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Original Article

Exosomes derived from non-canonical WNT5A-expressing cells activate canonical WNT signaling in recipient cells

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Purpose: WNT proteins regulate key developmental and physiological processes. Recent studies have found that exosomes, which are small extracellular vesicles, are novel transport carriers of WNT signaling proteins. In the present study, we isolated exosomes from conditioned media of canonical ligand WNT3A- and non-canonical ligand WNT5A-expressing HEK293T cells and analyzed their bioactivity.

Methods: Exosomes were isolated by differential centrifugation followed by size exclusion chromatography. Isolated exosomes were subjected to Western blot analysis and nanoparticle tracking analysis for their cargo contents and size distribution, respectively.

Results: Most enriched exosomes measured 120-140 nm and expressed canonical exosomal markers, including ALIX, CD63, and CD81. Expression of either WNT3A or WNT5A led to an increase in the total number of secreted exosomes. While WNT3A and active β-catenin were both recovered in exosomes derived from WNT3A-producing cells, only active β-catenin was detected in exosomes recovered from WNT5A-producing cells. Intriguingly, both types of exosomes activated β-catenin-dependent response in recipient cells, as evidenced by accumulation of cytosolic active β-catenin and transcriptional activation of T-cell factor/lymphoid enhancer factor (TCF/LEF) responsive elements.

Conclusion: These results suggest that WNT3A- and WNT5A-producing cells share a common mechanism for packaging of active β-catenin in exosomes.

Keywords: WNT signaling, WNT3A, WNT5A, exosomes, active β-catenin

1. Introduction

WNTs represent highly conserved secreted glycoproteins that regulate key developmental and physiological processes [1]. Wnt signaling involves two major pathways: a canonical WNT (cWNT) pathway that is β-catenin-dependent and a non-canonical Wnt (ncWNT) pathway that is β-catenin-independent. In the cWNT pathway, in the absence of cWNT ligand, β-catenin is phosphorylated by the complex composed of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β. Phosphorylated β-catenin is then subjected to ubiquitin-dependent proteasome degradation. The cWnt signaling pathway is triggered when canonical WNT ligands, such as WNT3A, bind to Frizzled receptors (Fzd) and low-density lipoprotein receptor-related protein 5/6 co-receptors (LRP5/6) to form complexes. This initiates the recruitment of Axin and dishevelled to the plasma membrane and disassembly of the β-catenin degradation complex, leading to accumulation of dephosphorylated β-catenin (active β-catenin, ABC) in the cytoplasm. This is followed by translocation of

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β-catenin into nucleus, where it binds to T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors and activates the transcription of cWnt target genes [1,2].

Several WNT ligands trigger β-catenin-independent pathways (ncWnt signaling pathways), including Wnt-Ca^{2+} pathway and planar cell polarity (PCP) pathway, leading to direct cell migration during embryogenesis [3-5]. WNT5A is a noncanonical ligand that can complex with Fzd2 and receptor tyrosine kinase-like orphan receptor 2 (ROR2) to activate Rac1 in a β-catenin-independent manner. Another study has indicated that Wnt5A induces hetero-oligomerization of ROR1/ROR2, which recruits and activates guanine exchange factors (GEFs), in turn activating RhoA and Rac1 [6]. In addition, WNT5A competes with WNT3A for binding to Fzd2, thereby inhibiting WNT3A-dependent LRP6 phosphorylation and β-catenin accumulation, as well as cWnt signaling in cells [7].

WNTs can be secreted into the extracellular space through secretory vesicles or extracellular vesicles. Exosomes are small extracellular vesicles (sEVs) (30–200 nm) of endocytic origin that are secreted by most cell types [25]. Exosomes have been shown to carry important signaling molecules, such as proteins, mRNAs, microRNAs, and long non-coding RNAs, and play a role in intercellular communication [8,9]. Exosomes carrying members of WNT signaling pathway, such as WNT3A, WNT5A and ABC, have been reported to induce Wnt signaling activity in recipient cells [10-15]. The aim of this study was to evaluate the signaling activity of exosomes derived from cWNT ligand WNT3A- or ncWNT ligand WNT5A-expressing HEK293T cells. Exosomes were isolated from conditioned media by size exclusion chromatography (SEC). Although WNT5A has been classified as an ncWNT ligand, isolated exosomes were enriched with ABC, leading to the activation of cWNT/β-catenin signaling in recipient cells.

2. Materials and methods

2.1. Cell culture and transfection

HEK293T cells were routinely maintained in DMEM (Life Technologies, #11960051) supplemented with 10% fetal bovine serum (FCS, Gibco, # A31606). The fetal bovine serum used for exosome purification experiments was depleted of bovine exosomes, by centrifugation at 100,000 × g for 18 h. For transient expression of WNT3A or WNT5A, the cells were transfected using NTR II (T-pro Biotechnology Inc., # JT97-N002M) the day after plating. The conditioned media and the transfected cells were collected and processed 48 hr post transfection. The vectors expressing human WNT3A (pcDNA-WNT3A) and human WNT5A (pcDNA-WNT5A) were purchased from Addgene.

2.2. Cell fractionation

Cell lysates were prepared by the addition of RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (Goal Bio Inc.) and protein phosphatase inhibitors (Goal Bio Inc.) and were incubated on ice for 10 min. After sonication, cell homogenates were subjected to centrifugation at 10,000 x g at 4°C for 10 min and soluble cell lysates were collected. The protein concentrations of cell lysates were determined using Bradford dye binding assay (Bio-Rad Inc., #5000006) with bovine serum albumin (Sigma, #A3608) as standard.

2.3. Isolation of exosomes from cell conditioned media

Conditioned media (about 30 ml) were removed 48 hr post-transfection and subjected to two consecutive centrifugation steps, 10 min at 300 x g and 30 min at 16,000 x g, to remove dead cells and cell debris, respectively. Supernatants were further concentrated to 500 µl using centrifugal filters (MWCO, 10 kDa, Ultracel, Amicon Inc., # UFC901008). Then, the concentrated supernatants were loaded onto a washed qEV size exclusion chromatography column (qEV original, Izon Science Ltd., #SP1) and 500 µl fractions were collected in 15 separate tubes as per the manufacturer's instructions. A portion of the separated fractions (30 µl) was subjected to Western blot analysis for the detection of exosomal markers (ALIX and CD81) and WNT family members. The fractions containing exosomes were pooled and further concentrated to 300 µl using Nanosep® centrifugal device (MWCO 10K,

Pall Inc., #OD010C34). Before concentration, EV-Save™ (Fujifilm Inc., #058-09261) was added to the pooled exosomal fractions to prevent adsorption of exosomes on filter membranes of centrifugal device.

2.4. Western blot

As previously described [16], 20 μg of proteins or 30 μl of exosomes were separated on 10% polyacrylamide gels and blotted onto PVDF membranes. After blocking with BlockPro™ blocking buffer (Visual Protein Biotechnology, # BP01-1L) at room temperature for 1 hr, membranes were incubated overnight with the primary antibody in blocking buffer (5% skim milk in PBS with 0.1% Tween 20) at 4°C. The membranes were then washed with PBST six times and incubated at room temperature for 1 hr with peroxidase-labeled anti-rabbit or anti-mouse IgG. Subsequently, the membranes were developed using an enhanced chemiluminescence system (T-pro LumiLong Plus chemiluminescence detection kit, T-pro Inc., # JT96-K004M) according to the manufacturer's instructions. The antibodies used in this study included rabbit monoclonal anti-ABC antibody (Cell Signaling Technology, #8814, 1:1000), rabbit polyclonal anti-WNT3A antibody (Genetex, # GTX128101, 1:1000), rabbit polyclonal anti-WNT5A antibody (Genetex, # GTX111187, 1:1000), mouse monoclonal anti-α-tubulin antibody (Novus, # NB100-690, 1:1000), rabbit polyclonal anti-ALIX antibody (Proteintech, # 12422-1-AP, 1:3000), rabbit polyclonal anti-CD63 antibody (Proteintech, # 25682-1-AP , 1:3000), mouse monoclonal anti-CD81 antibody (Santa Cruz Biotechnology, #sc-166029, 1:2000), mouse monoclonal anti-β-catenin antibody (BD Biosciences, #610153, 1:1000), mouse monoclonal anti-GSK3β antibody (BD Biosciences, #610201, 1:1000), rabbit polyclonal anti-calnexin antibody (Genetex, #GTX109669, 1:1000), goat anti-mouse antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch, # 115-035-003, 1:5000), and goat anti-rabbit antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch, # 111-035-003, 1:5000).

2.5. Nanoparticle tracking analysis (NTA)

NTA was performed at the Center for Micro/Nano Science and Technology at National Cheng Kung University, Taiwan. Aliquots of the exosomal preparations were resuspended in PBS and analyzed at similar dilutions using NanoSight LM10HS instrument (Malvern). For each sample, three videos of 60 s each were recorded at 25°C and a concentration of 20–100 particles per frame to calculate mean particle concentration and size.

2.6. SuperTopFlash (STF) reporter assay

As previously described [16], HEK 293T cells were cultured on 24-well tissue culture plates and transfected with STF plasmids (20 ng per well). *Renilla* luciferase, driven by the thymidine kinase gene promoter, was included as a transfection control (10 ng per well). A portion of exosomes was removed and filter-sterilized using 0.2 μm syringe filter (4 mm Diameter Syringe Filters, 0.2 μm, Corning Inc.). After transfection for 4 hr, sterilized exosomes were added to the cells and incubated at 37°C for an additional 24 hr. Cell lysates were prepared by the addition of passive lysis buffer included in the Dual-Luciferase Reporter Assay System kit (Promega, #E1910) and clarified by centrifugation at 10,000 x g and 4°C for 15 min. Supernatants were then subjected to activity determination of firefly luciferase and *Renilla* luciferase according to the manufacturer's protocol.

2.7. Statistical Analysis

Each experiment was performed in triplicate. Data were analyzed using unpaired Student's t-test. Results are expressed as mean ± SD. A value of $p < 0.05$ is considered statistically significant.

3. Results

3.1. Expression of WNT5A attenuates canonical WNT signaling activity in cells

Expression of WNT3A led to accumulation of ABC, a hallmark of WNT activation [17]. However, expression of WNT5A induced less cytosolic ABC than control vector (Fig. 1A). In addition, co-expression of WNT5A with STF, a reporter plasmid carrying TCF/LEF-binding sites in the upstream regulatory regions of luciferase [18], in HEK293T cells induced repressed TCF/LEF-dependent reporter activity in a dose-dependent manner (Fig. 1B). This

indicated that expression of WNT5A attenuates cWNT signaling activity in HEK293T cells, which is consistent with the findings of previous reports [19,20].

3.2. Isolated exosomes from WNT3A- or WNT5A-expressing cells contain active β-catenin

We next exploited the differences in exosomal cargo of WNT3A-expressing cells and WNT5A-expressing cells. To obtain exosomes from conditioned media of HEK293T cells, control vector-cells, WNT3A-transfected cells, and WNT5A-transfected cells were cultured in media containing exosome-free fetal bovine serum. To isolate exosomes produced by cells, conditioned media were subjected to a series of centrifugation steps and further fractionated by SEC [21-23] (Fig. 2). The fractions were analyzed for the presence of exosomal markers ALIX and CD81

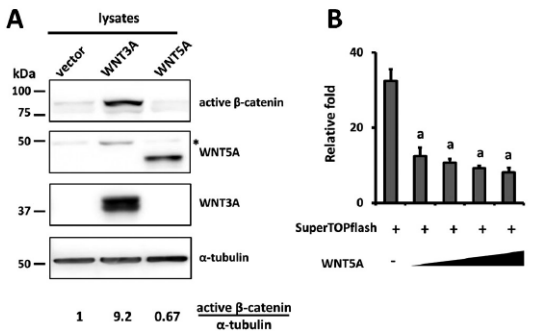

Fig. 1 Expression of WNT5A inhibits canonical Wnt signaling activity in HEK293T cells. (A) Cell lysates from control vector- or WNT3A- or WNT5A- transfected HEK293T cells were analyzed for endogenous soluble dephosphorylated β-catenin (active β-catenin).α-tubulin was used as a loading control. (B) SuperTopFlash plasmids were co-transfected with increasing amounts of WNT5A-expressing plasmids. Renilla luciferase, driven by thymidine kinase gene promoter, was included as a transfection control. [a] $p < 0.05$.

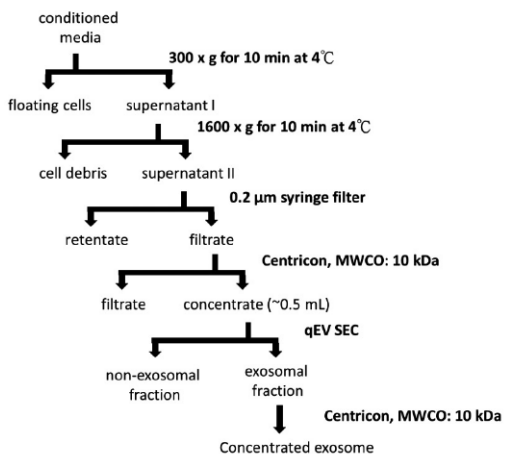

Fig. 2 The isolation scheme of exosomes from conditioned media. Supernatants of control vector- or WNT3A-transfected or WNT5A- transfected cell lines were cultured for 48 h and isolated by serial centrifugations and size exclusion chromatography.

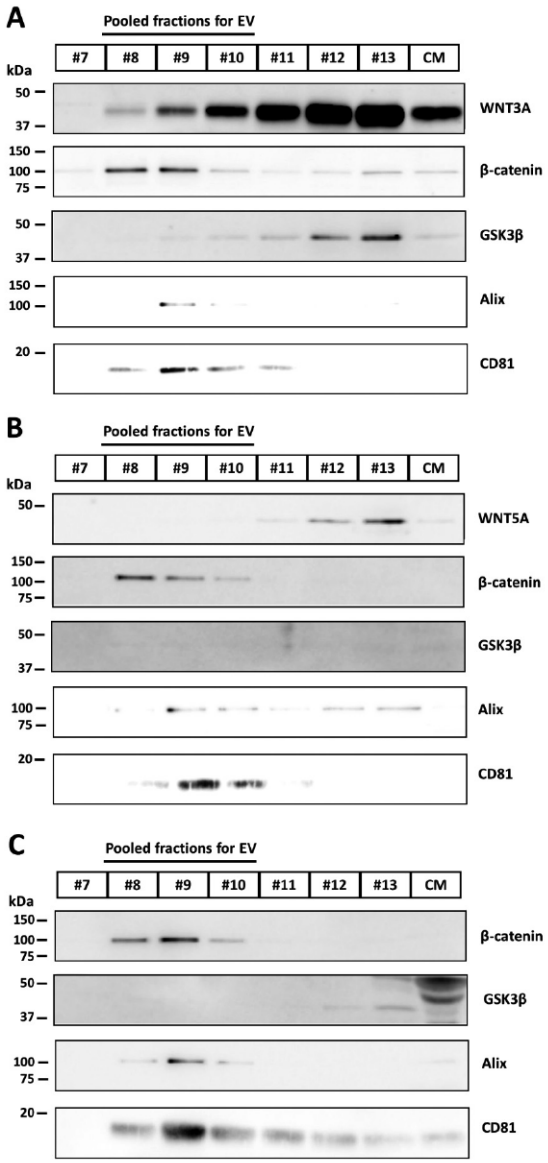

Fig. 3 Western blot of size exclusion chromatography fractionated conditioned media. Thirty µl of the selected fractions (fraction#7-13) were analyzed by Western blotting and tested for the presence of specific exosomal marker proteins (ALIX and CD81) and Wnt signaling pathway components.

[24]. In the fractions from WNT3A conditioned media, WNT3A ligand, β-catenin, and GSK3β were co-eluted with exosomal markers (Fig. 3A), indicating that these WNT family members are enriched in exosomes derived from WNT3A-expressing cells. On the contrary, only β-catenin co-migrated with exosomal markers in the fractions from control vector and WNT5A conditioned media (Fig. 3B, C). The fractions with exosomal markers were pooled and concentrated for subsequent analysis. The lack of non-exosomal markers (calnexin) in exosomal fractions (Fig. 6A) confirmed the purity of the isolated exosomes.

The isolated exosomes were further quantified by NTA (Fig. 4, 5), which revealed a typically heterogeneous exosomal population that ranged from 30 to 450 nm in diameter. The most enriched exosomes ranged from 120 to 140 nm (CTL, 127.2 + 8.3 nm; WNT3A, 123.6 + 9.0 nm; CTL, 139.1 + 11.2 nm; WNT5A, 130.9 + 9.0 nm) (Fig. 5C, D). Taken together, our isolated exosomes exhibited a high degree of purity and satisfied the size, structure, and molecular criteria of exosome vesicles [25].

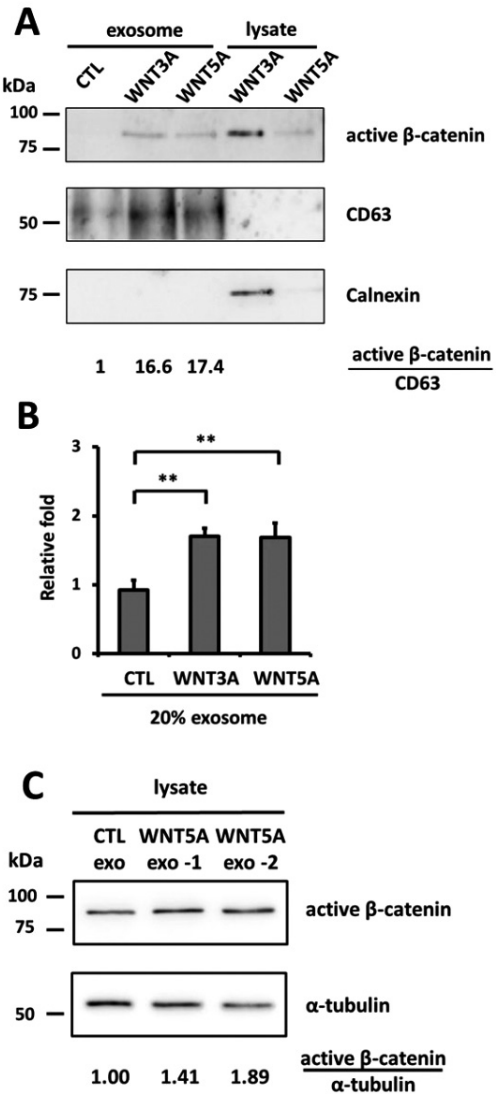

Fig. 6 Isolated exosomes from either WNT3A- or WNT5A-expressing cells enhance Wnt signaling activity in recipient cells. (A) Exosomes and cell lysates from control vector- or WNT3A- or WNT5A- transfected HEK293T cells were analyzed for ABC, CD63, and calnexin (a negative marker for exosome). (B, C) HEK293T transfected with the SuperTopFlash reporter vectors and phRG-TK were incubated with isolated exosomes. The cell lysates were subjected to determination of reporter activity (B) and Western blot for ABC (C). Renilla luciferase, driven by the thymidine kinase gene promoter (phRG-TK), was included as a transfection control. ** $p < 0.01$. α-tubulin was used as a loading control on Western blot.

3.3. Expression of either WNT3A or WNT5A in HEK293T cells induces an increase in the total number of secreted exosomes

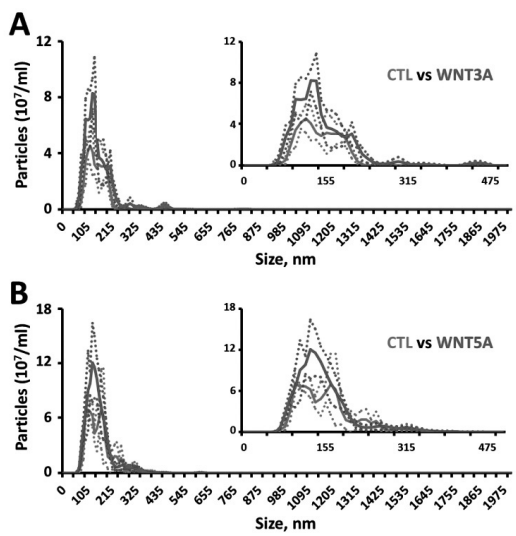

Fig. 4 Nanoparticle tracking analysis (NTA) of exosomes isolated from cell culture supernatants. (A, B) Representative results of NTA showing the size distribution of isolated exosomes. Red line indicates WNT3A or WNT5A; blue line indicates control exosomes. Solid line indicates the average, dashed line indicates the standard error. The y-axis shows the number of particles/ml and the x-axis shows the diameter of particles (unit: nm).

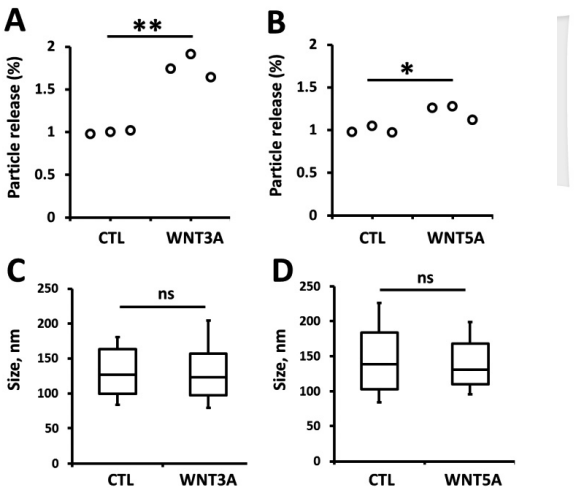

Fig. 5 (A, B) Dot plots illustrating the total numbers of particles in exosomal preparations from vector-, WNT3A- or WNT5A-transfected cells, relative to those obtained from preparations originating from control cells (taken as 100%), as determined by NTA. (E, F) Sizes of the particles (box plot), profiled by NTA, in exosomal fractions obtained from cells, expressing either empty vector (Mock), WNT3A, or WNT5A. The box plots indicate 75th percentile (top line of the box), median (middle line of the box), 25th percentile (bottom line of the box), 10th and 90th percentiles (whiskers). ns, nonsignificant; * $p < 0.05$; ** $p < 0.01$.

The results of NTA further revealed that expression of either WNT3A or WNT5A in HEK293T cells leads to an increase in the total number of exosomes secreted in comparison to the cells transfected with control vectors (Fig. 5A, B). Exosome size was not affected in comparison with control exosomes (Fig. 5C, D).

3.4. Isolated exosomes from WNT3A- or WNT5A-expressing cells contain active β-catenin and induce Wnt signaling activity in recipient cells

Previous studies have shown that bioactive WNT ligand and β–catenin are enriched in exosomes [11-13]. In the present study, both WNT3A ligand and ABC were detected in exosomes from WNT3A-transfected cells (Fig. 3A). Although WNT5A was not found in the exosomal fraction, ABC was enriched in exosomes secreted from WNT5A-transfected cells. More than 10-fold increases in ABC (normalized with CD63 markers) were detected in exosomes derived from WNT3A-expressing cells and WNT5A-expressing cells (Fig. 6A).

Since ABC was present in isolated exosomes, we next investigated whether exosomal β–catenin has any bioactive effect on cWNT signaling in recipient cells. The addition of WNT3A exosomes to HEK293T cells transfected with STF enhanced tcf1-dependent reporter activity in comparison with the addition of control exosomes. Intriguingly, addition of exosomes derived from WNT5A-expressing cells led to an increase in reporter activity in recipient cells (Fig. 6B). Both types of exosomes induced cytosolic β-catenin accumulation, a hallmark of the activated Wnt/β-catenin pathway [17] (Fig. 6C). Collectively, these results indicated that exosomes derived from WNT3A- or WNT5A-expressing cells are biologically active and enhance Wnt/β-catenin activity in HEK293T cells.

4. Discussion

In the present study, we isolated exosomes from conditioned media of cWNT3A- or ncWNT5A-expressing cells by SEC. The presence of canonical exosome protein markers, including ALIX, CD81, and CD63, and the absence of calnexin demonstrated a pure exosome preparation. Based on the results of NTA, the diameter of vesicles fell between 30 and 450 nm and that of most enriched exosomes between 120 and 140 nm. Based on biophysical characteristics and molecular features, we demonstrated the presence of exosomes with high purity in our preparation. In addition, expression of either WNT3A or WNT5A in HEK293T cells led to an increase in the total number of secreted exosomes. The detailed molecular mechanisms of the induction of secreted exosomes in WNT-producing cell line merit further investigation.

In WNT-producing cell lines, WNTs are secreted in the form of soluble factors or extracellular vesicles [11,13,26]. In the present study, WNT3A and WNT5A were expressed in HEK293T cells, a commonly used cell line for studying WNT signaling. In line with previous findings [11], WNT3A proteins were detected in both exosomal and non-exosomal fractions of conditioned media in HEK293T cells. However, WNT5A proteins were mostly detected in non-exosomal fraction. Previous studies have indicated that WNT secretion modes vary among cell lines.

In Madin-Darby canine kidney (MDCK) cells expressing WNTs, WNT3A, WNT5A, and WNT11 proteins are mostly recovered in non-exosomal fractions of conditioned media, while in Caco-2 colon cancer cells expressing WNT3A, WNT5A, or WNT11, almost all WNTs are detected in exosomal vesicles [13].

Extracellular vesicles, such as exosomes, play a role in intercellular communication, an important mechanism by which cells interact with one another. Our data showed that exosomes derived from WNT3A-producing HEK293T cells effectively activate WNT/β-catenin signaling in recipient HEK293T cells. It is interesting to note that exosomes isolated from WNT5A-producing HEK293T cells have similar effect on activation of cWNT signaling activity in recipient cells, leading to the accumulation of ABC and transcriptional activation of TCF/LEF responsive elements. This differs from exosomes derived from WNT5A-expressing L cells (mouse fibroblast cell line), which contain WNT5A proteins and have minimal effect on the activity of cWNT/β-catenin in recipient cardiac fibroblast cells [26]. It is likely that the exosomes derived from WNT5A-expressing HEK293T cells do not contain WNT5A ligands that might inhibit cWNT signaling. Additionally, the exosomes were enriched with ABC, thereby activating cWNT signaling activity in recipient cells. A previous study has indicated that exosome release of β-catenin is dependent on CD9 and CD82, since overexpression of CD9 or CD82 in HEK293T cells leads to dramatic enrichment of β-catenin in exosomes [12]. In addition, 14-3-3 family proteins have been shown to participate in the mechanism responsible for packaging of β-catenin in exosomes [10]. We observed an abundance of 14-3-3 family proteins in exosomes derived from either WNT3A- or WNT5A-expressing cells (unpublished result). A recent study has also indicated that Dvl-2, an essential component of both the cWNT and ncWnt pathways, forms a complex with members of the 14-3-3 protein family [10] . Taken together, it is reasonable that activation of cWNT (for example by WNT3A) or ncWNT signaling (for example by WNT5A) causes DVL-2 to associate with the 14-3-3 protein family, thereby packaging β-catenin in exosomes. Further studies to measure the levels of CD9, CD82, and 14-3-3 proteins in WNT3A- or WNT5A-producing cells are needed to explore the molecular mechanism underlying the exosomal packaging of ABC in cells.

In summary, we found that expression of either WNT3A or WNT5A in HEK293T cells leads to an increase in the total number of secreted exosomes. Although WNT3A is a cWNT ligand and WNT5A is an ncWNT ligand, both types of exosomes are enriched with ABC, leading to the activation of cWNT/β-catenin signaling activity in recipient cells. These results suggested that WNT3A-producing cells and WNT5A-producing cells share a common mechanism for packaging of ABC in exosomes.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Reference

1. Baarsma HA, Konigshoff M, Gosens R. The WNT signaling pathway from ligand secretion to gene transcription: molecular mechanisms and pharmacological targets. Pharmacol Ther 2013;138:66-83.
2. Liu C, Li Y, Semenov M, et al. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 2002;108:837-47.
3. Kikuchi A, Yamamoto H. Tumor formation due to abnormalities in the beta-catenin-independent pathway of Wnt signaling. Cancer Sci 2008;99:202-8.
4. Veeman MT, Axelrod JD, Moon RT. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev Cell 2003;5:367-77.
5. Kohn AD, Moon RT. Wnt and calcium signaling: beta-catenin-independent pathways. Cell Calcium 2005;38:439-46.

6. Yu J, Chen L, Cui B, et al. Wnt5a induces ROR1/ROR2 heterooligomerization to enhance leukemia chemotaxis and proliferation. J Clin Invest 2016;126:585-98.
7. Sato A, Yamamoto H, Sakane H, Koyama H, Kikuchi A. Wnt5a regulates distinct signalling pathways by binding to Frizzled2. EMBO J 2010;29:41-54.
8. Tkach M, Thery C. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. Cell 2016;164:1226-32.
9. Pegtel DM, Gould SJ. Exosomes. Annu Rev Biochem 2019;88:487-514.
10. Dovrat S, Caspi M, Zilberberg A, et al. 14-3-3 and beta-catenin are secreted on extracellular vesicles to activate the oncogenic Wnt pathway. Mol Oncol 2014;8:894-911.
11. Gross JC, Chaudhary V, Bartscherer K, Boutros M. Active Wnt proteins are secreted on exosomes. Nat Cell Biol 2012;14:1036-45.
12. Chairoungdua A, Smith DL, Pochard P, Hull M, Caplan MJ. Exosome release of beta-catenin: a novel mechanism that antagonizes Wnt signaling. J Cell Biol 2010;190:1079-91.
13. Harada T, Yamamoto H, Kishida S, et al. Wnt5b-associated exosomes promote cancer cell migration and proliferation. Cancer Sci 2017;108:42-52.
14. Menck K, Klemm F, Gross JC, Pukrop T, Wenzel D, Binder C. Induction and transport of Wnt 5a during macrophage-induced malignant invasion is mediated by two types of extracellular vesicles. Oncotarget 2013;4:2057-66.
15. Menck K, Sonmezer C, Worst TS, et al. Neutral sphingomyelinases control extracellular vesicles budding from the plasma membrane. J Extracell Vesicles 2017;6:1378056.
16. Chang CF, Hsu LS, Weng CY, et al. N-Glycosylation of Human R-Spondin 1 Is Required for Efficient Secretion and Stability but Not for Its Heparin Binding Ability. Int J Mol Sci 2016;17.
17. van de Wetering M, Sancho E, Verweij C, et al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241-50.
18. Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr Biol 2003;13:680-5.
19. Ishitani T, Kishida S, Hyodo-Miura J, et al. The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. Mol Cell Biol 2003;23:131-9.
20. Mikels AJ, Nusse R. Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. PLoS Biol 2006;4:e115.
21. Gamez-Valero A, Monguio-Tortajada M, Carreras-Planella L, Franquesa M, Beyer K, Borras FE. Size-Exclusion Chromatography-based isolation minimally alters Extracellular Vesicles' characteristics compared to precipitating agents. Sci Rep 2016;6:33641.
22. de Menezes-Neto A, Saez MJ, Lozano-Ramos I, et al. Size-exclusion chromatography as a stand-alone methodology identifies novel markers in mass spectrometry analyses of plasma-derived vesicles from healthy individuals. J Extracell Vesicles 2015;4:27378.
23. Boing AN, van der Pol E, Grootemaat AE, Coumans FA, Sturk A, Nieuwland R. Single-step isolation of extracellular vesicles by size-exclusion chromatography. J Extracell Vesicles 2014;3.
24. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol 2002;2:569-79.
25. Thery C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 2018;7:1535750.
26. Dzialo E, Rudnik M, Koning RI, et al. WNT3a and WNT5a Transported by Exosomes Activate WNT Signaling Pathways in Human Cardiac Fibroblasts. Int J Mol Sci 2019;20.