of PDCD6IP in the exosomes from the GCF of periodontitis patients were lower than healthy subjects (49).

Thus, changes in contents of exosomes such as miRNAs and proteins were considered to play an important role in signal transduction in inflammation. In conclusion, stimulation by inflammatory cytokines resulted in differential responses on relative amounts of exosomal miRNAs from Saos2 cells and HGF. These results suggest that the distinct roles of osteoblasts and gingival fibroblasts in the progression of periodontitis.

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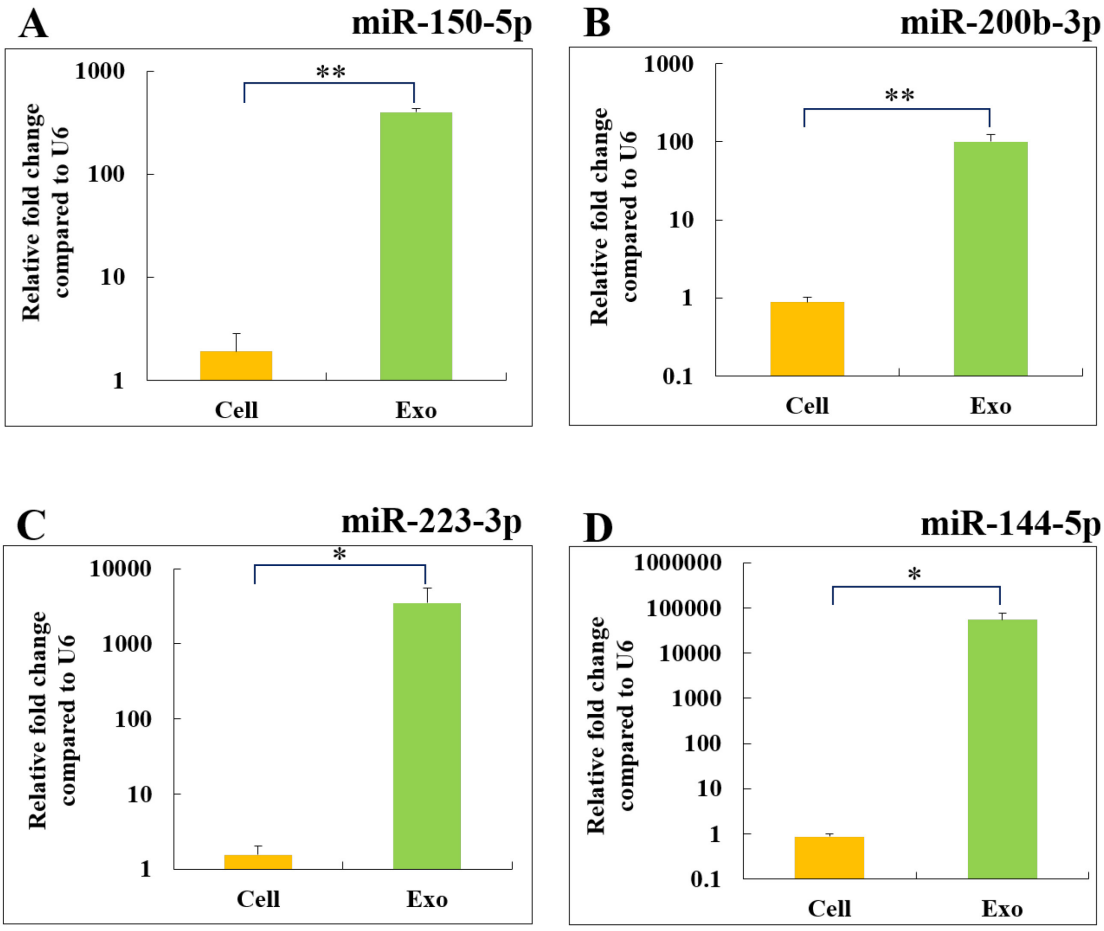

Fig.1 Comparison of miRNAs levels between Saos2 cells and the exosomes. Relative amounts of miR-150-5p (A), miR-200b-3p (B), miR-223-3p (C) and miR-144-5p (D) in the Saos2 cells and in the exosomes from conditioned media were measured by real-time PCR. Quantitative analyses of the three datasets are shown with standard error (SE). *P < 0.05, **P < 0.01, significant difference versus control.

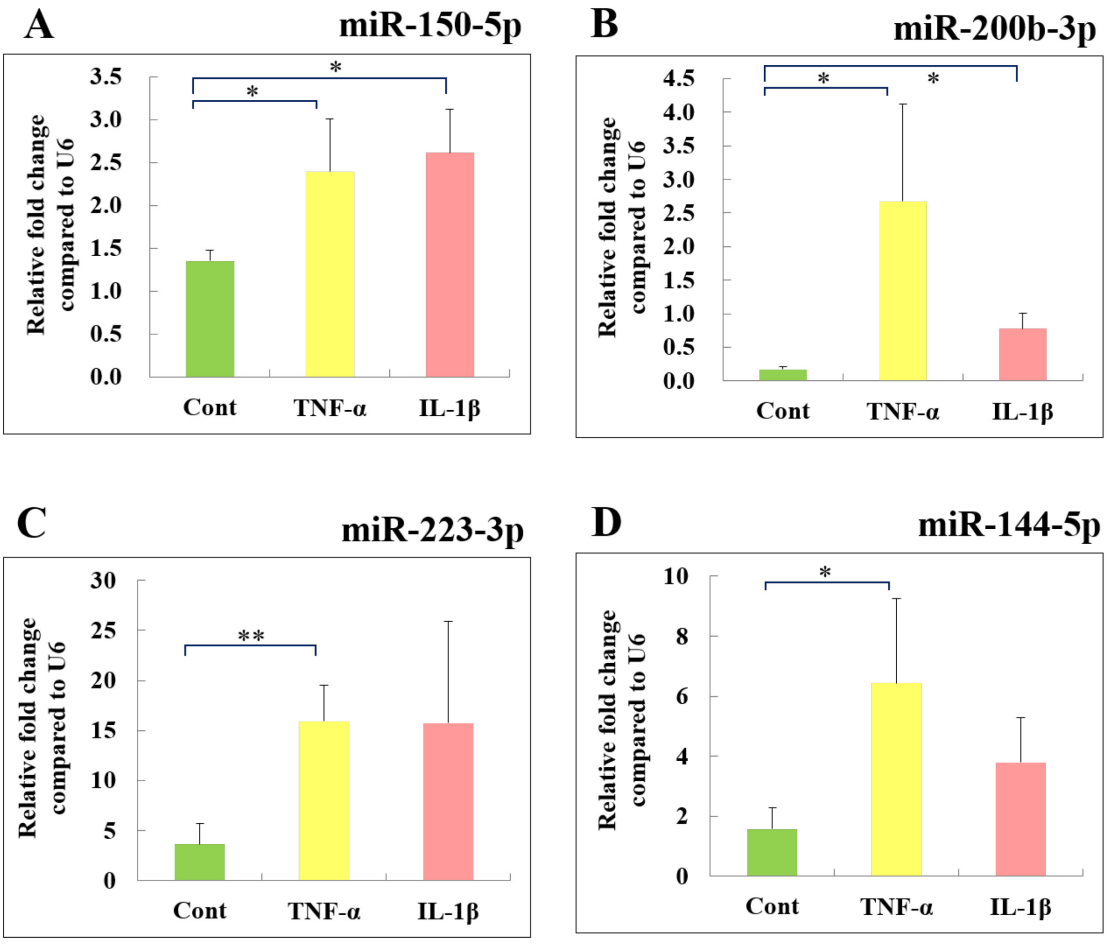

Fig. 2 Effects of cytokines to the amounts of miRNAs in the exosomes from Saos2 cells. Relative amounts of miR-150-5P (A), miR-200b-3P (B), miR-223-3P (C) and miR-144-5P (D) in the exosomes from conditioned media of Saos2 cells after stimulation by TNF-α (10 ng/ml) or IL-1β (1 ng/ml) were measured by real-time PCR. Cont indicates the experimental condition without TNF-α or IL-1β stimulation as a control. Quantitative analyses of the three datasets are shown with SE. *$P < 0.05$, **$P < 0.01$, significant difference versus control.

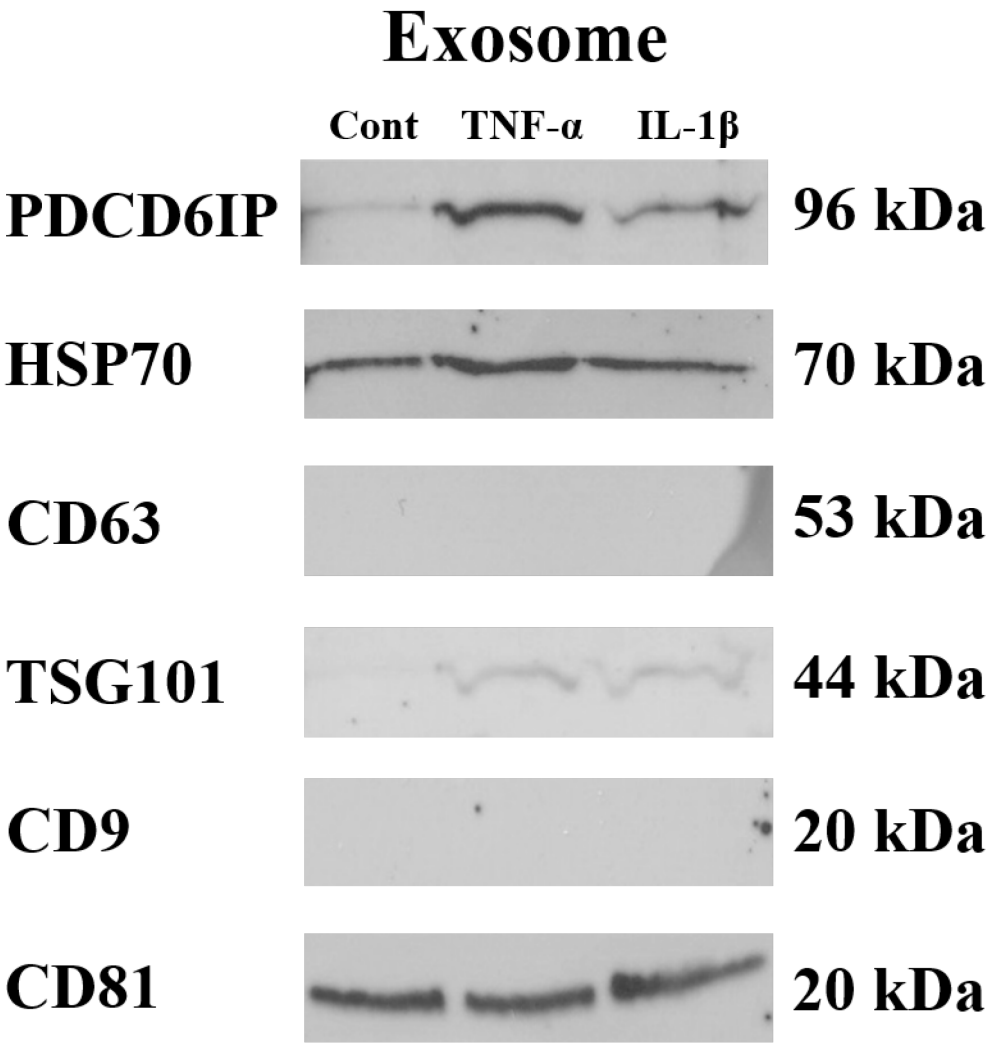

Fig. 3 Western blot analyses of maker proteins in the exosomes. PDCD6IP, HSP70, CD63, TSG101, CD9 and CD81 in the exosomes from conditioned media of Saos2 cells after stimulation by TNF-α (10 ng/ml) or IL-1β (1 ng/ml) were analyzed by Western blotting. Cont indicates the experimental condition without TNF-α or IL-1β stimulation as a control.

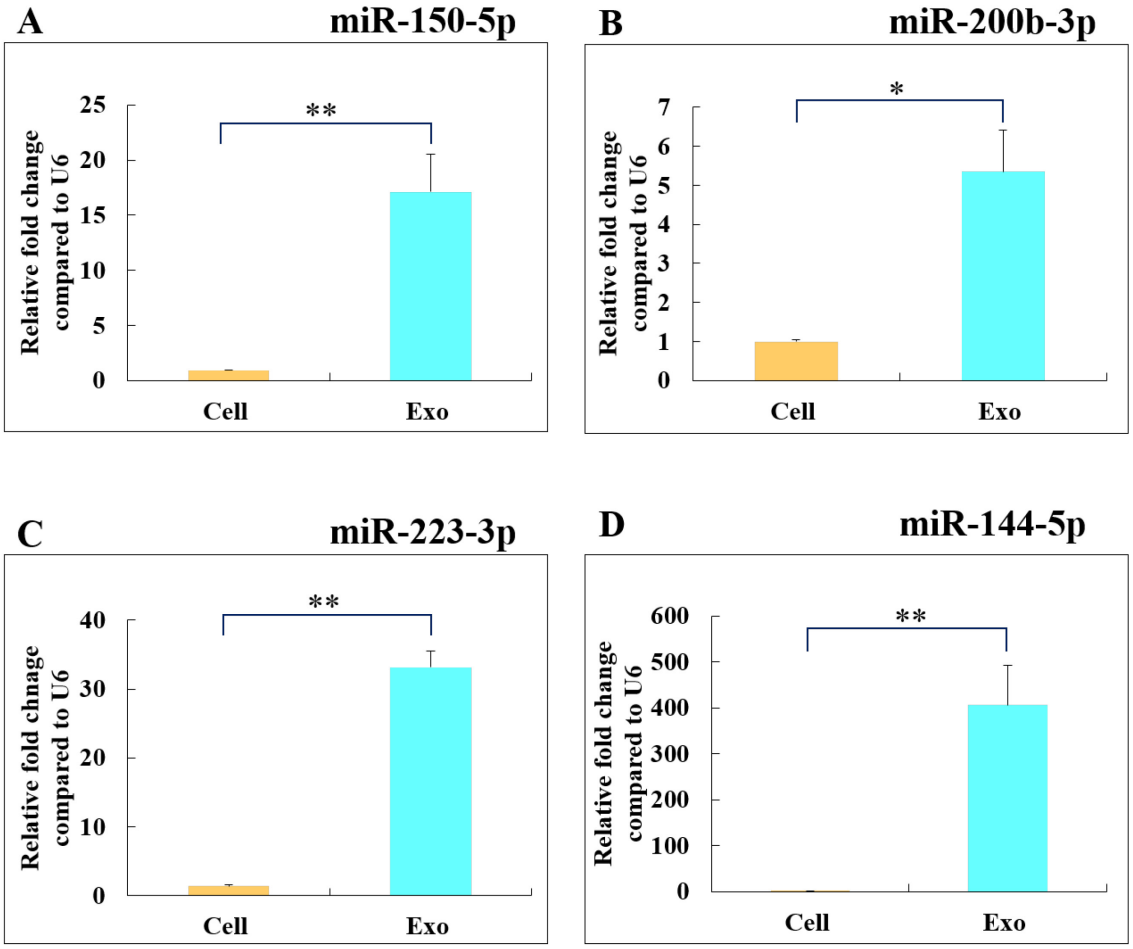

Fig. 4 Comparison of miRNAs levels between HGFs and the exosomes. Relative amounts of miR-150-5p (A), miR-200b-3p (B), miR-223-3p (C) and miR-144-5p (D) in the HGFs and in the exosomes from conditioned media were measured by real-time PCR. Quantitative analyses of the three datasets are shown with standard error (SE). Significant difference versus control, *P < 0.05, **P < 0.01.

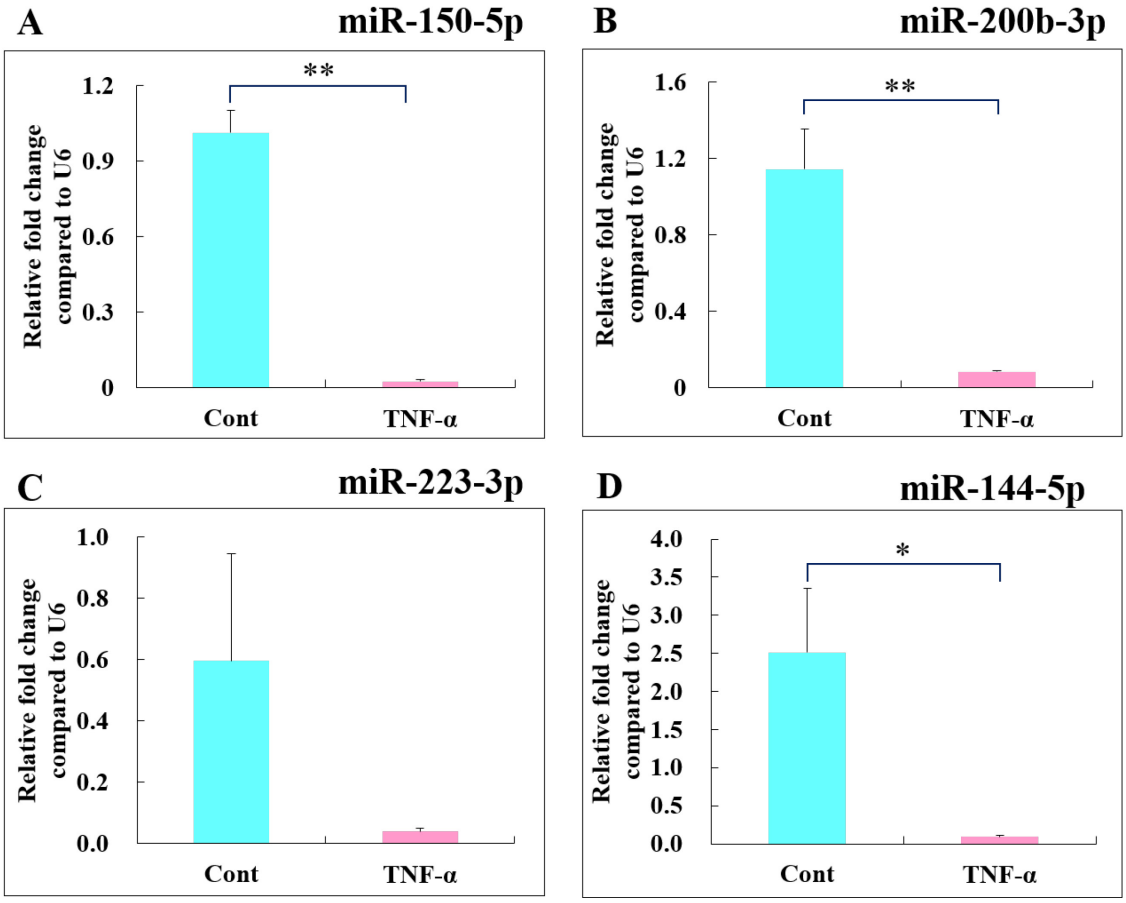

Fig. 5 Effects of TNF-α on the amounts of miRNAs in the exosomes from HGFs. Relative amounts of miR-150-5P (A), miR-200b-3P (B), miR-223-3P (C) and miR-144-5P (D) in the exosomes from conditioned media of HGFs after stimulation by TNF-α (10 ng/ml) were measured by real-time PCR. Cont indicates the experimental condition without TNF-α stimulation as a control. Quantitative analyses of the three datasets are shown with SE. Significant difference versus control, *P < 0.05, **P < 0.01.

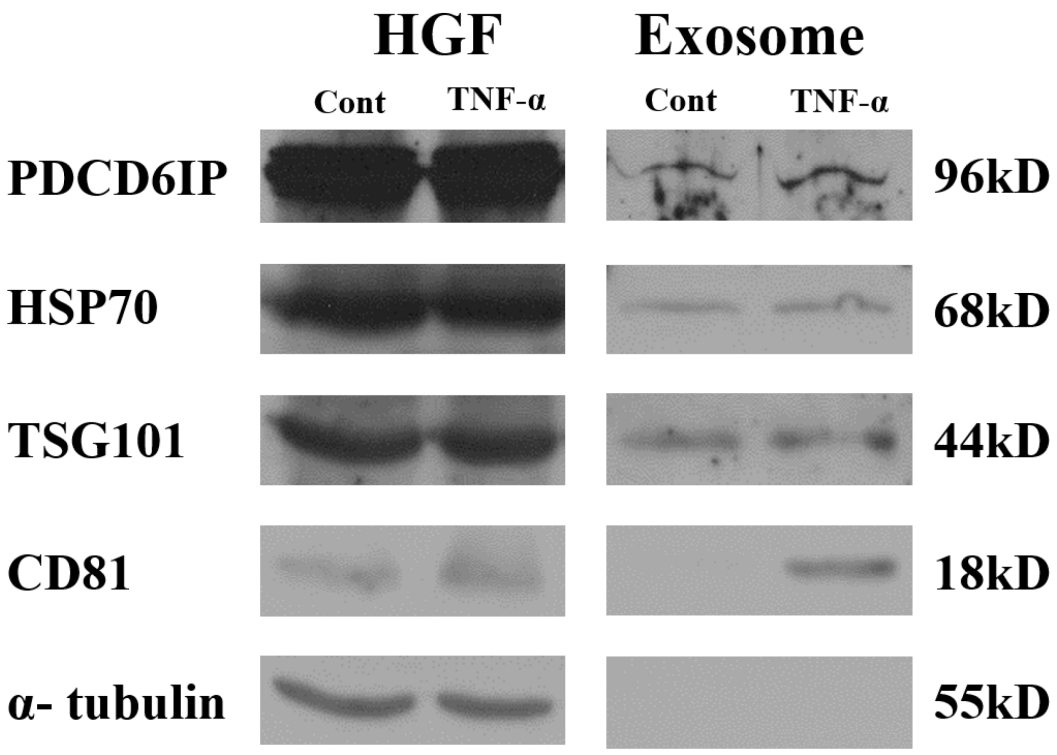

Fig. 6 Western blot analyses of maker proteins. Expressions of the maker proteins in the HGF and the exosomes were analyzed with/without TNF-α (10 ng/ml) stimulation by Western blotting. Cont indicates the experimental condition without TNF-α as a control. Molecular weight of each protein is shown on the right.