Combined Analysis of Surface Protein Profile and microRNA Expression Profile of Exosomes Derived from Brain Microvascular Endothelial Cells in Early Cerebral Ischemia

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ABSTRACT: Endothelial cell damage is an important pathological basis for the deterioration of acute ischemia stroke. Our previous studies have been exploring the mechanism of blood–brain barrier (BBB) endothelial cell injury in the early stage of cerebral ischemia. Exosomes act as an important intercellular player in neurovascular communication. However, the characteristic of exosomes derived from BBB endothelial cells in early ischemic stroke is poorly understood. We exposed cultured brain microvascular endothelial cells (bEnd.3) to 3 h oxygen glucose deprivation (OGD) to mimic early cerebral ischemia in vitro and compared miRome and surface protein contents of exosomes derived from bEnd.3 cells by miRNA sequencing and the proximity barcoding assay (PBA). A total of 346 differentially miRNA (159 upregulated and 187 downregulated) were identified via miRNA-Seq in bEnd.3 cells after exposure to OGD for 3 h. Moreover, Gene Ontology (GO) and KEGG pathway analyses showed that cell proliferation- and angiogenesis-associated miRNAs were significantly affected. The abnormal changes in top eight miRNAs were further verified by a quantitative polymerase chain reaction (qPCR). PBA experiments showed that the numbers of exosomes carrying the following proteins increased significantly under ischemia, including bFGF, CD146, EPHA2, ABCB5, and ITGB2. These proteins were related to angiogenesis, cell proliferation, and cell inflammation. The network analysis combining PBA data with miRNA-Seq data showed that 79 miRNAs were related to 24 membrane proteins and predicted that there were surface proteins associated with a variety of miRNA molecules, such as ITGA9, XIAP, ADAM1, ITGA2, ITGA3, PDPN, and ITGB1. Meanwhile, there were miRNAs related to various surface proteins including miR-410-3p, miR-378b, and miR-1960. Taken together, our data demonstrated for the first time the changes of exosomal miRNAs and surface protein profiles derived from ischemic microvascular endothelial cells, which may provide new therapeutic targets for BBB protection in ischemic stroke.

1. INTRODUCTION
Acute ischemic stroke is a serious threat to human health with high morbidity and high mortality.1 Tissue-type plasminogen activator (tPA) is the only approved drug for ischemic stroke by the US Food and Drug Administration (FDA). Severe cerebral edema and dangerous cerebral hemorrhage are the two potentially fatal neurovascular complications in tPA thrombolytic therapy, of which blood–brain barrier (BBB) injury is the common pathological basis.2 Brain capillary endothelial cells are one of the key structural components of the BBB, which could not only maintain the normal barrier function of the BBB but also sense ischemic injury.3 Dysfunction of the BBB endothelial cells has been extensively studied, which leads to the high permeability of the BBB, vasogenic edema, and vasospasm and thus aggravates ischemic brain damage.2 However, how the BBB endothelial cells sense ischemic injury and interact with surrounding cells consisting of the neurovascular unit, such as pericytes, astrocytes, and neurons, remains to be explored.

Exosomes are small membrane-coated extracellular vesicles with a diameter of 30–100 nm secreted by a variety of cells. They contain various molecular constituents including protein and RNAs from maternal cells and can act as a medium bridging intercellular communication.4 Accumulating evidence has shown that exosomes can provide a novel way to alleviate ischemic brain damage through improving angiogenesis,
inhibiting cell apoptosis, and decreasing inflammation. Exosomes derived from both neuronal and non-neuronal cells could improve neuron growth and promote axon regeneration of injured neurons. Neural stem cell-derived exosomes can improve nerve function damage and reduce brain edema after stroke. Astrocyte-derived exosomes inhibit neuronal apoptosis and glial scar formation and improve nerve plasticity and nerve function. In previous studies, ischemia-induced intercellular exosome communication is mostly concentrated on neurons and astrocytes; however, there are few reports on the characteristic profile of exosomes in the brain microvascular endothelial cells after early ischemia and exosome-mediated signal transmission between endothelial cells and other cells in the brain.

MiRNAs are emerging as key players in the pathogenesis of stroke and have been implicated in the regulation of cell death, cell metabolism, inflammatory, and angiogenic processes. Growing therapeutic potential of specific miRNAs, such as miR-15a/16-1, miR-126, miR-223, and miR-124, has been identified in the treatment of stroke. Exosome surface membranes proteins derived from parent cells reflect the physiological, pathological, and functional status of the cells from which they are derived and can be used as disease biomarkers in the future. However, these exosome surface protein markers may not be detectable because of their low abundance. Recently, Wu et al. reported a new single exosome analysis technique with high sensitivity and good specificity that can identify the surface protein composition of a single exosome through antibody DNA conjugates and next-generation sequencing and thus expand the heterogeneity research of exosomes.

In this study, we used in vitro oxygen glucose deprivation (OGD) models to mimic ischemia and investigated the contents in miRNAs and membrane surface proteins associated with exosomes purified from conditioned media (CM) of ischemic bEnd.3 cells using the miRNA sequence and proximity barcoding assay (PBA), respectively, and analyzed the correlation between miRNA and surface proteins. This is a study to identify the characteristics of exosomes derived from brain microvascular endothelial cells in early ischemia, moreover, to conduct a preliminary integrative analysis between exosome miRNA and membrane surface proteins in the ischemic condition.

2. RESULTS

2.1. Characterization of Exosomes Derived from Mice Brain Microvascular Endothelial Cells. To determine the properties of brain microvascular endothelial cell-derived exosomes under the ischemic condition, we assessed the characterization of exosomes isolated from bEnd.3 cells exposed to 3 h OGD. We used ultracentrifugation or ExoQuick-TC total exosomes kits to isolate exosomes from cell media of bEnd.3 cells. Exosomes were identified by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and western blotting. First, we demonstrated that bEnd.3 cells generated exosome-enriched particles with a mean diameter of 118.4 ± 9.4 nm in the control group versus 132.6 ± 10.1 nm in the OGD group (Figure 1A) using a dynamic light scattering analysis system.
(NTA-based exosome detection system). The TEM image showed that exosomes were present as small membrane vesicles with the typical nanoparticle size range (Figure 1B). In addition, the exosomes were found to express exosomal markers including CD9, CD63, and Alix by western blotting (Figure 1C). These results indicated that the particles isolated from CM of bEnd.3 cells were verified to be exosomes.

2.2. Quantitative Exosome miRNA Profiling. To study the effect of exposure to OGD on the noncoding RNA profile of exosomes derived from bEnd.3 cells, we conducted NGS experiments with triplicate biological replicates extracted from OGD-treated bEnd.3 cells and control cells. The extracted RNAs sized between 17 and 70 nt were used to construct small RNA cDNA libraries. The libraries were sequenced on the Illumina HiSeq 2500 platform. By comparing the filtered reads...
with the authoritative miRNA/rRNA/snRNA/snoRNA database, we obtained functional categorization profiling of the miRNA sequences, and the miRNAs of the control group and the OGD group were 7.33 ± 2.30 and 6.00 ± 1.00%, respectively (Figure 2A). The majority of reads ≥19 nt corresponded to mature miRNAs. The obtained miRNA sequences were compared with publicly available data on the miRDeep2 and miRBase V21 database. For each miRNA sequence-based profile, the expression level of miRNAs was estimated by the number of sequence reads. After quartile normalization, we identified 159 upregulated and 187 downregulated miRNAs (log2(Fold Change)) ≥ 1 and P < 0.05 (Figure 2B,C). The top ten significantly differentially expressed miRNAs between control and OGD groups are listed in Table 1. Consequently, the target gene prediction and enrichment analysis of differential miRNAs between control bEnd.3-derived and OGD bEnd.3-derived exosomes were performed. To characterize the predominant pathways, KEGG pathway enrichment analysis was executed to compare the specific miRNA targets to the whole reference gene background. Thirty signal pathways showing the most statistical difference were selected in our KEGG analysis data (Figure 2D). Among these 30 pathways, metabolic pathways, PI3K-Akt signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, Wnt signaling pathway, focal adhesion, mammalian target of rapamycin (mTOR), forkhead box O (FoxO), endocytosis, calcium signaling pathway, and protein cyclic guanosine monophosphate-kinase G (cGMP-PKG) signaling pathways may contribute to endothelial cell survival, cell metabolism, and exosomal transportation. Next, we used the GO approach to analyze the enrichment of candidate target genes. The top ten significant terms of three GO categories, including biological process, cellular component, and molecular function, were presented (Figure 3A). Based on the correlation between miRNA and mRNA, we performed coexpression network analysis to screen the important miRNAs involved in endothelial cells injury induced by OGD. The result of network analysis indicated that miRNAs with high degrees were mmu-miR-379-5p, mmu-miR-122-5p, mmu-miR-494-3p, mmu-miR-412-5p, mmu-miR-369-3p, mmu-miR-409-3p, mmu-miR-214-3p, and mmu-miR-127-3p (Figure 3B). These top eight miRNAs were all downregulated in bEnd.3 cells subjected to OGD treatment, and of note, mmu-miR-412-5p and mmu-miR-379-5p were located in the core position of the network. Moreover, we found that mmu-miR-412-5p and mmu-miR-379-5p had a common target gene Slco3a1, and mmu-miR-494-3p and mmu-miR-369-3p had a common target gene Elod1.

To validate the inspection result, we performed a quantitative real-time polymerase chain reaction (qRT-PCR), and the data showed that these miRNAs were indeed downregulated in the OGD group compared with the control group (Figure 4). These results indicated that the miRNAs enriched in the exosomes of brain microvascular endothelial cells may participate in the modulation of cell survival, proliferation, and angiogenesis under ischemic conditions.

### 2.3. Profiling of Surface Proteins of Exosomes by PBA

Special membrane proteins on the surface of exosomes can be used as specific markers for the separation of exosomes; however, their potential significance as a disease biomarker is ignorable in view of the limitations of single exosomal feature detection. Therefore, we applied PBA to investigate the profiling of cell surface proteins of bEnd.3 cell-derived exosomes and examined whether exosomes from OGD-treated cells have distinct surface protein patterns compared with nons ischemic cells. To test this, we performed dimension reduction of individual exosomes from two groups based on their surface protein compositions using T-distributed Stochastic Neighbor Embedding (t-SNE) analysis. The results showed that OGD treatment significantly changed the distribution of exosomal surface proteins compared with the nons ischemic control group (Figure 5A). As shown in Figure 5B, there were 11751 exosomes expressing only one surface protein in the control group, while there were 25820 such exosomes (∼1.2-fold increase) in the OGD group. We also compared the numbers of the exosomes expressing two surface proteins and three surface proteins between the OGD-treated group and the control group, and the results are shown in Figure 5B. There were 6518 exosomes expressing two proteins and 858 exosomes expressing three proteins recorded in the OGD-treated group compared with 1872 two-protein exosomes and 223 three-protein exosomes recorded in the control group. These data indicated that OGD significantly increased the number of exosomes secreted by bEND3 cells. By comparing the surface protein profiles of the two groups, the pyramid diagrams of the U1 pattern (one-protein exosome), U2 pattern (two-protein exosome), and U3 pattern (three-protein exosome) are presented, as shown in Figure 5C–E. For one-protein exosomes, significant differences were observed in 21 proteins between the control group and OGD group, including UPA, TROP2, SURVIVIN, N-CADHERIN, MUC4, MUC1, MCP-1, LMP1, ITGB1, ITGB2, ITGAX, ITGAM, ITGAE, ITGA2B, ITGA2, IL-8, EGF, CD146, ANNEXIN, ALDH1, and ABCB5 (Figure 5C). As shown in Figure 5D, there were 28 combinations in the U2 pattern, for which the numbers of two-protein exosomes in the OGD group were significantly higher than those in the control group. There were five combinations in the U3 pattern showing significant difference between the OGD group and the control group, including CD146, FGF BASIC, ITGB2 (P = 0.01952); CD146, FGF BASIC, EPHA2 (P = 0.02313); CD146, CD340, CD166 (P = 0.03024); ABCB5, FGF BASIC, ITGB2 (P = 0.03024); and ABCB5, FGF BASIC, EPHA2 (P = 0.00011) (Figure 5E). Of note, the exosomes with more surface proteins identified were better distinguished, and these differential surface proteins were related to cell proliferation and angiogenesis, which might act as exosomal markers for endothelial cells challenged by ischemia.

### Table 1. Top Ten Significantly Differentially Expressed miRNAs between bEnd.3-Derived and OGD bEnd.3-Derived Exosomes

| miRNA_ID   | up/down | log 2 (fold change) | P-value   |
|------------|---------|---------------------|-----------|
| mmu-miR-412-5p | down    | −12.5054            | 1.44 × 10⁻⁴⁵ |
| mmu-miR-122-5p | down    | −9.1688             | 2.63 × 10⁻³⁷ |
| mmu-miR-369-3p | down    | −10.1621            | 8.20 × 10⁻⁵¹ |
| mmu-miR-127-3p | down    | −7.5257             | 4.96 × 10⁻⁴⁴ |
| mmu-miR-409-3p | down    | −8.4086             | 1.33 × 10⁻⁴³ |
| mmu-miR-379-5p | down    | −9.9317             | 6.07 × 10⁻⁴⁵ |
| mmu-miR-494-3p | down    | −9.4927             | 2.12 × 10⁻⁴⁰ |
| mmu-miR-214-3p | down    | −14.8316            | 4.00 × 10⁻⁴⁰ |
| mmu-miR-299b-3p | down  | −14.7051            | 1.43 × 10⁻³⁸ |
| mmu-miR-8095   | down    | −14.8813            | 9.00 × 10⁻³⁸ |
Figure 3. Gene Ontology and miRNA regulatory network analysis. (A) GO enrichment analysis of predicted targets genes to the differentially expressed miRNAs in bEnd.3 cells after OGD treatment. Green dots indicated biological process, red dots indicated cellular component, and blue dots indicated molecular function. (B) Interaction network of top eight miRNAs and target genes involved in ischemia-induced endothelial cells injury.
complications. Our previous study has shown that injury of BBB endothelial cells is a major cause of postischemic reperfusion-associated hemorrhagic reperfusion injury.20 However, the endothelial cell injury plays an important role in BBB dysfunction in the early stage of ischemia.20 In this study, we performed the first investigation of mapping the miRNA expression profile and surface protein profile of the exosomes derived from ischemic brain microvascular endothelial cells using integrated miRNome and surface membrane proteomic analyses. The major findings are as follows: (1) OGD promotes the release of exosomes in endothelial cells, and significant differences of exosomal miRNome and surface proteomics are observed between OGD-treated cells and control cells; (2) the exosomal miRNAs and surface proteins identified in OGD-treated endothelial cells are closely associated with angiogenesis, cell proliferation, and cell inflammation; and (3) there is a clear correlation between miRNAs and surface proteins identified in the exosomes isolated from ischemic brain microvascular endothelial cells. These data indicate that the exosomal miRNAs and surface proteins may serve as new biomarkers for brain microvascular endothelial cell damage in ischemic stroke.

The exosomes are involved in the transmission of information between BBB cell components and the regulation of BBB integrity by delivering functional proteins, mRNA transcription products, and miRNAs to recipient cells.21 There is evidence that exosomes can act as sensors for neuroinflammation.22 Gao et al. found that endothelial colony-forming cell (ECFC)-generated exosomes played a positive role in BBB integrity in mice after traumatic brain injury.23 Exosomes are also secreted and transferred between glia and neurons in the nervous system, exerting extensive effects on nerve activation, development, and regeneration.24 More recently, miR-132 was reported to be transported from neurons to cerebral vascular endothelial cells by releasing exosomes and regulating the integrity of cerebral blood vessels.25 In this study, we used the nanoparticle tracking method to investigate whether OGD affects exosome size and concentration. Although we did not find significant differences in the size and concentration of exosomes between control and OGD-treated endothelial cells, the numbers of exosomes were significantly increased after OGD treatment, which is consistent with a previous report in which the vascular endothelial cells showed increased exosome secretion in response to hypoxia.26 Using quantitative approaches to analyze exosomal miRNA contents, we further demonstrated that the exosomes derived from OGD-treated bEnd.3 cells

Figure 4. qRT-PCR validation of exosomal miRNA sequencing data. Endothelial cells were treated with OGD for 3 h and total RNA was extracted for qRT-PCR to analyze the expression of miR-379-5p (A), miR-122-5p (B), miR-494-3p (C), miR-412-5p (D), miR-369-3p (E), miR-409-3p (F), miR-214-3p (G), and miR-127-3p (H). The results were normalized to the U6 values for each sample, and fold changes were shown as means ± standard deviation (SD) in three independent experiments (*P < 0.05). *P < 0.05 from one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test for pairwise comparisons.

2.4. Correlation between Exosomes miRNAs and Surface Proteins. The exosomes have been shown to regulate cell growth, cell migration, and cell-to-cell communication through the cargos they carry including proteins, mRNA, miRNA, and other signal molecules; however, it remains unknown whether there is a potential correlation between miRNAs and surface proteins in exosomes. To investigate the relationship between miRNAs and membrane proteins of exosomes derived from OGD-treated endothelial cells, we performed integrated analyses. As shown in Figure 6, there were 79 miRNAs closely related to 24 membrane proteins. Combining with miRNA sequencing data analysis (especially the top eight miRNAs), we found that miR-379-5p was associated with ITGAX and miR-494-3p and miR-409-3p were related to ADAM10. There were several other surface proteins associated with a variety of miRNA molecules, such as ITGA9, XIAP, ADAM1, ITGA2, ITGA3, PDPN, and ITGB1 in OGD-treated endothelial cells. Meanwhile, several miRNAs were relevant to surface proteins including miR-410-3p, miR-378b, and miR-1960. These results clearly indicated that OGD treatment enhanced the correlation between miRNA expression and surface proteins of exosomes derived from brain microvascular cells, in which miRNAs might regulate the distribution of exosomal surface proteins, while the surface proteins possibly contributed to the miRNA sorting process.

3. DISCUSSION

The BBB is composed of endothelial cells, pericytes, astrocytes, tight junctions, and the basement membrane.18 Accumulating evidence shows that injury of BBB endothelial cells is a major cause of postischemic reperfusion-associated hemorrhagic complications.19 Our previous study has shown that endothelial cell injury plays an important role in BBB dysfunction in the early stage of ischemia.20 However, the exosomes derived from endothelial cells involved in the cellular response to OGD have not been fully addressed. In this study, we performed the first investigation of mapping the miRNA expression profile and surface protein profile of the exosomes derived from ischemic brain microvascular endothelial cells...
Figure 5. Surface protein profiling of individual exosomes derived from bEnd.3 cells after OGD. (A) t-SNE plots of all samples split and autoclustered by flowSOM (each sample down-sampled to the size of 10,000), which visualized the marker intensity of individual exosomes with one identified protein type. (B) Comparison of exosome numbers in two groups. U1, exosomes with one protein; U2, exosomes with two proteins; U3, exosomes with three proteins. Pyramid diagram showing the numbers of one-protein exosomes (C), two-protein exosomes (D), and three-protein exosomes (E). Red columns indicate the control group, and blue columns indicate the OGD group. Filter out records with 0 exosome. Representative P values were marked in the right column.
were clearly distinguishable from the exosomes isolated from control cells. We further analyzed the eight miRNAs that were most significantly affected by OGD treatment and found that these miRNAs regulate a variety of functions, including cell proliferation and migration (miRNA-122-5p and miR-409-3p), vascular structure formation (miR-412-5p), nerve fiber regeneration (miR-379-5p), cell apoptosis (miR-494-3p), and cell adhesion (miR-127-3p).

The exosomes from different sources can be distinguished by identifying their surface protein combinations. Haqqani et al. found that the exosomes derived from human brain microvascular endothelial cells contained at least 1179 proteins, including 60 marker proteins, such as Alix, TSG101, CD81, and CD9. Currently, the exosomal proteins are analyzed as a whole, while few studies have been carried out to analyze the characteristics of the surface proteins in a single exosome, in which the heterogeneity of exosomes would be largely ignored. In our study, the type and number of surface proteins on every single exosome were resolved by the PBA technique, which helped to classify exosomes into different subgroups. By comparing the surface protein profiles identified in the exosomes between control and OGD-exposed exosomes, we observed significant increases in a bunch of surface proteins in the exosomes derived from OGD-treated endothelial cells, such as FGFBASIC, CD146, EPHA2, ABCB5, and ITGB2. These proteins have been shown to promote angiogenesis, such as FGFBASIC, CD146, SURVIVIN, TROP2, and EGF, cell proliferation, such as EPHA2 and N-CADHERIN, or inflammation regulation, such as MCP-1 and IL-8. Our data that OGD treatment induces an increase in the numbers of exosomes carrying proangiogenic surface proteins, such as CD146, FGFBASIC, and ITGB2 or CD146, FGFBASIC, and EPHA2 suggest that OGD may activate the endothelial cells a compensatory mechanism to promote the establishment of microcirculation through cell-to-cell communication in endothelial cells in response to ischemic stimuli. Our data also raise an important possibility that the specific surface protein combinations identified in exosomes may serve as biomarkers for evaluating the ischemic status of brain microvascular endothelial cells.

Emerging evidence has shown that the miRNAs are not randomly integrated into exosomes but rather through a sorting mechanism to guide specific miRNAs into exosomes. The sorting mechanism for exosomal miRNA is associated with proteins that act as an assembly protein to participate in exosomal miRNA composition, such as AGO2 and GW182. In other words, there are certain proteins that deliberatively control the sorting process of exosomal miRNA. Here, our data show that OGD enhances the association of a variety of miRNA molecules with surface proteins identified in the exosomes derived brain endothelial cells, such as miR-410-3p (associated with ITGA2 and ADAM1), miR-378b (associated with ITGA2 and CD36), and miR-1960 (associated with ITGB8 and ITGA9). However, in view of the limitations of the PBA technique, which can only be qualitative but not...
quantitative, it is uncertain that the presence of membrane surface proteins might promote the aggregation of miRNAs or it needs to reach a certain expression threshold to achieve this effect, which needs to be further investigated in future studies. In turn, it also remains to be determined whether the exosomal miRNAs contribute to the localization of specific proteins to the exosomal membrane in future studies.

4. CONCLUSIONS

In conclusion, our present study has demonstrated that ischemia in vitro promotes the release of exosomes and the changes of exosomal miRNA and surface protein profiles in brain microvascular endothelial cells, and these changes may represent a compensatory mechanism to promote angiogenesis, cell proliferation, and inflammatory regulation. Moreover, there is a clear correlation between these exosomal miRNAs and surface proteins. Screening and identifying the types and characteristics of the exosomes secreted by ischemic endothelial cells may provide a new way to evaluate the functional status of cerebrovascular endothelial cells in ischemic stroke.

5. EXPERIMENTAL SECTION

5.1. Cell Culture. Mouse brain microvascular endothelial cells (bEnd.3, American Type Culture Collection, Manassas, VA) were cultured in 75 cm² flasks. The bEnd.3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin at 37 °C in a humidified incubator gassed with 5% CO₂ /95% air. The cells were subcultured and allowed to grow to confluence before exposure to OGD treatment.

5.2. Oxygen Glucose Deprivation Model. To mimic the ischemic condition in vitro, the bEnd.3 cells were exposed to OGD as described previously. In brief, bEnd.3 cells were seeded on Nunc cell culture dishes, then incubated in glucose-free medium (Gibco, Carlsbad, CA), and subsequently moved into a humidified air-tight chamber (Billups-Rothenberg Inc., Del Mar, CA) equilibrated with 95% N₂ and 5% CO₂ for 15 min. The chamber was then sealed and kept at 37 °C for 2 h. The oxygen concentration was controlled below 0.2% by an oxygen analyzer (Sable Systems, Las Vegas, NV). The medium of OGD treatment was FBS-free, and the medium of the control group was exosome-depleted FBS (Ohio Technology Corp., Ltd., Shanghai, CN). After OGD treatment, the conditioned medium was collected to isolate exosomes for further analysis.

5.3. Exosome Isolation from Cell Culture Supernatants. Exosomes were isolated from bEnd.3 culture supernatant by ultracentrifugation or the Total Exosome Isolation (from cell culture media) kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. In brief, conditioned media (CM) were collected from 30% confluent bEnd.3 cells in sterile conditions and filtered using a filter unit (Millipore, Billerica, MA) with a 0.22 μm membrane to remove intact cells and debris. The CM was then ultracentrifuged at 120 000g for 2 h at 4 °C using an ultrahigh-speed centrifuge (Beckman, Brea, CA). The resulting pellets were washed with cold phosphate-buffered saline (PBS) and then ultracentrifuged one more time as above. Finally, the collected pellets were resuspended in PBS and immediately stored at −80 °C until subsequent usage.

5.4. Nanoparticle Tracking Analysis. Nanoparticle tracking analysis (NTA) was performed by a NanoSight LM 10 instrument (Malvern Instruments Ltd., U.K.) following the manufacturer's instructions. Briefly, 1 mL of sample was drawn and then slowly injected into the sample pool, the laser module was put back on the base, and then the probe of the OMEGA thermometer was put into the copper hole of the laser module panel. The focal lengths of the appropriate Screen Gain and Camera Level were adjusted. The measurements were performed by selecting the appropriate measurement method and measurement parameters. NTA acquisition settings were optimized between sample readings.

5.5. Transmission Electron Microscopy. The collected exosome pellet was diluted in 30 μL of PBS and kept at −20 °C until transmission electron microscopy (TEM) analysis. Each 10 μL droplet of aspirate samples was placed on the copper mesh, allowed to settle for 1 min, and then rinsed by PBS. Phosphotungstic acid (10 μL) on the copper mesh was absorbed and precipitated for 1 min. The floating liquid was absorbed by filter paper and kept at room temperature for 2 min. The preparation was examined using a transmission electron microscope (JEM-1200EX, Japan Electronics Corporation, Japan).

5.6. Western Blotting. Total proteins were extracted from the cultured cells using RIPA lysis buffer. The protein concentration was detected by the BCA Protein Assay reagent kit (Thermo Fisher Scientific, Waltham, MA). The cell protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Then, nonspecific binding was blocked by 5% nonfat milk in trisbuffered saline with Tween-20 (P9416; Sigma-Aldrich) for 1 h at room temperature. Membranes were incubated with primary antibodies, including anti-CD63 (ab108950; Abcam), anti-Alix (ab117600; Abcam), and anti-CD81 (ab79559; Abcam), for overnight at 4 °C. Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at 25 °C before incubating with the enhanced chemiluminescence (ECL) reagent (Thermo-Pierce, Rockford, IL). The protein levels were quantitated after normalization to β-actin.

5.7. Exosome miRNA Sequencing and Construction of Libraries. Total RNA from exosomes was used for miRNA sequencing. Library preparation and miRNA sequencing were performed by Ribobio (Guangzhou, China). Briefly, the total RNA of the sample or the purified sRNA fragments that were connected the 3′ end and the 5′ end successively were then reverse-transcribed to CDNA for PCR amplification. Afterward, the target fragment library was recovered by gel cutting, the qualified library was sequenced using the Illumina HiSeq. 2500 platform, and only small RNAs ranging from 18 to 30 nt were used for library construction.

5.8. Real-Time Quantitative PCR Analysis. To validate the miRNA sequencing data, quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using RNA obtained from the control group (n = 3) and the OGD group (n = 3). Total RNA was purified from bEnd.3 cells with Trizol reagent (Invitrogen). Real-time PCR was performed using the TaqMan miRNA assay kit protocol on an AB7500 fast real-time PCR machine. The expression levels of miRNAs were measured in terms of the threshold cycle value and normalized to the U6 level as an internal control. The specific primers were purchased from GeneCopoeia Biotechnology (Guangz-
5.9. Proximity Barcoding Assay. Profiling surface proteins on individual exosomes were detected by the proximity barcoding assay (PBA) as described previously. Briefly, 240 cell surface protein antibodies conjugated with DNA sequence coding tags (antibody tags) were used to recognize all exosome surface proteins in CM samples. The same single exosome-specific DNA coding sequence (exosome tag) was added to all antibody tags on the same exosome through the proximity coding reaction. All encoded sequences are amplified by PCR to obtain the exosome tag—antibody tag DNA sequence, and then, the sample tag—exosome tag—antibody tag DNA sequence was obtained using the sample tag (sample tag) primer to continue the amplification. According to the Illumina sequencing technology requirements, the DNA fragment library was established, DNA testing was performed with Illumina NextSeq. 500 and Illumina NextSeq FC-404-2005 kits, and the read DNA sequence information was the sample tag—exosome tag—antibody tag DNA sequence. The composition of individual exosome surface proteins was identified and quantified; thereby, the classification of all individual exosomes was analyzed.

5.10. Statistical Analysis. The data of statistical analysis was analyzed using SPSS 20.0 software. All values were presented as mean ± standard error (SEM). Differences between groups were compared by either an unpaired Student’s t-test or one-way ANOVA followed by Tukey’s posthoc test for P values. The numbers of exosomes with given combinations of proteins were calculated using the Fisher test at a confidence level of 0.05, and related operations were implemented using R script. The t-SNE algorithm used was included in the R package Rtsne. The network of miRNAs and surface proteins was constructed using Cytoscape software (version 3.8.0). P < 0.05 was considered to be statistically significant.

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This work was performed and accomplished by all authors. D.Y., Z.L., G.G., X.L., and Y.W. contributed to the execution of the research project. Z.L. and W.L. contributed to data analysis. Y.Z. and W.L. participated in experimental design and manuscript preparation. All authors approved the final manuscript.

Notes

The authors declare no competing financial interest.

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ABCB5, ABC subfamily B member 5; ADAM1, fertilin α; ALDH1, aldehyde dehydrogenase 1; BBB, blood–brain barrier; cGMP-PKG, protein cyclic guanosine monophosphate-kinase G; CM, conditioned media; DMEM, Dulbecco’s modified Eagle’s medium; ECL, electrochemiluminescence; EGF, epidermal growth factor; Elovİ5, fatty acid elongase 5; FBS, fetal bovine serum; FoxO, forkhead box protein O; IL-8, interleukin-8; ITGAX, integrin αX; ITGAM, integrin αM; ITGAE, integrin αE; ITGAB2, integrin αB; ITGAI2, integrin α2; ITGB1, integrin β1; ITGB2, integrin β2; ITGB8, integrin β8; ITGAI9, integrin α9; LMP1, latent membrane protein 1; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; mTOR, mammalian target of rapamycin; MUC4, mucin-4; MUC1, mucin-1; NTA, nanoparticle tracking analysis; OGD, oxygen glucose deprivation; PBA, proximity barcoding assay; Slco3a1, solute carrier organic anion transporter family member 3A1; TEM, transmission electron microscopy; tPA, tissue-type plasminogen activator; TROP2, tumor-associated calcium signal transducer 2; UPA, ulipristal acetate

**ABBREVIATIONS**

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