Secretion of small/microRNAs including miR-638 into extracellular spaces by sphingomyelin phosphodiesterase 3

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Abstract. A recent study demonstrated that intracellular small/microRNAs are released from cells, and some of these extracellular RNAs are embedded in vesicles, such as ceramide-rich exosomes, on lipid-bilayer membranes. In the present study, we examined the effects of sphingomyelin phosphodiesterase 3 (SMPD3), which generates ceramide from sphingomyelin, on the release of small/microRNAs from intracellular to extracellular spaces. In these experiments, SW480 human colorectal and HuH-7 human hepatocellular cancer cells were cultured for 48 h in serum-free media. Culture supernatants were then collected, and floating cells and debris were removed by centrifugation and filtration through a 0.22-µm filter. Extracellular small RNAs in purified culture supernatants were stable for 4 weeks at room temperature, after 20 freeze-thaw cycles and exposure to pH 2.0, and were resistant to ribonuclease A degradation. Amino acid sequence analyses of SMPD3 showed high homology between mammals, indicating evolutionary conservation. Therefore, to investigate the mechanisms of cellular small/microRNA export, SW480 and HuH-7 cells were treated with the SMPD3 inhibitor GW4869 in serum-free media. Culture supernatants were collected for microarray and/or reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments. The number of microRNAs in culture supernatants was decreased following treatment with GW4869. Among these, extracellular and intracellular miR-638 were dose-dependently decreased and increased, respectively. These data suggest that SMPD3 plays an important role in the release of microRNAs into extracellular spaces.

Introduction

MicroRNAs of 20-25 nucleotides act as post-transcriptional regulators of gene expression, and their localization suggests that they primarily function in the cytoplasm. Recently, a number of microRNAs were found in extracellular spaces (1), and some of these were embedded in extracellular vesicles such as exosomes (2). However, it has been suggested that some extracellular microRNAs form complexes with Argonaute 2 (Ago2), high-density lipoprotein (HDL) and other RNA-binding proteins (3-6). Therefore, microRNAs may be present in various bound forms in extracellular spaces. Diagnostic biomarkers were recently identified in body fluids such as serum, plasma, urine, milk and saliva (7-11). However, these data require further validation in focused studies of extracellular microRNA stability.

Exosomes are extracellular vesicles, ~40-200 nm in diameter, which are secreted from epithelial (15), endothelial (16), cancer (17), dendritic (18), and mesenchymal stem cells (19), as well as B lymphocytes (20). Exosome secretion has been identified in human and mouse cells in vitro (1). However, few studies have demonstrated RNA secretion in other organisms.

Although the mechanisms of exosome biogenesis remain to be adequately defined, current models suggest that exosomes are formed within multivesicular bodies (MVBs) (21), which are formed during maturation of early into late endosomes, with concomitant and corresponding accumulation of intraluminal vesicles (ILVs) (22). Endosomal sorting complexes required for transport (ESCRT) machinery are also responsible for generating vesicles in MVBs through a process known as endosome budding (23). In addition, ceramide is reportedly involved in an ESCRT-independent process of exosome generation (24). Ceramide, which is generated from sphingomyelin by neutral sphingomyelinase 2 (nSMase2), is found in lipid components of exosome membranes (25), and is encoded by the sphingomyelin phosphodiesterase 3 (SMPD3).
gene. Although this enzyme has been shown to be involved in the secretion of small RNAs such as microRNAs (26), which small/microRNAs are released following the actions of nSMase2 remains to be determined.

In the present study, we investigated the stability of extracellular small RNAs against external factors including ribonuclease A (RNase A), long-term incubation, freeze-thaw, and pH change using HuH-7 human hepatocellular cancer cells. In addition, we examined the evolutionary conservation of SMPD3 in mammals and determined the effects of an SMPD3 inhibitor on the release of small/microRNAs from HuH-7 and SW480 human colorectal cancer cells.

Materials and methods

Cell lines and culture. HuH-7 human hepatocellular cancer cells (JCRB0403) were purchased from the Health Science Research Resources Bank (Osaka, Japan). The human colorectal cancer cell line SW480 (CCL-228) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HuH-7 cells were cultured in Dulbecco's minimal essential medium (D-MEM; Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum and 100 µl/ml penicillin, and 100 µg/ml streptomycin. SW480 cells were cultured in RPMI-1640 medium (Wako) supplemented with 10% FBS, 100 µl/ml penicillin, and 100 µg/ml streptomycin. The cells were cultured at 37˚C in a 5% CO₂ atmosphere.

Purification of culture supernatants. SW480 and HuH-7 cells were plated on collagen-coated 10-cm dishes at 1x10⁶ cells/dish in culture media. After 72 h, the culture media were discarded and the cells were washed three times in serum-free culture media. Serum-free culture media supplemented with the SMPD3 inhibitor GW4869 (Sigma-Aldrich, St. Louis, MO, USA) at final concentrations of 0, 1.0, 3.3, and 10.0 µM were then added at 10 ml per dish, and the cells were cultured for 48 h. Cell culture media were then collected and centrifuged at 300 x g at 4˚C for 3 min to remove floating cells. Supernatants were centrifuged at 2,000 x g at 4˚C for 15 min and were collected in new tubes. Culture supernatants were also centrifuged at 12,000 x g at 4˚C for 35 min to remove cell debris, and the supernatants were collected in new tubes and filtered using 0.22-µm filters. Extracellular RNAs in the supernatants were then isolated using Isogen II (NipponGene, Tokyo, Japan).

RNA extraction. Extracellular and intracellular RNAs from SW480 or HuH-7 cells were isolated using Isogen II (NipponGene) according to the manufacturer’s instructions. The sizes of extracted RNAs were determined using an Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico kits (both from Agilent Technologies, Foster City, CA, USA) according to the manufacturer's instructions.

Microarray analysis. Species of extracellular microRNAs were distinguished by labeling with Hy5 fluorescent dye using a miRCury LNA™ microRNA Hy5 Power labeling kit (Exiqon, Copenhagen, Denmark). Microarray analyses were then conducted using a Toray microRNA microarray system. Toray 3D-Gene human miRNA oligo chips (Toray, Tokyo, Japan) were hybridized with Hy5-labeled microRNAs in hybridization solution at 32˚C for 16 h using a hybridization oven. Hybridized microarray chips were then washed in a wash buffer according to the manufacturer's instructions, and images of fluorescent signals were captured using a Toray 3D-gene scanner 3000 (Toray).

Reverse transcription polymerase chain reaction (RT-PCR) and RT quantitative PCR (RT-qPCR). To investigate SMPD3 mRNA expression in SW480 and HuH-7 cells, cDNAs were synthesized from isolated RNAs using High Capacity cDNA reverse transcriptase kits according to the manufacturer's instructions. Subsequently, qPCR for mRNAs was performed using 2X Power SYBR-Green master mix, 10.0 µM forward and reverse primers (Table I), and a StepOne Plus real-time PCR system (all from Life Technologies), under the following conditions: 10 min at 95˚C, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec. GAPDH was used as an internal control. Expression levels were determined using the comparative Ct method and were normalized to those from SW480 cells. Amplified fragments were then detected on 4% agarose gel electrophoresis containing ethidium bromide and images of fluorescent signals were captured using a ChemiDoc XRS system and Quantity One software (both from Bio-Rad, Hercules, CA, USA).

Expression levels of extracellular and intracellular microRNAs from SW480 and HuH-7 cells were analyzed using cDNAs that were synthesized from microRNAs using TaqMan microRNA RT kits and the prescribed 5X RT primer (both from Life Technologies) according to the manufacturer's instructions. Subsequently, qPCR for microRNAs was performed using FastStart TaqMan probe master (Roche Diagnostics, Basel, Switzerland), a 20X probe, and a StepOne Plus real-time PCR.
system (Life Technologies) under the following conditions: 10 min at 95˚C, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec. RNAs were isolated from 200-µl aliquots of culture supernatants following the addition of 1 µl of 5 nM cel-miR-39. Cel-miR-39 was used as an external control and U6 small nuclear RNA (snRNA) was used as an internal control. Expression levels were determined using the comparative Ct method.

Multiple alignments of SMPD3 amino acid sequences. Amino acid sequences for Homo sapiens SMPD3, NP_061137.1; Pan troglodytes SMPD3, XP_001167790.1; Mus musculus SMPD3, NP_067466.1; and Bos taurus SMPD3, NP_001179292.1, were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov), and were subjected to multiple alignment analysis using Genetyx 10 software (Genetyx, Tokyo, Japan).

Statistical analysis. Data are presented as the mean ± standard error of the mean (SEM). Multiple group comparisons were performed using one-way analysis of variance (ANOVA), followed by post hoc pair-wise comparisons of significant differences using Dunnett's test. Differences were considered significant when P<0.01.

Results and Discussion

Extracellular small RNAs are stable against changes in various conditions. Encapsulation of released cellular small RNAs in exosomes likely allows high stability against changes in several conditions (12-14). Accordingly, small RNAs in purified supernatants from HuH-7 cells were stable through RNase A treatment, long-term incubation, cycles of freezing and thawing and pH changes.

In experiments conducted in this study, serum-free culture supernatants from HuH-7 cells were purified by centrifugation and filtration and were incubated with RNase A at a final concentration of 0.4 µg/ml for 10 min at 37°C. After extraction of total RNAs from culture supernatants, a peak for small RNAs of 25-200 nt was detected using an Agilent bioanalyzer (Fig. 1A). However, in culture supernatants, small RNAs were stable after incubation at room temperature for 4 weeks, 20 cycles of freezing and thawing (room temperature to -80°C), and reduction of pH to 2.0 (Fig. 1B-D). These data indicate high stability of small RNAs in culture supernatants.

Evolutionary conservation of SMPD3 in mammals. SMPD3 is reportedly involved in the secretion of microRNAs (26). The present analyses of various mammalian SMPD3 sequences (Homo sapiens, Pan troglodytes, Mus musculus and Bos taurus) indicated high sequence homology (Fig. 2), with an amino acid sequence identity of 99.69, 91.02 and 89.50% between Homo sapiens and Pan troglodytes, Mus musculus, Bos Taurus, respectively. Moreover, two hydrophobic segments, two palmitoylation sites and the catalytic domain were highly conserved between examined mammals (Fig. 2). These analyses suggest that SMPD3 may have similar functions across these species.

Small/microRNAs, such as miR-638, are secreted into extracellular spaces via a ceramide-dependent mechanism. Although nSMase2 produces ceramide from sphingomyelin (25), it is reportedly involved in the secretion of small RNAs such as microRNAs (26). Thus, we investigated the relationship between small RNA secretion and SMPD3 mRNA expression in SW480 and HuH-7 cells. In the RT-qPCR experiments, SMPD3 mRNA expression in HuH-7 cells was 28.62-fold higher than that in SW480 cells (Fig. 3A). Moreover, peak RNA release from HuH-7 cells was higher than that of SW480 cells (Fig. 3B), and corresponded with high SMPD3 mRNA expression.

In the present study, concentrations of small RNAs in culture supernatants were determined following treatment of HuH-7 or SW480 cells with non-competitive SMPD3 inhibitor GW4869 at a final concentration of 10.0 µM. In these experiments, small RNA contents were markedly decreased after 48 h (Fig. 3B), suggesting that SMPD3 is important in small RNA secretion.

In subsequent experiments, the amounts and species of microRNAs in culture supernatants from GW4869-treated HuH-7 cells were analyzed using a Toray microRNA micro-
array system. MicroRNA expression profiles following treatment with 0 and 10.0 µM GW4869 for 48 h are shown in a scatter plot (Fig. 3C). Various microRNAs, including miR-638, were decreased in culture supernatants from GW4869-treated cells (Table II).

Subsequent RT-qPCR experiments showed a significant decrease in miR-638 expression in the SW480 and HuH-7 cells and supernatants following treatment with GW4869 (P<0.01). Specifically, extracellular miR-638 expression in HuH-7 cells was decreased 3.92- and 16.67-fold in the presence of 3.3 and 10.0 µM GW4869, respectively. In SW480 cells it was decreased 2.29-, 11.36- and 113.14-fold in the presence of 1.0, 3.3 and 10.0 µM GW4869, respectively (Fig. 3D-E). By contrast, intracellular miR-638 expression was significantly increased 2.43-, 5.41- and 15.81-fold in the presence of 1.0, 3.3, and 10.0 µM GW4869 in HuH-7 cells, and 5.01-, 7.00-, and 9.57-fold, respectively, in SW480 cells when compared with expression in the presence of 0 µM GW4869 (P<0.01; Fig. 3D-E). These data suggest that SMPD3 plays an important role in the release of a number of microRNAs, including miR-638, and that microRNAs accumulated in cells following the inhibition of exosome membrane formation by GW4869.

Small RNAs secretions are involved in the formation of exosomes, the regulation of vesicle trafficking, and the plasma membrane fusion of MVBs (28,29). Exosome transport is considered a highly controlled process that involves a number of Rab GTPases. Accordingly, Rab11 overexpression has been shown to stimulate exocytosis (30), and Rab27a and Rab27b have been shown to control exosome secretion by regulating vesicle transport of MVBs to plasma membranes (31). In addition, secretion of exosomes containing small RNAs requires
fusion of MVBs to plasma membranes, potentially involving soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein complexes (32). Future studies are required to clarify the mechanisms of RNA and exosome release into extracellular spaces.

In conclusion, extracellular small RNAs are comparatively stable due to their presence in exosomes. Moreover, the high evolutionary conservation of SMPD3 indicates an important role in the release of miR-638 and other microRNAs into extracellular spaces.
Table II. The microRNA expression in culture supernatants of HuH-7 cells treated with 0 or 10.0 µM GW4869 using a Toray microRNA microarray system.

| microRNAs       | Raw signal intensity | microRNAs       | Raw signal intensity |
|-----------------|----------------------|-----------------|----------------------|
|                 | 0 µM GW4869          | 10.0 µM GW4869  |                      |
| hsa-miR-3960    | 65,098.0             |                 |                      |
|                 | 8,112.9              |                 |                      |
| hsa-miR-4787-5p| 26,349.0             |                 |                      |
|                 | 1,593.3              |                 |                      |
| hsa-miR-4508    | 23,348.0             |                 |                      |
|                 | 1,349.6              |                 |                      |
| hsa-miR-3665    | 22,420.0             |                 |                      |
|                 | 1,538.6              |                 |                      |
| hsa-miR-4488    | 21,004.0             |                 |                      |
|                 | 207.2                |                 |                      |
| hsa-miR-762     | 20,776.0             |                 |                      |
|                 | 1,527.1              |                 |                      |
| hsa-miR-4739    | 18,737.0             |                 |                      |
|                 | 671.5                |                 |                      |
| hsa-miR-4516    | 16,181.0             |                 |                      |
|                 | 1,884.0              |                 |                      |
| hsa-miR-4505    | 12,498.5             |                 |                      |
|                 | 443.2                |                 |                      |
| hsa-miR-3648    | 12,080.7             |                 |                      |
|                 | 175.5                |                 |                      |
| hsa-miR-4466    | 11,807.8             |                 |                      |
|                 | 1,714.7              |                 |                      |
| hsa-miR-4488    | 11,136.4             |                 |                      |
|                 | 2,385.7              |                 |                      |
| hsa-miR-3196    | 11,008.8             |                 |                      |
|                 | 1,971.5              |                 |                      |
| hsa-miR-2861    | 10,473.1             |                 |                      |
|                 | 1,617.8              |                 |                      |
| hsa-miR-638     | 10,180.6             |                 |                      |
|                 | 587.0                |                 |                      |
| hsa-miR-1908    | 9,862.2              |                 |                      |
|                 | 559.4                |                 |                      |
| hsa-miR-4725-3p| 9,736.2              |                 |                      |
|                 | 502.2                |                 |                      |
| hsa-miR-4294    | 9,309.6              |                 |                      |
|                 | 3,504.9              |                 |                      |
| hsa-miR-3656    | 8,857.1              |                 |                      |
|                 | 964.6                |                 |                      |
| hsa-miR-4467    | 8,139.1              |                 |                      |
|                 | 446.2                |                 |                      |
| hsa-miR-4745-5p| 7,940.2              |                 |                      |
|                 | 495.4                |                 |                      |
| hsa-miR-4734    | 7,933.7              |                 |                      |
|                 | 615.8                |                 |                      |
| hsa-miR-4497    | 7,932.5              |                 |                      |
|                 | 451.3                |                 |                      |
| hsa-miR-744     | 6,356.6              |                 |                      |
|                 | 244.6                |                 |                      |
| hsa-miR-4327    | 6,287.8              |                 |                      |
|                 | 272.2                |                 |                      |
| hsa-miR-4723-5p| 6,007.8              |                 |                      |
|                 | 416.2                |                 |                      |
| hsa-miR-663     | 5,899.5              |                 |                      |
|                 | 576.6                |                 |                      |
| hsa-miR-3621    | 5,509.6              |                 |                      |
|                 | 3,186.3              |                 |                      |
| hsa-miR-4454    | 4,467.7              |                 |                      |
|                 | 112.9                |                 |                      |
| hsa-miR-1268    | 4,115.5              |                 |                      |
|                 | 711.4                |                 |                      |
| hsa-miR-1246    | 3,965.1              |                 |                      |
|                 | 3,866.2              |                 |                      |
| hsa-miR-3940-5p| 3,925.4              |                 |                      |
|                 | 936.0                |                 |                      |
| hsa-miR-4492    | 3,916.0              |                 |                      |
|                 | 251.9                |                 |                      |
| hsa-miR-3178    | 3,777.1              |                 |                      |
|                 | 459.8                |                 |                      |
| hsa-miR-1469    | 3,516.2              |                 |                      |
|                 | 639.1                |                 |                      |
| hsa-miR-4530    | 3,463.2              |                 |                      |
|                 | 242.0                |                 |                      |
| hsa-miR-1228*   | 3,319.6              |                 |                      |
|                 | 628.7                |                 |                      |
| hsa-miR-4459    | 2,894.8              |                 |                      |
|                 | 286.1                |                 |                      |
| hsa-miR-4687-3p| 2,760.3              |                 |                      |
|                 | 700.9                |                 |                      |
| hsa-miR-4463    | 2,668.5              |                 |                      |
|                 | 478.1                |                 |                      |
| hsa-miR-1915    | 2,516.5              |                 |                      |
|                 | 185.4                |                 |                      |
| hsa-miR-1260b   | 2,456.4              |                 |                      |
|                 | 295.3                |                 |                      |
| hsa-miR-4281    | 2,407.2              |                 |                      |
|                 | 344.2                |                 |                      |
| hsa-miR-149*    | 2,356.0              |                 |                      |
|                 | 257.3                |                 |                      |
| hsa-miR-4749-5p| 2,305.3              |                 |                      |
|                 | 176.4                |                 |                      |
| hsa-miR-1275    | 2,241.6              |                 |                      |
|                 | 215.3                |                 |                      |
| hsa-miR-1268b   | 2,210.7              |                 |                      |
|                 | 350.2                |                 |                      |
| hsa-miR-3141    | 1,979.9              |                 |                      |
|                 | 250.6                |                 |                      |
| hsa-miR-4417    | 1,809.8              |                 |                      |
|                 | 425.3                |                 |                      |
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References

1. Valadi H, Ekström K, Bossios A, Sjostrand M, Lee JJ and Lötvall JO: Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9: 654-659, 2007.

2. Hu G, Drescher KM and Chen XM: Exosomal miRNAs: biological properties and therapeutic potential. Front Genet 3: 56, 2012.

3. Arroyo JD, Chevillet JR, Kroh EM, et al: Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 108: 5003-5008, 2011.

4. Turchinovich A, Weiz L, Langheinz A and Burwinkel B: Characterization of extracellular circulating microRNA. Nucleic Acids Res 39: 7223-7233, 2011.

5. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD and Remaley AT: MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol 13: 423-433, 2011.

6. Wang K, Zhang S, Weber J, Baxter D and Galas DJ: Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res 38: 7248-7259, 2010.

7. Brase JC, Wuttig D, Kuner R and Sultmann H: Secretory mechanisms and intercellular transfer of microRNAs is a novel mechanism of genetic exchange between vesicles in human plasma. Proc Natl Acad Sci U S A 108: 566, 2012.

8. Huang Z, Huang D, Ni S, Peng Z, Sheng W and Du X: Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. Int J Cancer 127: 118-126, 2010.

9. Moon PG, Lee JE, You S, et al: Proteomic analysis of urinary exosomes from patients of early IgA nephropathy and thin basement membrane nephropathy. Proteomics 11: 2459-2475, 2011.

10. Michael A, Bajracharya SD, Yuen PS, et al: Exosomes from human saliva as a source of microRNA biomarkers. Oral Dis 16: 34-38, 2010.

11. Lässer C, Alikhani VS, Ekström K, et al: Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. J Transl Med 9: 9, 2011.

12. Taylor DD and Gercel-Taylor C: MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 110: 13-21, 2008.

13. Kosaka N, Izumi H, Sekine K and Ochiya T: microRNA as a new immune-regulatory agent in breast milk. Silence 1: 7, 2010.

14. Ge Q, Zhou Y, Lu J, Bai Y, Xie X and Lu Z: miRNA in plasma exosome is stable under different storage conditions. Molecules 19: 1568-1575, 2014.

15. Karlsson M, Lundin S, Dahlgren U, Kahu H, Pettersson I and Telemo E: ‘Tolerosomes’ are produced by intestinal epithelial cells. Eur J Immunol 31: 2892-2900, 2001.

16. Muturi HT, Dreesen JD, Nilewski E, et al: Tumor and endothelial cell-derived microvesicles carry distinct CEACAMs and influence T-cell behavior. PLoS One 8: e74654, 2013.

17. King HW, Michael MZ and Gleade JM: Hypoxic enhancement of exosome release by breast cancer cells. BMC Cancer 12: 421, 2012.

18. Montecalvo A, Larregina AT, Shufesky WJ, et al: Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 119: 756-766, 2012.

19. Lee JK, Park SR, Jung BK, et al: Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. PLoS One 8: e84256, 2013.

20. Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yosio O and Geuze HJ: Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. J Biol Chem 273: 20121-20127, 1998.

21. Bobrie A, Colombo M, Raposo G and Ther Y: Exosome secretion: molecular mechanisms and roles in immune responses. Traffic 12: 1659-1668, 2011.

22. Hanson PI and Cashikar A: Multivesicular body morphogenesis. Annu Rev Cell Dev Biol 28: 337-362, 2012.

23. Raiborg C and Stenmark H: The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature 458: 445-452, 2009.

24. Trajkovic K, Hsu C, Chiantia S, et al: Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science 319: 1244-1247, 2008.

25. Marchesini N, Luberto C and Hannun YA: Biochemical properties of mammalian neutral sphingomyelinase 2 and its role in sphingolipid metabolism. J Biol Chem 278: 13775-13783, 2003.

26. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsu K and Ochiya T: Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem 285: 17442-17452, 2010.

27. Lee DH, Kim SH, Ahn KH, et al: Identification and evaluation of neutral sphingomyelinase 2 inhibitors. Arch Pharm Res 34: 229-236, 2011.

28. Rodriguez-Boulan E, Kreitzer G and Müssch A: Organization of vesicular trafficking in epithelia. Nat Rev Mol Cell Biol 6: 233-247, 2005.

29. Jahn R and Fasshauer D: Molecular machines governing exocytosis of synaptic vesicles. Nature 490: 201-207, 2012.

30. Savina A, Vidal M and Colombo MI: The exosome pathway of vesicular trafficking in epithelia. Nat Rev Mol Cell Biol 6: 233-247, 2005.

31. Marchesini N, Luberto C and Hannun YA: Biochemical properties of mammalian neutral sphingomyelinase 2 and its role in sphingolipid metabolism. J Biol Chem 278: 13775-13783, 2003.

32. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsu K and Ochiya T: Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem 285: 17442-17452, 2010.

33. Lee DH, Kim SH, Ahn KH, et al: Identification and evaluation of neutral sphingomyelinase 2 inhibitors. Arch Pharm Res 34: 229-236, 2011.

34. Rodriguez-Boulan E, Kreitzer G and Müssch A: Organization of vesicular trafficking in epithelia. Nat Rev Mol Cell Biol 6: 233-247, 2005.

35. Jahn R and Fasshauer D: Molecular machines governing exocytosis of synaptic vesicles. Nature 490: 201-207, 2012.

36. Savina A, Vidal M and Colombo MI: The exosome pathway of vesicular trafficking in epithelia. Nat Rev Mol Cell Biol 6: 233-247, 2005.

37. Ostrowski M, Carmo NB, Krum R and Müsch A: The exosome pathway of vesicular trafficking in epithelia. Nat Rev Mol Cell Biol 6: 233-247, 2005.