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Comparison of exosomesisolationmethods

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***Abstract*:** *Exosomes are 40~120 nm extracellular vesicles released from a multitude of cell types, and widely exist in various body fluids. Exosomescan conveyseveral types of important biological functional molecules, as well as participate in diverse cellular functions.Exosomeshad been foundin some in vitro studies of cultured cancer cells, and exosome release was later reported to be involved in various cancer types. The close relationship between exosomes and cancer provides a new strategy for the effective treatment of cancer. However,much of the information about new research advances in exosomeshas been obtained from heterogeneous or impure exosomes. The information consists ofconfounding interpretation of the findings,due to which the related research progress has beenrestricted.Therefore, the isolation and purification of exosomes is of great significance for the analysis of exosomes and subsequent diagnosis in cancer treatment.In this paper, recent advances in exosome isolation as well as purification are reviewed, and the development of exosome researchin cancer diagnosis is prospected.*

***Keywords*:** *Exosomes,Tumor,Isolation, Purification*

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I. Introduction

Tumor is one of the most important diseases threatening human health and survival.Early diagnosis of tumors can reduce the mortality of patients.However, there is still lack of effective methods for early diagnosis of tumor in clinical practice, and there is an urgent need to look for biomarkers that can be applied in early diagnosis of tumors.

With the continuous progress of relevant technologies, increasing number of researchers have focused on the role of exosomes in tumorigenesisas well as tumor development, and their clinical applications[1,2].Originally,exosomes were found in the supernatant of sheep reticulocytes cultivated in vitro. Exosomesare the homogenous vesicles of actively secreting cells, which have a lipid bilayer structure. They have a diameter of 40-100 nm in electronic microscopy, with a density of 1.13-1.19 g/ml and can be sedimented at 100,000×g[3,4].About 20 years ago,exosomes wereregarded as"garbage bags" that clean up discarded organelles during cells maturation process. Exosomes are involved in many physiological as well as pathological processes by playing an important role in intercellular communication [5-10]. In vitro studies have shown that exosome secretion has a close relationship with tumor, particularly associated with many processes like tumorigenesis, tumor development, tumor metastasis, angiogenesis, anti-tumor immunity and tumor immune escape[5]. Therefore, exosomes are expected to be utilized as biomarkers for the diagnosis of tumorigenesis, which has a broad clinical application prospect.

Recently, relevant researches on exosomeshave been emerging in various fields. In many studies of exosomes in cancer patients, it has been found that exosomes secreted by cancer cells contain characteristic molecules that can be used to distinguish them from other exosomes and can be used as molecular markers for cancer diagnosis.However,much of the informationwasobtained from heterogeneous or impure exosomesand confounded interpretation of findings, due to which related researches have been restricted. Hence, in order to get purer exosomes for subsequent analysisthere is an urgent need to formulate strategiesfor exosomeisolationas well as purification.The information obtained from purer exosomeswould help to appropriately interpret theunambiguous biologicalfunctions at the levels of both protein and RNA. Therefore, this paper summarized the exosomesseparation as well as purification strategies and prospected their applications in cancer diagnosis.

1. Isolation and Purification of Exosomes

Exosome isolation and purification process is often influencedby cellular debris, cell vacuoles and protein aggregates. The unique physical and chemical properties of exosomes can be exploited to achieve the isolation as well as purification of exosomes, such as a smaller size,phospholipid bilayer membrane structure and specific transmembrane proteins.

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1.1 Differential Centrifugation

Differentialcentrifugation was developed by Johnstone et al[11].Initially, ithad been used to isolate exosomes from maturing sheep reticulocytes. Cells were collected by centrifugation at 8,000×g for 10 minand thesupernatant was recentrifuged at 100,000×g for 90 min tocollect the released vesicles. Then, the strategy was optimized by Thery et al[12]to eliminatelarge cells,large dead cells and large cell debrisby using of successive centrifugations at increasing speeds(Fig.1). At each of thesesteps, the pellet is thrown away, and the supernatant is used for the following step. The final supernatant is thenultracentrifuged at 100,000×g for 70 minto collect the smallvesicles that correspond to exosomes. The pellet is washed in a large volume of PBS, toeliminate contaminating proteins, and then ultracentrifugedfor one last time at the same high speed.

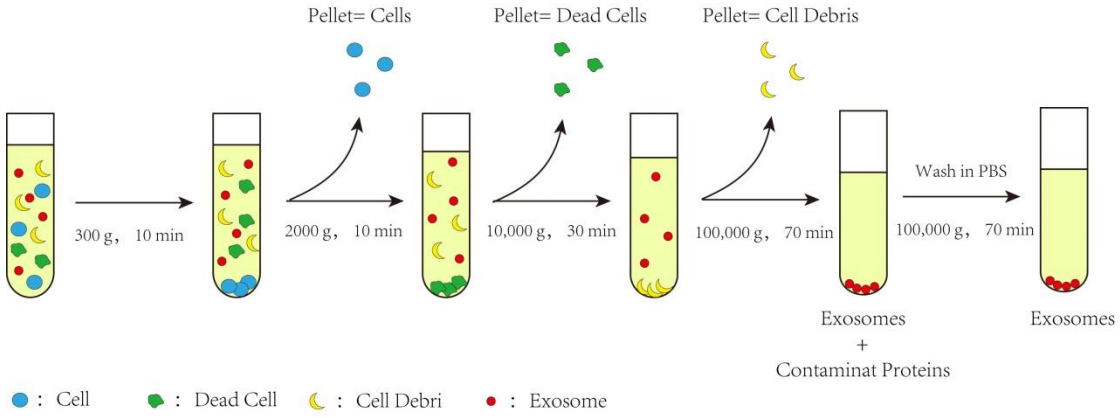

Figure.1 Exosome purification procedure based on differential centrifugation. The speed and length of each centrifugation are indicated.After each of the first three centrifugations, pellets (cells, dead cells, cell debris) are discarded,and thesupernatant is kept for the next step. In contrast to the first three centrifugation steps, pellets (exosomes + contaminant proteins,exosomes) are kept after the two 100,000 × g centrifugation steps, and supernatants are discarded.

Differential centrifugation a standard and very common method for the isolation of exosomes from biological fluids and cultures medium. However, the method is less efficient when viscous biological fluids are used forisolation, such as plasma and serum.Therefore, it is necessaryto dilutethem, and increase the speed or time of centrifugation.

In addition, there are still many shortcomings in the differential ultracentrifugation method.1)Due to the inhomogeneity of the cell secretion vesicles, the precipitation often contains other types of secretory vesicles andaggregated proteins, resulting in the isolation of less pure exosomes.2) The repeated ultra-highspeed centrifugation procedure can result in destruction of exosomes structures and lowrecovery rate of samples.3) The method usually needs a mass of initial samples.It is not suitable for tracing samples and proteomic analysis of precious samples.

1.2DensityGradientCentrifugation

To solve the problem of lesspureexosomes in differential centrifugation, the strategy of density gradient centrifugation was developed by Escola et al[13, 14]. Samples hadbeen processed by differential centrifugation.For further purification of exosomes, thepellet was resuspended in 5 mL of 2.5 M sucrose, 20 mMHepes/NaOH, pH 7.2.A linear sucrose gradient is layered on the top of the exosomessuspension in a SW27 tube (Beckman Instruments, Inc.),and ultracentrifuged at 100,000×g for several hours.Proteinswill be sedimented on the bottom of the centrifuge tube, while exosomeswith the lipid bilayer structure will be sedimented on the equidensity area (1.10 ~ 1.18 kg/L), consequently purerexosomes will be obtained.

Briefly,in density gradient centrifugation the sample and the gradient material are ultracentrifugedtogether,subsequentlythe different components in the sample are deposited into respective equidensity areas. By this approach,thelow density exosomes canbe isolated from other vesicles, particles and contaminants, as well asmore pureexosomeswill beobtained.There are stillsome disadvantages, such as, the tedious preparation, time-consuming procedure and isolation of fewer exosomes.

1.3 Immunological Isolation

There are special membrane proteins on the surface of exosomesmembrane, such as CD63, CD81, CD82,CD9,Alix and so on,which can be used as specific markers for the isolation ofexosomes [15].Antibodycoated magnetic beads were effectively used to isolate exosomes from antigen presenting cells [16]. Antibodies canalso

beimmobilized in chromatographic stationary phase, and microfluidic devices.Using the specific interaction between antibodies and protein markers, the specific enrichment ofexosomes can be realized, such as inimmunomagneticbeadmethod (Fig.2).Therefore,it's a method of using carrier with antibodies directed against exosomes membranes proteins instead of isolating exosomesby ultracentrifugation.In another way, exosomes were first isolated on the base of ultracentrifugation andthen, the isolated exosomes were further purified with magnetic beads precoated with the antibody. Thereafter, beads were incubated by rotation top end over with 20μg of exosomes for 1 h at 4 °C. Afterwashing four times, beads and exosomes were resuspended in PBSfor further experiments [17].

Recently, Wako hasdevelopedMagCapture, a reagent kitof affinity purification based on membrane surface phosphatidylserine (PS).Higher purity of the exosomes can be obtained by using the metal chelating reagent with neutral pH for the competitive eluting, which is applicable to the following biological analysis. However, the nonspecific adsorption of these stroma results in the mixing of acquired exosomes with interfering proteins.

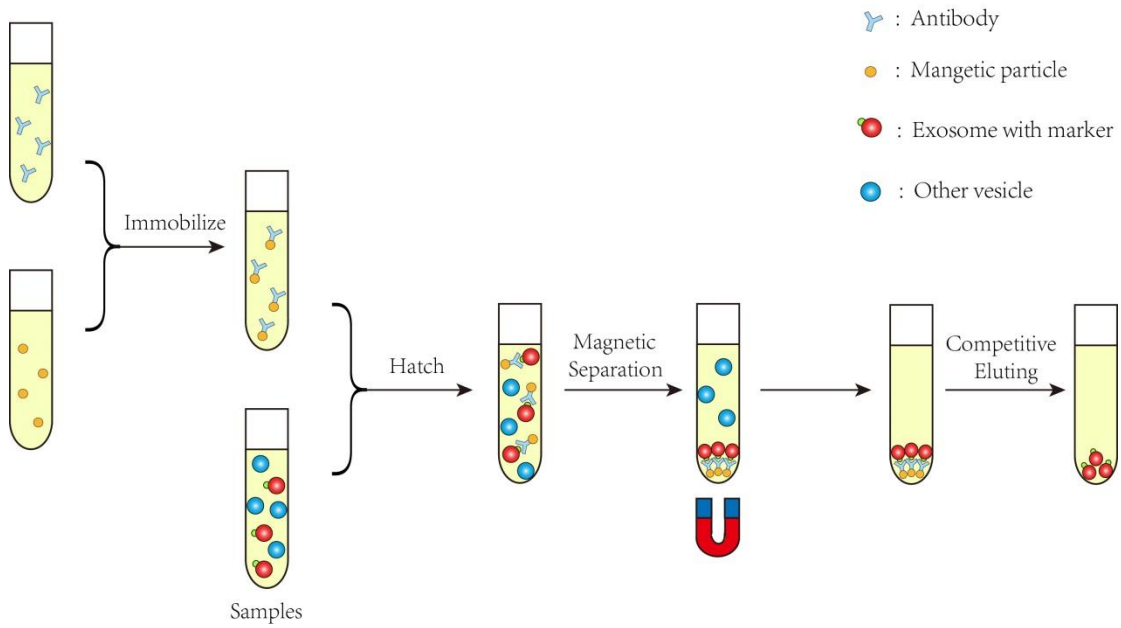

Figure.2Immunomagnetic bead method. Antibodies were immobilized on the immune magnetic beads, and then incubated with samples. Antibody reacts with antigens on the surface of exosomes membrane. Exosomesbound with magnetic beads were placed in a strong magnetic field to separate them from other unbound vesicles.Backed away from the magnetic fieldand obtained pure exosomes by competitive eluting.

In conclusion,immunological isolationhas the advantages ofhigh specificity and preservingvesicle integrity of obtained exosomes,which is also suitable for the qualitative as well as quantitative analysisof exosomes.However, there are also several shortcomings, for example, itis not appropriate for the isolation of larger amounts of exosomes, and the obtained exosomesare still mixed with interfering proteins.

1.4 Polymer-based Precipitation

The mechanismofexosomes deposited bypolymeris based on the principle that water molecules are retained by polymerresulting in thedecreaseofexosomessolubility.Thus, less-soluble exosomescanbesedimentedand collected under the condition of low speed centrifugation [18]. However, this mechanism needs to be further studied. Polymer-based precipitation technique includes the following steps.1) Mixing the biological fluid with polymer-containing precipitation solution;2) Incubation at 4℃;3)Centrifugation at low speed. Many companies have developed a series of kits for different source of samples [19-21](cell cultures, blood samples, urine samples, etc.) to achieve rapid isolation and purification of exosomes. These commercial kits tend to be expensive,so it's not suitable for the process of large-scale samples. Furthermore, people found that the exosomes obtained from the commercial kits were often mixed with interfering proteins, which will affect the subsequent analysis. Therefore, the commercial kits based on polymer precipitationare worth further optimizing and improving.

One of the most common polymers used for polymer-based precipitation is polyethylene glycol (PEG). Meckes et al [22]proposed a method based on PEG (ExtraPEG).0.8 kg/L PEG (with average molecular weight of 6000) were incubatedwith culture medium, which was followed by low-speed centrifugation to obtain the crude exosomes, and then ultra-high speed centrifugation was done to clean further.Zhang et al [23]optimized the relative

molecular mass and content of PEG, salinity and other parameters, meanwhilethe best conditions forexosomesenrichment were determined. Then, 0.1 kg/L PEG (with averagemolecular weight of 10, 000)was used for PEG precipitating twice, to further remove the interference ofserum proteins.

This method obtainedexosomes with high recovery rate and puritywhichare suitable for the subsequent analysis ofproteomics and sequencing. Biological samplesoften contain plenty of proteins, which inevitably result in the presence of proteins in the finalproduct precipitatedby PEG. Therefore, the subsequent cleaning stepsare very important for the reliable identification of exosomes.

1.5 Filtration and Volume Exclusion Chromatography

Exosomeswere isolated by the ultrafiltration membrane of different molecular weight cut off (MWCO).MWCO is the largest relative molecular mass molecule which is free to pass through a sort of porous material.Exosomes are homogenous membrane vesicles withgreater relative molecular mass than the general proteins, thereforeexosomescan beseparated from other large molecules by the different size of the MWCO membranes [24].The operation can keep the biological activity of the exosomes, but there also exist the problem of low productivity.

Volume exclusion chromatography isolates exosomesby the relative relation between the pore size of gel pore and sample molecular size.The large molecules in the sample are not able to enter the gel hole and can only pass through the chromatographic column among the porous gel particles.The small molecules can enter most of the holes in the gel, which trap in the column and wash out more slowly [25].The grain diametersofexosomesare larger than protein and lipid, soit can pass through detached columns quickly. In this way, the high purity exosomesare isolatedfrom impurities effectively.The column cannot be used multiple times because itcausesserious pollution from proteins and lipids, which limits the application of this method.

II. Discussion

With the development of research, the new functions of exosomeswill be revealed.It is very important for people to understand its role in physiological and pathological processes.The existing isolationmethodscannot guarantee the content, purity, and bioactivity of the exosomesat the same time.The advantagesanddisadvantagesofthose approaches described above have beensummarizedin Table.1. Selecting the appropriateisolation and purification methodsunder different conditions is beneficial to obtain the higher purity and keep the activity of exosomes.

Table.1 Methods of exosomeisolation and purification

Isolation methods	Mechanism	Advantages	Disadvantages	Range of application
Differential centrifugation	Different particles have different sedimentation velocity	A standard and common method, large sample sizeprocessed and simple operation	Lowpurity, low recovery rate and not suitable for proteomic analysis of precious samples.	Biological fluids and media, which have low viscosity
Density gradient centrifugation	Different particles have different sedimentation coefficients	Low-density exosomes can be isolated, higher purity of exosomes	Tedious preparation, timeconsuming, isolation of fewer exosomes,	Various biological fluids including plasma and serum.
Immunological isolation	The antibody specific reaction of exosomes with protein markers	High specificity,preserve vesicle integrity[26]and suitable for qualitative and quantitative analysis	Mixing with interfering proteins and not suitable forpurificationoflarge number of samples	Isolates only a subpopulation of marker-positive exosomes [12].
Polymer-based precipitation	The solubility of exosomes is lowered by polymer, and sedimented under low centrifugal speed	High efficiency and rapid	Co-precipitation of proteins exist in the product	Various biological fluids including cell cultures, blood, urine, etc.
Filtration	Using ultrafiltration membrane of different molecular weight cut off (MWCO)	Simple operation, time-saving and maintain the biological activity of the exosomes	Lower productivity	Isolated from other large molecules by the different size of membranes
Volume exclusion chromatography	The relative relation between the pore size of gel pore and sample molecularsize	High purity and Doesn't affectexosome's structure	Timeconsuming and column can't be reused	The grain diameter of proteins and lipids are smaller than the exosomes

Generally speaking, exosomes can be isolated directlyfrom biological fluids by using the methods of differential centrifugation, density gradient centrifugation andimmunological isolation.However,much of the exosomes have low purity and mix with interfering proteins, which will affect the subsequent analysis.On the other hand, exosomescan be further purified by using the methods of polymer-based precipitation, filtration and volume exclusion chromatography. In short, what method will be used depend on the specific application scenarios. For example, 1) Immunological isolation require specialized antibodies, cost is higher, but not allexosometypescanbe captured; 2) PEG precipitatingtwice will remove serum proteins; 3) Exclusion chromatography can be able to obtain more pureexosomes, and separated from impurities, but the volume of processing samples will be restricted. Furthermore, according to the isolation of different objects, several different methods can be combined. The exosomesare first isolated crudely,then purified finely to obtain a higher purity, but the complex steps will decrease the production of exosomes. With the research going deeper, more and more good ways will come up. The best method would be that which isolates exosomes fast, have low cost, and gives high purity output.

III. Prospect

Exosomes play an important role in the pathogenesis of various diseases including tumors.Several methods have been developed to isolate and purify exosomes from biological fluids.Centrifugal technology is still a common means, meanwhile, other methodsshow promising results, such as filtration and immunological isolation. Now there are also methods combined with other different methods to isolate and purify more pure exosomes.At present, it is inevitable to avoid mixing of interferon proteins in exosomes obtained by several major isolation methods.The exosomes have the phospholipid bilayer membrane structure, which is distinguished from all the interfering proteins. Developingan enriched material with a specific affinity for the phospholipid bilayer may be an effective scheme of improving exosomespurity and enrichment efficiency.

As a new tumor marker, tumor-derived exosomes have a promising application in early diagnosis, disease monitoring and prognosis evaluation of tumors[26]. However, the separation technology of exosome is not perfect at present, and it is difficult to obtain exosome with high purity, which causes certain difficulties for subsequent detection as well as analysis. Therefore, several methods of exosome separation and purification reviewed in this paper are of great significance for subsequent exosomeanalysis and detection.The application of exosomes in tumor diagnosis and treatment are promising, but how to realize its clinical application value still needs the joint efforts of researchers.

References

[1]. SIMONS M，RAPOSE G. Exosmes-vesicular carriers for intercellular communication[J]. CurrOpin Cell Biol, 2009, 21（4）:575-581.

[2]. RAPOSO G，STOORVOGEL W. Extracellular vesicles：exosomes，microvesicles，and friends[J]. J Cell Biol，2013，200（4）：373-383

[3]. Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis, and function[J]. Nature Reviews Immunology, 2002, 2(8):569-579.

[4]. Olver C, Vidal M, et al. Proteomic Analysis of Secreted Exosomes[J]. Sub-cellular biochemistry, 2007, 43:99-131.

[5]. D'SOUZA-SCHOREY C，CLANCY J W. Tumorderived microvesicles：shedding light on novel microenvironment modulators and prospective cancer biomarkers[J]. Genes Dev，2012，26（12）：12871299.

[6]. Tauro B J, Greening D W, Mathias R A, et al. Two Distinct Populations of Exosomes Are Released from LIM1863 Colon Carcinoma Cell-derived Organoids[J]. Molecular & Cellular Proteomics Mcp, 2013, 12(3):587.

[7]. Mahaweni N M, Kaijen-Lambers M E H, Dekkers J, et al. Tumour-derived exosomes as antigen delivery carriers in dendritic cell-based immunotherapy for malignant mesothelioma[J]. Journal of Extracellular Vesicles, 2013, 2(2):22492.

[8]. Valencia K, Luisravelo D, Bovy N, et al. miRNA cargo within exosome-like vesicle transfer influences metastatic bone colonization[J]. Molecular Oncology, 2014, 8(3):689-703.

[9]. El A S, Lakhal S, Mäger I, et al. Exosomes for targeted siRNA delivery across biological barriers[J]. Advanced Drug Delivery Reviews, 2013, 65(3):391-397.

[10]. Record M, Carayon K, Poirot M, et al. Exosomes as new vesicular lipid transporters involved in cell–cell communication and various pathophysiologies[J]. BiochimicaEtBiophysicaActa, 2014, 1841(1):108-120.

[11]. Johnsen K B, Gudbergsson J M, Skov M N, et al. A comprehensive overview of exosomes as drug delivery vehicles — Endogenous nanocarriers for targeted cancer therapy[J]. Biochimica Et BiophysicaActa, 2014, 1846(1):75-87

[12]. Johnstone R M, Adam M, Hammond J R, et al. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes)[J]. Journal of Biological Chemistry, 1987, 262(19):9412-9420.

[13]. Théry C, Amigorena S, Raposo G, et al. Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids[M]. Current Protocols in Cell Biology. John Wiley & Sons, Inc. 2006:Unit 3.22.

[14]. Escola J M, Kleijmeer M J, Stoorvogel W, et al. Selective Enrichment of Tetraspan Proteins on the Internal Vesicles of Multivesicular Endosomes and on Exosomes Secreted by Human B-lymphocytes[J]. Journal of Biological Chemistry, 1998, 273(32):20121.

[15]. Raposo G, Nijman H W, Stoorvogel W, et al. B lymphocytes secrete antigen-presenting vesicles[J]. Journal of Experimental Medicine, 1996, 183(3):1161-1172.

[16]. Mathivanan S, Simpson R J. ExoCarta: A compendium of exosomal proteins and RNA[J]. Proteomics, 2009, 9(21):4997–5000.

[17]. Clayton A, Court J, Navabi H, et al. Analysis of antigen presenting cell derived exosomes based on immuno-magnetic isolation and flow cytometry[J]. Journal of Immunological Methods, 2001, 247(2):163-174.

[18]. Jansen F H, Krijgsveld J, Van R A, et al. Exosomal secretion of cytoplasmic prostate cancer xenograft-derived proteins[J]. Molecular & Cellular Proteomics Mcp, 2009, 8(6):1192.

[19]. Zeringer E, Barta T, Li M, et al. Strategies for isolation of exosomes[J]. Cold Spring Harbor Protocols, 2015, 2015(4):319.
[20]. Vaidyanathan R, Naghibosadat M, Rauf S, et al. Detecting Exosomes Specifically: A Multiplexed Device Based on Alternating Current Electrohydrodynamic Induced Nanoshearing[J]. Analytical Chemistry, 2014, 86(22):11125-11132.
[21]. Amarnath S, Foley J E, Farthing D E, et al. Bone Marrow-Derived Mesenchymal Stromal Cells Harness Purinergenic Signaling to Tolerize Human Th1 Cells In Vivo[J]. Stem Cells, 2015, 33(4):1200-1212.
[22]. Beer L, Zimmermann M, Mitterbauer A, et al. Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells: Impact of Released Proteins and Exosomes for Tissue Regeneration[J]. Scientific Reports, 2015, 5:16662.
[23]. Rider M A, Hurwitz S N, Jr D G M. ExtraPEG: A Polyethylene Glycol-Based Method for Enrichment of Extracellular Vesicles[J]. Scientific Reports, 2016, 6:23978.
[24]. Weng Y, Sui Z, Shan Y, et al. Effective isolation of exosomes with polyethylene glycol from cell culture supernatant for in-depth proteome profiling[J]. Analyst, 2016, 141(15):4640.
[25]. Lai R C, Arslan F, Lee M M, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury[J]. Stem Cell Research, 2010, 4(3):214.
[26]. Chen T S, Arslan F, Yin Y, et al. Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human ESC-derived MSCs[J]. Journal of Translational Medicine, 2011, 9(1):47.
[27]. PITT J M，ANERÉ F，AMIGORENA S，et al. Dendritic cell-derived exosomes for cancer therapy[J]. J Clin Invest，2016，126（4）：1224-1232.

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