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Exosomes Derived From Hypoxic Colorectal Cancer Cells Promote Angiogenesis Through Wnt4-Induced β-Catenin Signaling in Endothelial Cells

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Cancer cell-derived exosomes have been actively released into the tumor microenvironment with pleiotropic roles in tumor growth and metastasis, including angiogenesis and immune modulation. However, the functions and underlying mechanisms of exosomes shed by colorectal cancer (CRC) cells under hypoxic conditions remain unknown. Here we found that exosomes derived from hypoxic CRC cells promoted the proliferation and migration of endothelial cells. Suppression of exosome secretion through RAB27a knockdown in CRC cells inhibited exosomal-induced proliferation and migration of endothelial cells. Furthermore, we discovered that these exosomes enriched with Wnt4 were dependent on HIF1α. Exosomal Wnt4 increased β-catenin nuclear translocation in endothelial cells. The induction of β-catenin signaling is critical for the proliferation and migration of endothelial cells, which could be abolished by the inhibitor ICG001. The in vivo animal study further revealed the tumor-promoting effects of CRC cell-derived exosomes with enhanced tumor growth and angiogenesis. Taken together, our study indicates that CRC cells promote angiogenesis through exosome-mediated Wnt/β-catenin signaling in endothelial cells under hypoxia, which might be a new mechanism in CRC development.

Key words: Colorectal cancer (CRC); Exosome; Wnt; Catenin; Angiogenesis

INTRODUCTION

Colorectal cancer (CRC), especially metastatic CRC, has attracted many researchers' attention in recent years as one of the most common causes of cancer-related deaths[1,2]. Though the development of targeted therapies, including EGFR-targeted therapy and angiogenesis-targeted therapy, has gained significant progress in patient survival, a large number of issues remain unresolved[3]. In particular, elucidating how CRC cells regulate angiogenesis under a hypoxic tumor microenvironment is crucial for effective angiogenesis-targeted therapy in metastatic CRC.

Exosomes are nanosized membrane vesicles with a diameter between 30 and 100 nm, which are derived from endosomal compartment invaginations and release dependent on RAB27[4–6]. As in other types of tumors, CRC cell-derived exosomes have important roles in tumor progression including invasion, angiogenesis, immune modulation, and distal metastasis by effectively delivering microRNAs, mRNAs, and proteins[7–10]. However, the functions and underlying molecular mechanisms of hypoxic CRC cell-derived exosomes are unknown.

Wnt/β-catenin signaling directs fundamental processes during metazoan development and can be aberrantly activated in cancer[11–13]. Wnt stimulation induces the recruitment of the scaffold protein Axin from an inhibitory destruction complex to a stimulatory signalosome[14]. Wnt4 is a member of the Wnt family with secreted signal protein that participates in carcinogenesis[15–17]. The upregulation of Wnt4 is observed in gastric cancer[18]. Wnt4 regulates the proliferation of breast cancer stem cells in response to progesterone[19].

In this study, we set out to reveal the functions of hypoxic CRC cell-derived exosomes. We found that exosomes released by hypoxic CRC cells promoted the proliferation and migration of endothelial cells. In addition, we discovered that these exosomes were enriched with Wnt4. Exosomal Wnt4 increased β-catenin nuclear translocation in endothelial cells. The induction of β-catenin signaling is critical for the proliferation and migration of endothelial cells. The in vivo animal study further revealed the tumor-promoting effects of CRC cell-derived exosomes with enhanced tumor growth and angiogenesis. Altogether, our study revealed that CRC cells promoted angiogenesis through exosome-mediated Wnt/β-catenin signaling in endothelial cells under hypoxia, which might be a novel target for CRC treatment.

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MATERIALS AND METHODS

Exosome Isolation

In order to isolate exosomes, CRC cells HT29 and HCT116 were treated with 250 μM Cocl2 for 48 h, and the supernatant was collected. We then centrifuged the supernatant twice (1,000×*g*×10 min and 3,000×*g*×30 min to deplete the cell or fragments) and then added the total exosome isolation kit (Life Technologies, Carlsbad, CA, USA) overnight, and again centrifuged for 10,000×*g*× 1 h. Exosomes were resuspended in PBS and stored at 80°C. The concentration of exosome was detected by the BCA Protein Assay.

Western Blot

To analyze the expression of exosomal marker CD63, Western blot assays were performed using the following primary antibodies: rabbit anti-human CD63 (ab59479; 1:1,000; Abcam, Cambridge, MA, USA) and mouse anti-actin (1:10,000; Millipore, Billerica, MA, USA). Briefly, tissues were lysed with RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate] containing protease inhibitors (CompleteMini; Roche); 20–30 μg of lysate samples was separated on 8%–12% SDS-PAGE gels and transferred to PVDF membranes. The membranes were incubated with primary antibodies overnight at 4°C. Incubation of the primary antibody was followed by incubation with an HRP-conjugated secondary antibody. The bound antibodies were detected using an ECL kit (PI32209; Pierce, Rockford, IL, USA).

Lentiviral Vector-Mediated HIF1α or RAB27a Knockdown

Hypoxia-inducible factor-1α (HIF1α) shRNA sequence was 5′-CAGAAATGGCCTTGTGAAA-3′. RAB27a shRNA sequence was 5′-GCTTAACGACAGCGTTC TT-3′. After 48 h, the knockdown efficiency was confirmed via quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot.

Lentiviral vectors for human shRNA carrying a green fluorescent protein (GFP) sequence were constructed by Hanyin Co. (Shanghai, P.R. China). The recombinant knockdown lentivirus and the negative control (NC) lentivirus (GFP-lentivirus; Hanyin Co.) were prepared and titered to 10^9 TU (transfection unit)/ml. To obtain the stable HIF1α knockdown cell line, cells were seeded in six-well dishes at a density of 2×10^5 cells per well. The cells were then infected with the same titer virus with 8 μg/ml polybrene on the following day. Approximately 72 h after viral infection, GFP expression was confirmed under a fluorescence microscope, and the culture medium was replaced with a select medium containing 4 μg/ml puromycin. The cells were then cultured for at least 14 days. The puromycin-resistant cell clones were isolated, amplified in medium containing 2 μg/ml puromycin for 7 to 9 days, and transferred to a medium without puromycin. The clones were designated as KD or NC cells.

CCK8 Analysis

Cell counts were determined using the cell counting kit 8 (CCK8) (Dojindo, Japan). A total of 1×10^3 GBM cells were plated into 96-well culture plates in triplicate, and cell growth was determined daily for 5 days using a tetrazolium salt-based colorimetric assay (Dojindo Molecular Technologies) according to the manufacturer's protocol. Absorbance was measured at 450 nm. Three independent experiments were performed.

Transwell Assay

Cells were then plated in the top chamber of Transwell assay inserts (Millipore) with a membrane containing 8-μm pores in 200 ml of serum-free RPMI-1640 medium. The assays were conducted in triplicate. The inserts were then placed into the bottom chamber of a 24-well plate containing RPMI-1640 with 10% FBS as a chemoattractant. After 24 h, the top layer of the insert was scrubbed with a sterile cotton swab to remove any remaining cells. The invading cells on the bottom surface were stained with 0.1% crystal violet, examined, counted, and imaged using digital microscopy. The number of cells in five random fields of each chamber was counted, and an average number of cells was calculated.

Xenograft Animal Model

US National Institutes of Health and institutional guidelines for animal welfare and experimental conduct were followed. The animal study was approved by the Institutional Animal Care and Use Committee of Shengjing Hospital. HT29 cells were implanted subcutaneously in anesthetized 6-week-old athymic nude mice with a subsequent treatment of exosomes. Tumor size and weight were monitored.

Statistical Analysis

All statistical analyses were performed using SPSS for Windows v.17.0 (SPSS, Chicago, IL, USA). All results were considered significant at two-sided value of $p<0.05$.

RESULTS

Hypoxic Colorectal Cancer Cell-Derived Exosomes Induced Proliferation and Migration of Endothelial Cells

To investigate the function of hypoxic CRC cell-derived exosomes in CRC progression, these exosomes were isolated from two different CRC cells. The morphology

of exosomes was observed under transmission electron microscopy (Fig. 1A). The diameter of the exosomes ranged from 30 to 100 nm (Fig. 1A). The results of Western blotting showed that, compared with cell lysates, the exosomes were enriched with exosomal marker CD63 (Fig. 1B), which indicated the effective isolation of exosomes.

To find out the effects of hypoxic CRC cell-derived exosomes on endothelial cells, we analyzed the proliferation ability of HUVECs treated with exosomes isolated from hypoxic CRC cells or normoxic CRC cells. The results of the CCK8 assay showed that both hypoxic CRC cell-derived exosomes significantly promoted HUVEC cell proliferation compared with the exosomes isolated in a normoxic condition (Fig. 1C and D). Furthermore, the results of the Transwell assay showed that both CRC cell-derived exosomes incubated in hypoxia enhanced HUVEC migration ability relative to those in the normoxic control (Fig. 1E and F). These results showed that CRC cell-derived exosomes in hypoxic conditions promoted proliferation and migration of endothelial cells.

Inhibition of Exosome Secretion in Colorectal Cancer Cells Reduced Proliferation and Migration of Endothelial Cells

To further identify the importance of exosomes in hypoxic CRC cells inducing proliferation and migration of endothelial cells, we knocked down RAB27a in these cells. RAB27a was effectively suppressed (Fig. 2A). As a result, the secreted exosomes were reduced compared with the negative control (Fig. 2B). CRC cells with or without RAB27a knockdown were cocultured with HUVECs. Results of the CCK8 assay showed that RAB27a knockdown significantly reduced HUVEC proliferation compared with the negative control (Fig. 2C and D). Furthermore, the results of the Transwell assay showed that CRC cells with RAB27a inhibition reduced HUVEC migration ability relative to the negative control (Fig. 2E and F). These results showed that inhibition of exosome secretion reduced CRC cells mediating proliferation and migration of endothelial cells.

Hypoxic CRC-Derived Exosomal Wnt4 Enhanced Proliferation and Migration of Endothelial Cells

Since secreted hypoxic CRC-derived exosomes enhanced proliferation and migration of endothelial cells, we screened the angiogenetic factors that were released from cells, including VEGF and Wnt family members such as Wnt1, Wnt2, Wnt3a, Wnt4, Wnt5a, Wnt6, Wnt7b, Wnt10b, and Wnt11, in hypoxic exosomes. RT-PCR results found that the mRNA levels of Wnt4 were significantly higher in hypoxic CRC cells compared with the normoxic control (Fig. 3A and B). The Western blotting results further revealed higher levels of Wnt4 protein in hypoxic exosome-incubated HUVECs (Fig. 3C). Knockdown of Wnt4 in HUVECs abrogated hypoxic exosome-mediated proliferation enhancement (Fig. 3D–F). The results of the Transwell assay showed that Wnt4 inhibition reduced HUVEC migration ability relative to the negative control (Fig. 3G and H). These results showed that hypoxic CRC-derived exosomal Wnt4 enhanced proliferation and migration of endothelial cells.

Hypoxia Stimulates Exosomal Wnt4 Upregulation in an HIF1α-Dependent Way

Considering that the upregulated exosomal Wnt4 mRNA levels were under hypoxic conditions, we further studied whether hypoxia-induced Wnt4 expression was regulated by HIF1α, the most important hypoxic response factor. We knocked down HIF1α in both CRC cells (Fig. 4A and B). RT-PCR results showed that both cellular and exosomal Wnt4 levels were decreased after HIF1α knockdown (Fig. 4C and D). These results showed that hypoxia stimulated exosomal Wnt4 upregulation, which is dependent on HIF1α.

Exosomal Wnt4-Activated β-Catenin Signaling Was Required for Proliferation and Migration of Endothelial Cells

Wnt/β-catenin signaling is one of the most important pathways in cancer. We further examined the β-catenin signaling in HUVECs treated with hypoxic exosomes. The higher nuclear translocation of β-catenin was revealed in hypoxic exosome-treated HUVECs by Western blotting compared with control (Fig. 5A). To demonstrate the functional roles of β-catenin in hypoxic CRC cell-derived exosomes, we treated HUVECs with β-catenin inhibitor ICG001 simultaneously. The CCK8 assay showed that hypoxic CRC cell-derived exosomes induced proliferation of HUVECs, which was abolished by treatment with ICG001 (Fig. 5B and C). The results of the Transwell assay showed that ICG001 inhibited hypoxic CRC cell-derived exosomes induced by HUVEC migration (Fig. 5D and E). These results suggested that Wnt4-activated β-catenin signaling was required for proliferation and migration of endothelial cells.

The Role of CRC-Derived Exosomal Wnt4 Promoted CRC Growth and Angiogenesis

To investigate the role of CRC derived-exosomal Wnt4 in CRC development, HT29 cells were injected subcutaneously in nude mice to generate tumors with a diameter of 5 mm. Hypoxic and normoxic exosomes were then injected into the center of the xenograft tumors. The tumor volume was monitored every 3 days. The hypoxic exosome treatment promoted tumor growth compared

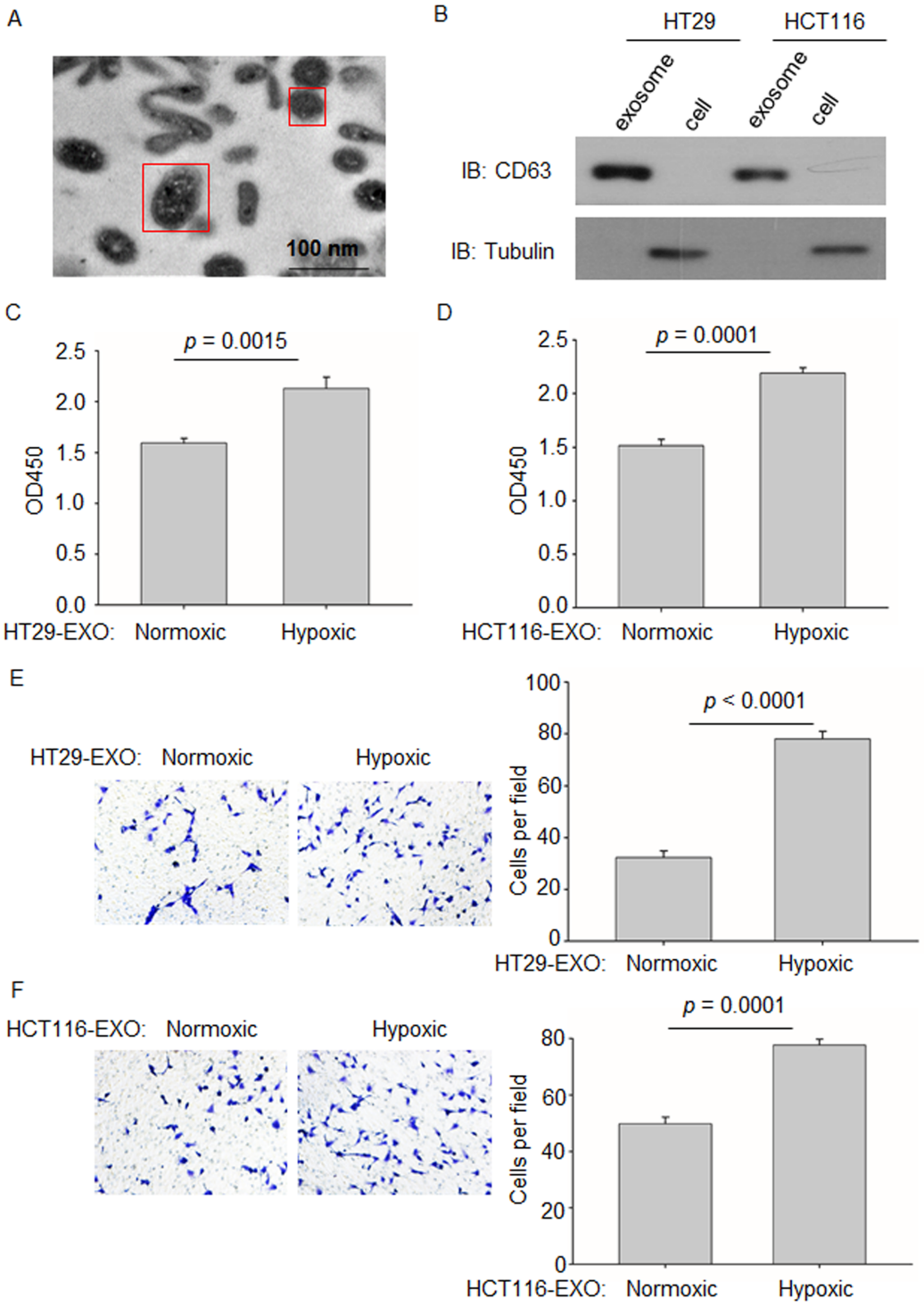


Figure 1. Hypoxic colorectal cancer cell-derived exosomes induced proliferation and migration of endothelial cells. (A) Representative electron micrograph of exosomes isolated from hypoxic CRC cells revealing the typical morphology and size (50–200 nm). Scale bar: 500 nm. (B) Western blot analysis showing the presence of CD63 and the absence of tubulin in exosomes derived from the hypoxic HT29 and HCT116 cells. (C) CCK8 analysis showed that exosomes derived from the hypoxic HT29 cells significantly induced endothelial cell proliferation compared to the normoxic control. (D) CCK8 analysis showed that exosomes derived from hypoxic HCT116 cells significantly induced endothelial cell proliferation compared to the normoxic control. (E) Transwell analysis showed that exosomes derived from hypoxic HT29 cells significantly induced endothelial cell migration compared to the normoxic control. (F) Transwell analysis showed that exosomes derived from hypoxic HCT116 cells significantly induced endothelial cell migration compared to the normoxic control.

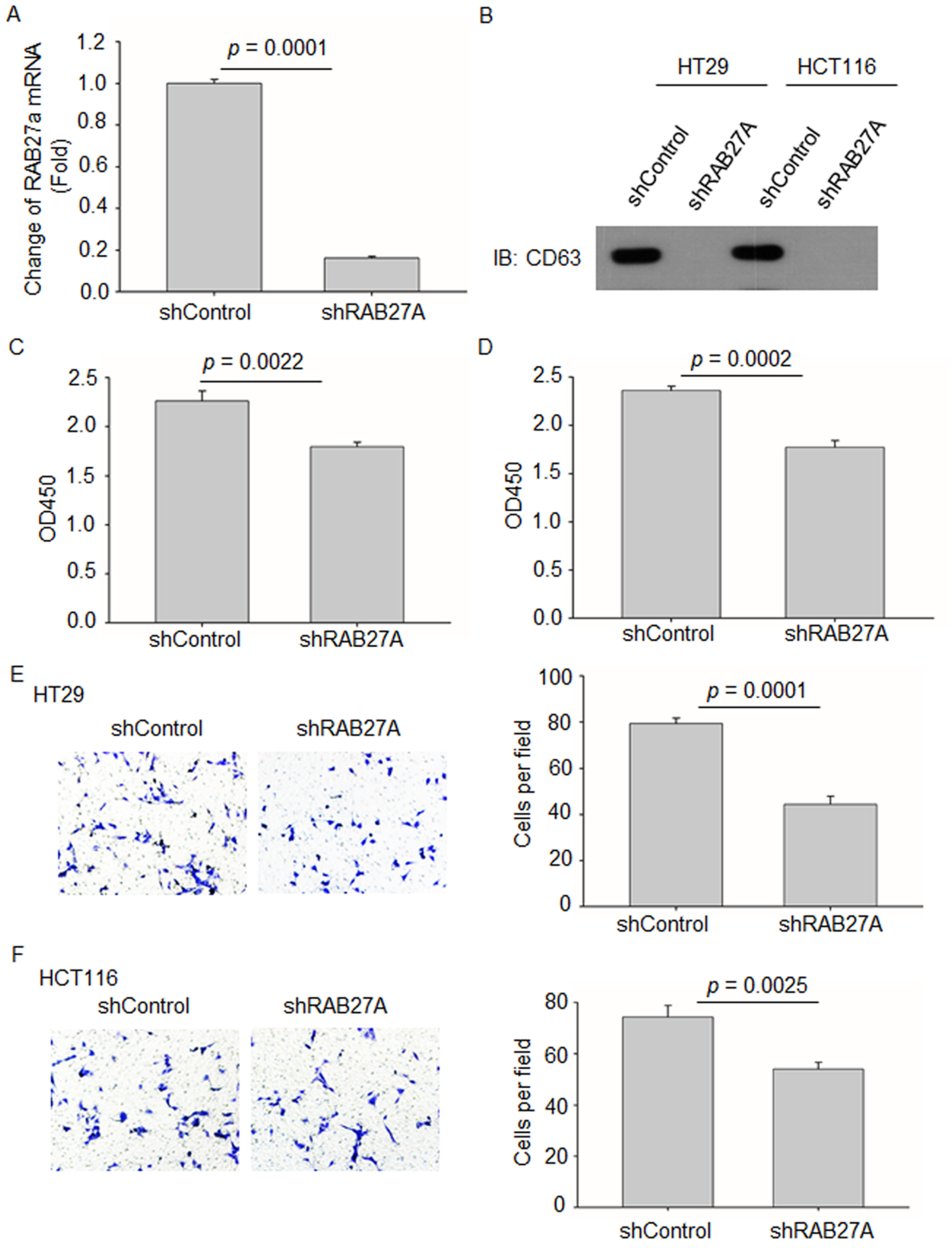
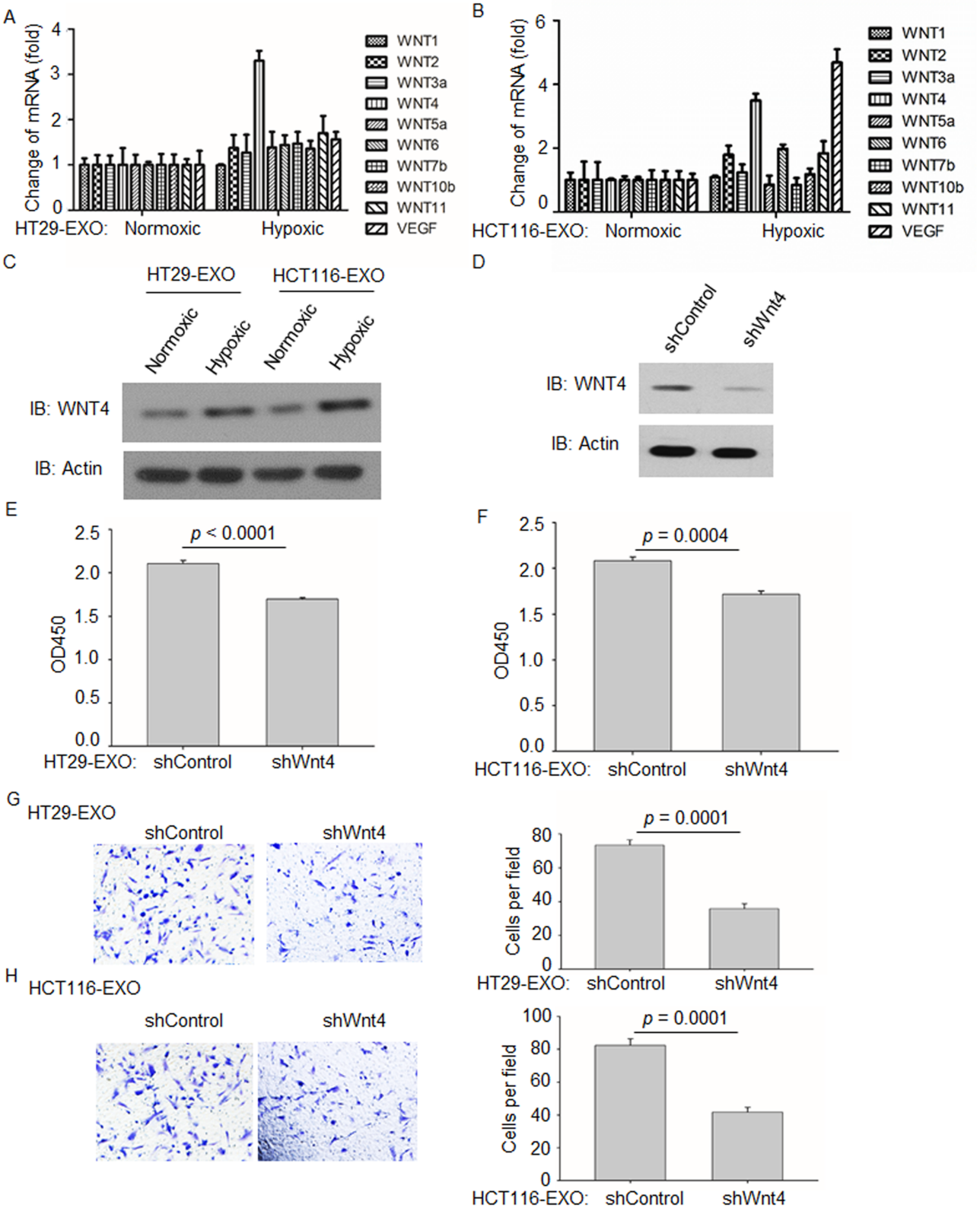


Figure 2. Inhibition of exosome secretion in hypoxic colorectal cancer cells reduced proliferation and migration of endothelial cells. (A) Real-time PCR analysis confirmed the efficient knockdown of RAB27a in HT29 and HCT116 cells. (B) Western blot analysis showing the presence of exosomal CD63 derived from hypoxic HT29 and HCT116 cells decreased after RAB27a suppression. (C) CCK8 analysis showed that exosomes derived from hypoxic HT29 cells with or without RAB27A suppression significantly induced endothelial cell proliferation compared to the normoxic control. (D) CCK8 analysis showed that exosomes derived from hypoxic HCT116 cells with or without RAB27a suppression significantly induced endothelial cell proliferation compared to the normoxic control. (E) Transwell analysis showed that exosomes derived from hypoxic HT29 cells with or without RAB27A suppression significantly induced endothelial cell migration compared to the normoxic control. (F) Transwell analysis showed that exosomes derived from hypoxic HCT116 cells with or without RAB27a suppression significantly induced endothelial cell migration compared to the normoxic control.


A
Change of mRNA (fold)
HT29-EXO: Normoxic Hypoxic
WNT1
WNT2
WNT3a
WNT4
WNT5a
WNT6
WNT7b
WNT10b
WNT11
VEGF
B
Change of mRNA (fold)
HCT116-EXO: Normoxic Hypoxic
C
HT29-EXO
HCT116-EXO
Normoxic
Hypoxic
IB: WNT4
IB: Actin
D
shControl
shWnt4
IB: WNT4
IB: Actin
E
p < 0.0001
OD450
HT29-EXO: shControl shWnt4
F
p = 0.0004
OD450
HCT116-EXO: shControl shWnt4
G
HT29-EXO
shControl
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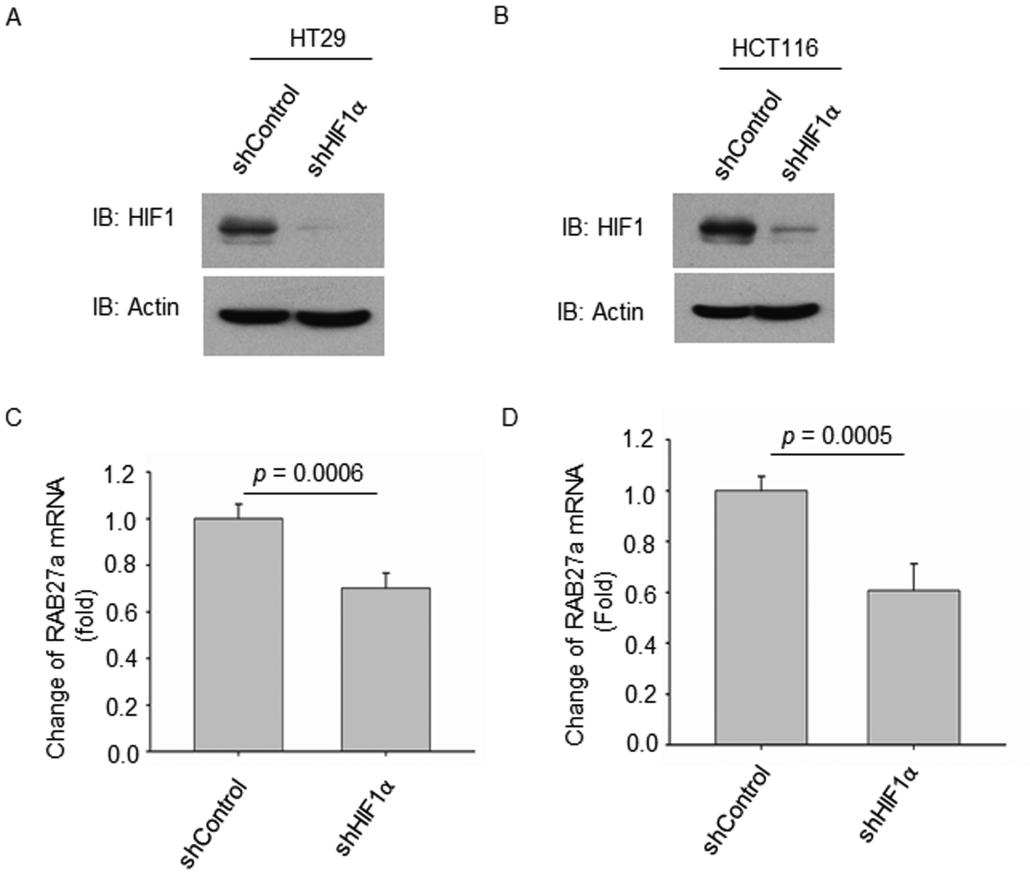

Figure 4. Hypoxia stimulated exosomal Wnt4 expression in an HIF1α-dependent way. (A) Western blot analysis showed that HIF1α expression was knocked down after being transfected with HIF1α-shRNA in HT29 cells. (B) Western blot analysis showed that HIF1α expression was knocked down after being transfected with HIF1α-shRNA in HCT116 cells. (C) Real-time PCR analysis of Wnt4 mRNAs in exosomes derived from HT29 with or without HIF1α knockdown under hypoxia. (D) Real-time PCR analysis of Wnt4 mRNAs in exosomes derived from HCT116 with or without HIF1α knockdown under hypoxia.

with normoxic exosome incubation (Fig. 6A and B). The tumor weights were higher in the hypoxic exosome-treated group (Fig. 6C). The immunohistostaining analysis of tumor tissues with vesicular marker CD31 found that the $CD31^+$ cells were increased more in the hypoxic exosome-treated group than in the normoxic exosome-treated group (Fig. 6D). These data, along with the above in vitro results, indicate that CRC cells may upregulate Wnt4 in exosomes under a hypoxic microenvironment, which induces endothelial cell proliferation and migration and thus promotes tumor growth and angiogenesis.

DISCUSSION

Recent studies have shown that exosomes mediate cell–cell communications through the transferring of RNAs and proteins, which play a significant role in a variety of

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Figure 3. Hypoxic CRC-derived exosomal Wnt4 enhanced proliferation and migration of endothelial cells. (A) Real-time PCR analysis of VEGF and Wnt family genes in exosomes derived from HT29 under hypoxia. Exosomes derived from normoxic HT29 cells served as control. (B) Real-time PCR analysis of VEGF and Wnt family genes in exosomes derived from HCT116 under hypoxia. Exosomes derived from normoxic HCT116 cells served as control. (C) Western blot analysis showing that WNT4 protein increased after being incubated with exosomes derived from hypoxic HT29 and HCT116 cells. (D) Western blot analysis showing that WNT4 expression was suppressed after being transfected with shRNA in HT29 and HCT116 cells. (E) CCK8 analysis showed that HUVECs treated with exosomes derived from hypoxic HT29 cells with Wnt4 knockdown proliferated slower than the negative control. (F) CCK8 analysis showed that HUVECs treated with exosomes derived from hypoxic HCT116 cells with Wnt4 knockdown proliferated slower than the negative control. (G) Transwell analysis showed that HUVECs treated with exosomes derived from hypoxic HT29 cells with Wnt4 knockdown migrated slower than the negative control. (H) Transwell analysis showed that HUVECs treated with exosomes derived from hypoxic HCT116 cells with Wnt4 knockdown migrated slower than the negative control.

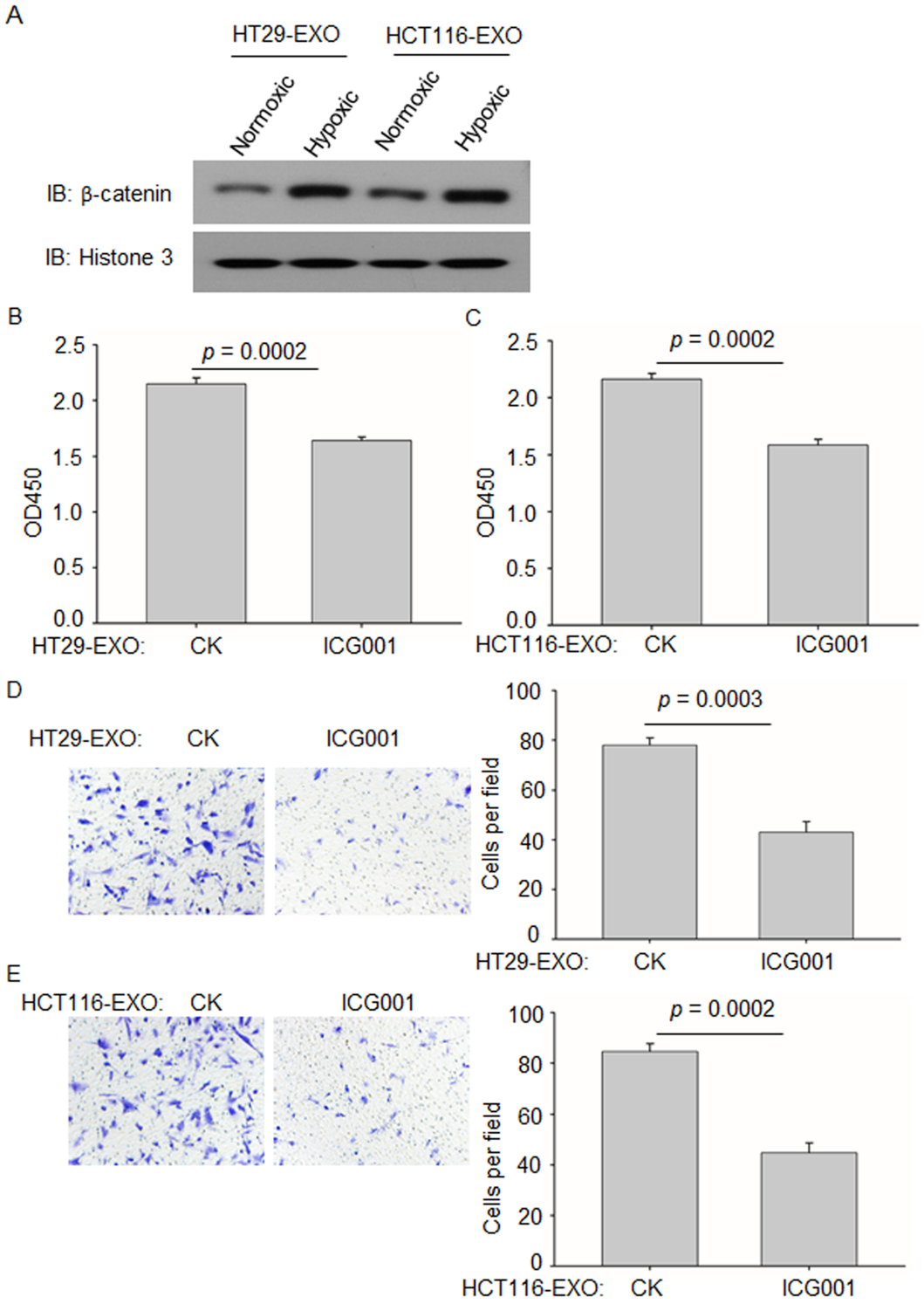

Figure 5. Exosomal Wnt4-activated β-catenin signaling is required for proliferation and migration of endothelial cells. (A) Western blot analysis showed that nuclear β-catenin was increased in HUVECs treated with exosomes derived from hypoxic HT29 and HCT116 cells. (B) CCK8 analysis showed that HUVECs treated with β-catenin signaling inhibitor ICG001 significantly abrogated the hypoxic HT29 cell-derived exosome-facilitated proliferation. (C) CCK8 analysis showed that HUVECs treated with β-catenin signaling inhibitor ICG001 significantly abrogated the hypoxic HCT116 cell-derived exosome-facilitated proliferation. (D) Transwell analysis showed that HUVECs treated with β-catenin signaling inhibitor ICG001 significantly abrogated the hypoxic HT29 cell-derived exosome-facilitated migration. (E) Transwell analysis showed that HUVECs treated with β-catenin signaling inhibitor ICG001 significantly abrogated the hypoxic HCT116 cell-derived exosome-facilitated migration.

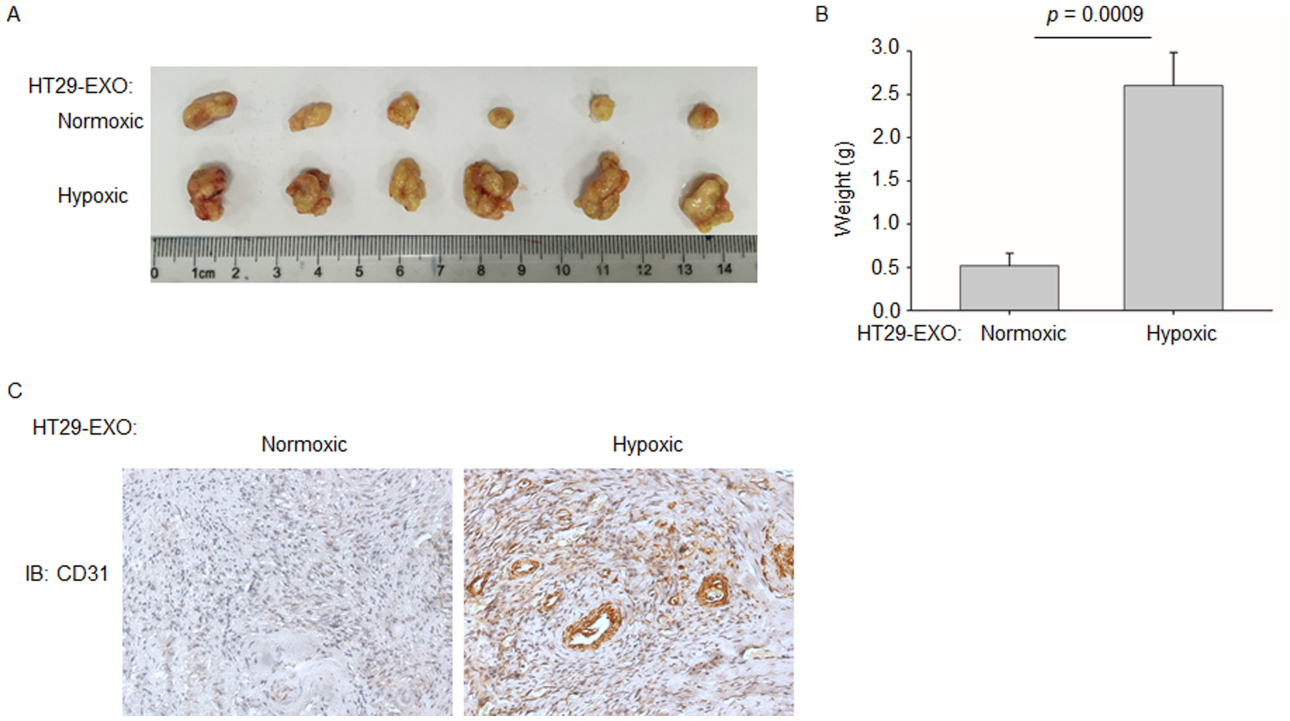

Figure 6. The role of CRC derived-exosomal Wnt4 promoted tumor growth and angiogenesis. (A) Photo of tumors inoculated with HT29 cells subcutaneously with exosome treatment. (B) Tumor weight of formed tumors inoculated with HT29 cells subcutaneously with exosome treatment. (C) Immunohistochemistry analysis of CD31 showed that angiogenesis was higher in hypoxic exosome-treated tumor. Magnification: 200×.

physiological and pathological processes, including cancer[20–23]. In this study, we investigated the function and associated pathways of hypoxic CRC cell-derived exosomes. We found that exosomes derived from hypoxic CRC cells promote angiogenesis through Wnt4-induced β-catenin signaling in endothelial cells. This is the first study, to our knowledge, revealing exosome-mediated Wnt/β-catenin signaling in endothelial cells by hypoxic CRC cells.

Hypoxia has been found to be an important driving force for tumor progression by regulating pathways involved in angiogenesis, invasion, metabolism, and genetic instability[24–27]. Though recent research found the function of exosomes in tumor progression, the cellular function and underlying mechanisms of hypoxic exosomes have not been well clarified. Exosomes derived from hypoxic oral squamous cell carcinoma cells[28], glioma cells[29], and leukemia cells[30] have been found to promote tumor progression by regulating phenotypic modulation of endothelial cells or normoxic tumor cells. Here we show that exosomes derived from hypoxic CRC cells promoted the proliferation and migration of endothelial cells. Suppression of exosome secretion through RAB27A knockdown in CRC cells inhibited exosome-induced proliferation and migration of endothelial cells. Consistent with previous reports, our results suggest that exosomes are important transducers between cells in the tumor microenvironment.

Exosomes are known to contain mRNAs, noncoding RNAs, and proteins. The horizontal transfer of mRNAs has been found in cancer[5,9]. In this study, we found that Wnt4 mRNA was transferred from hypoxic CRC cells through exosomes. Accumulating evidence revealed the importance of the Wnt signaling pathway in development and in diseases like cancer. Gross et al. demonstrated that Wnts are transported through endosomal compartments onto exosomes[30]. Menck et al. found that extracellular vesicles were involved in Wnt5a transportation during the process of macrophage-mediated invasion[31]. Zhang et al. showed that exosomes derived from huMSC contain Wnt4 proteins that prompt wound healing[32]. In this study, we found that Wnt4 mRNAs were enriched in exosomes instead of Wnt4 protein. This might be another way of exosome-mediated Wnt transfer. Moreover, the exosomal Wnt4s were upregulated by HIF1α. Circulating exosomes have been found to be influenced by HIF1α and HIF2α expression.

Wnt/catenin is a classic pathway in cancer[13]. Without Wnt signaling, β-catenin is degraded in the cytoplasm. With Wnt signaling, β-catenin is accumulated in the cytoplasm and translocated into the nucleus to function as a transcriptional cofactor[14]. We therefore examined

β-catenin signaling in endothelial cells. We revealed that hypoxic exosomes activated β-catenin signaling in endothelial cells. This might be partially mediated by Wnt4. As the exosomal components are very complicated, it requires further investigation to find out whether there are more molecules enhancing the activity of Wnt/β-catenin signaling in hypoxic CRC cell-derived exosomes.

In summary, we find the tumor-prompted function of hypoxic CRC cell-derived exosomes by delivering Wnt4 mRNAs to activate β-catenin in endothelial cells and to facilitate endothelial cell proliferation and migration. Our findings provide new implications for the cancer therapeutic strategy of exosome inhibition.

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