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STEM CELL-DERIVED EXOSOMES: BIOGENESIS, CHARACTERIZATION, ISOLATION AND PRECONDITIONING

Introduction. Exosomes are nanoscale extracellular vesicles of endosomal origin (approximately 30–150 nm) secreted by almost all cell types, which mediate intercellular communication by carrying proteins, lipids and nucleic acids. Because stem cell-derived exosomes reproduce much of the paracrine activity of the parent cell without the drawbacks of cell transplantation, they offer a promising option in cell-free regenerative medicine.

The aim of the study – to summarise current data on the structure, molecular markers, cellular sources and isolation of stem cell-derived exosomes, and on the preconditioning strategies used to modulate their properties.

Research Methods. The peer-reviewed literature was searched in the PubMed, Web of Science and Scopus databases, with priority given to primary experimental studies, widely cited reviews and the international reporting framework for extracellular vesicles.

Results and Discussion. The review distinguishes exosomes from microvesicles and apoptotic bodies; describes exosome structure (the lipid bilayer, the tetraspanins CD9, CD63 and CD81, the proteins ALIX and TSG101, and the internal contents); sets out identification by positive and negative markers; outlines the main stem cell types (mesenchymal cells from bone marrow, adipose tissue, umbilical cord and dental tissues; pluripotent, including induced pluripotent, cells; and neural and other progenitors) and how they are obtained; and compares the isolation methods (ultracentrifugation, size-exclusion chromatography, precipitation and filtration) with reported figures. Exosome composition depends strongly on the source cell and culture conditions.

Conclusions. Both the stem cell source and the preconditioning regimen (hypoxia, cytokines, three-dimensional culture and pharmacological agents) markedly influence exosome properties. Standardised production remain essential if exosomes are to progress from a research material toward a clinically applicable, cell-free therapeutic.

KEY WORDS: exosomes; extracellular vesicles; mesenchymal stem cells; exosome biogenesis; exosome isolation; preconditioning; regenerative medicine.

INTRODUCTION. Signalling between cells depends not only on diffusible molecules but also on a diverse assortment of membrane-bounded particles that are discharged into the extracellular space. This collection of particles is referred to as extracellular vesicles (EVs) and includes several subpopulations that differ in the manner of their formation; among them, the exosomes – the smallest species, generated within the endosomal compartment – have received the closest scrutiny [1; 2]. An exosome is enclosed by a phospholipid bilayer whose

composition resembles that of the plasma membrane and carries a defined assortment of proteins, lipids and nucleic acids inherited from the cell that produced it; once taken up by a target cell, it discharges this content and can thereby redirect the target cell's behaviour [2; 3]. The observation that such content can be conveyed between cells in a functional form, and can consequently alter the phenotype of the recipient, confirmed that exosomes act as authentic carriers of intercellular signals [4; 5].

For many years the regenerative benefit of mesenchymal and related stem cells was ascribed to their incorporation into damaged tissue; it has since become evident, however,

that much of this benefit is exerted at a distance and is conveyed by the exosomes these cells release [6; 7]. Recognition of this paracrine mechanism has stimulated the pursuit of “cell-free” treatments built upon stem cell-derived exosomes, several of which have entered preclinical testing and initial clinical trials [8; 9]. The advance of the field is nevertheless hampered by unsettled terminology and by the variability that stems from the many cell sources and preparation procedures in use. With this in mind, the present article reviews what is currently known of the architecture, molecular markers, cellular origins and separation of stem cell-derived exosomes, as well as the preconditioning approaches used to alter their composition and therapeutic behaviour.

RESEARCH METHODS. The material for this review was drawn from peer-reviewed publications located through searches of the PubMed, Web of Science and Scopus databases, combining the terms “exosome”, “extracellular vesicle”, “mesenchymal stem cell”, “induced pluripotent stem cell”, “exosome markers”, “exosome isolation” and “preconditioning”. Emphasis was placed on primary experimental work, on frequently cited review articles, and on the consensus reporting recommendations issued by the International Society for Extracellular Vesicles. Consistent with editorial policy, works originating in – or written in the language of – the aggressor states were omitted. The literature so

assembled was organised by theme rather than by type of study.

RESULTS AND DISCUSSION. The sections that follow deal in turn with the classification of extracellular vesicles, the makeup of the exosome, the markers relied upon for its identification, the leading stem-cell sources and the vesicles they release, the procedures by which exosomes are separated and examined, and the preconditioning approaches used to adjust their composition.

Types of extracellular vesicles

Three main categories of extracellular vesicle are customarily recognised, separated from one another by how they form and by their dimensions (Fig. 1, Table 1). The exosomes (roughly 30–150 nm) take shape inside the endosomal system as the internal vesicles of multivesicular bodies and are discharged when those bodies merge with the cell surface [2; 10]. Microvesicles (roughly 100–1 000 nm) instead form by the outward protrusion and pinching-off of the plasma membrane [10], while apoptotic bodies (on the micrometre scale, around 1–5 µm) are shed by cells undergoing programmed death [2]. Since these categories overlap in size and cannot be cleanly resolved from one another, current guidelines favour the neutral label “small extracellular vesicles” wherever an endosomal origin has not been directly proven [11]. To remain consistent with most of the works cited here, the word “exosome” is nonetheless used throughout, on the understanding that many

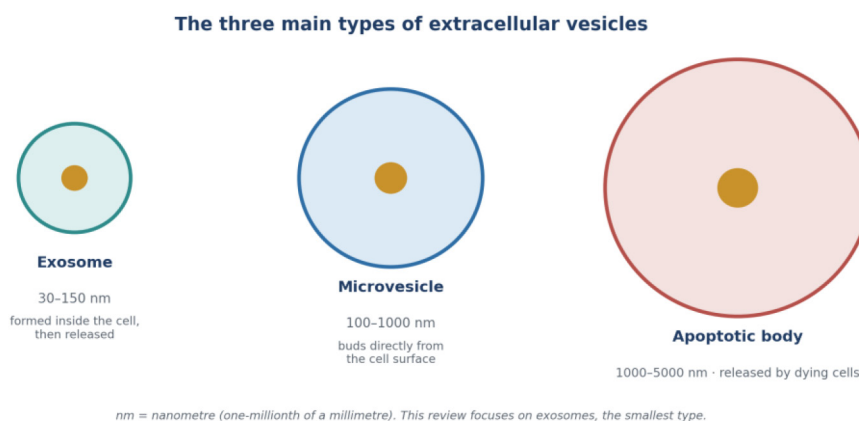


Fig. 1. The three main types of extracellular vesicles, shown by relative size [2; 10]

Table 1 – Main types of extracellular vesicles [2; 10]

Type of vesicle	Size	Where it comes from	Note
Exosome	30–150 nm	Formed inside the cell, then released	Smallest; the focus of this review
Microvesicle	100–1 000 nm	Buds directly from the cell surface	Overlaps exosomes in size
Apoptotic body	1 000–5 000 nm	Released by dying cells	Largest of the three

preparations are in fact mixed populations of small vesicles.

Structure of an exosome

The boundary of the exosome is a phospholipid bilayer whose lipid make-up departs from that of the plasma membrane, showing enrichment in cholesterol, sphingomyelin, glycosphingolipids and phosphatidylserine – features that lend the particle stability (Fig. 2) [12; 13]. Set into this membrane are various proteins, the most distinctive of which belong to the tetraspanin family; three of these – CD9, CD63 and CD81 – occur abundantly in many exosome samples and are commonly used to recognise them [2; 14]. The surface also carries adhesion molecules, integrins and other proteins that direct the attachment and uptake of the vesicle by recipient cells [10].

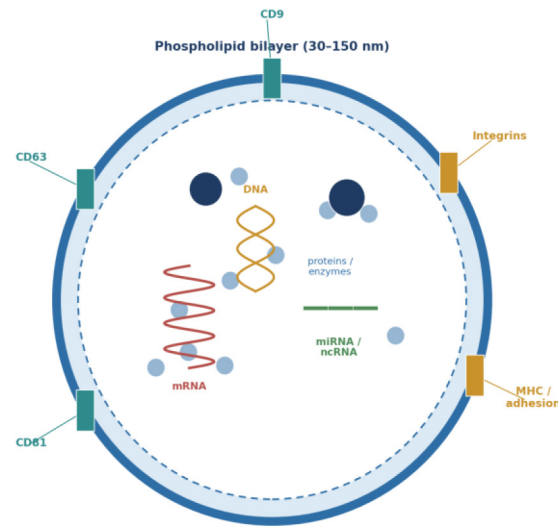


Fig. 2. The structure of an exosome [12; 14]

Within the vesicle lies a carefully selected portion of the parent cell's proteins and transcripts, together with cytosolic proteins, lipids and, notably, RNA – chiefly messenger RNA (mRNA) and microRNA (miRNA) able to influence gene expression in the receiving cell. DNA fragments have likewise been noted in some preparations, though their quantity and exact position within the vesicle continue to be disputed [10; 15]. Hand-curated databases now list many thousands of exosome-associated

proteins and nucleic acids from a wide range of cell types, indicating that the molecular contents are drawn selectively, rather than at random, from the cell of origin [15; 16]. Because these molecules are biologically active and their profile shifts with cell type, exosomes have drawn attention both as diagnostic indicators and as therapeutic carriers [17]. The reliance of the contents on the identity of the producing cell is a thread that runs through the remainder of this review.

Exosome surface markers

Because single vesicles cannot be distinguished by ordinary means, molecular markers are essential for confirming the identity of exosome preparations and for judging their quality [18]. Two kinds of marker are considered (Table 2). Those expected to be present – the positive markers – comprise the surface tetraspanins CD9, CD63 and CD81 alongside the internal proteins ALIX (ALG-2-interacting protein X) and TSG101 (tumour susceptibility gene 101), each of which takes part in the formation of exosomes [14; 18]. The negative markers, which belong to other parts of the cell, ought to be scarce; their presence in quantity signals that non-vesicular cellular material has been carried through with the vesicles.

Marker data must nevertheless be read with care. Careful quantitative work has shown that not one of CD9, CD63 or CD81 is found on every exosome, that none is confined to exosomes alone, and that the amount of each differs appreciably from one preparation and one cell source to the next [19; 20]. Sound practice therefore calls for examining several markers together and for showing both that the expected positive markers are enriched and that the negative ones are absent [18].

Stem cells and their exosomes

Understanding the stem cells that act as exosome sources is a necessary step toward understanding the vesicles they yield. A stem cell is characterised by two capacities: it can renew itself and can give rise to one or more specialised cell types. The chief cell populations used to produce exosomes are set out in Figure 3.

Table 2 – Exosome markers [14; 18; 19]

Marker group	Examples	What it tells us
Positive surface markers	CD9, CD63, CD81 (tetraspanins)	Common on exosomes; used to identify them
Positive inner markers	ALIX, TSG101	Take part in exosome formation; enriched inside the vesicle
Negative markers	Calnexin, GM130, cytochrome c	Belong to other cell parts; should be low or absent in a pure sample

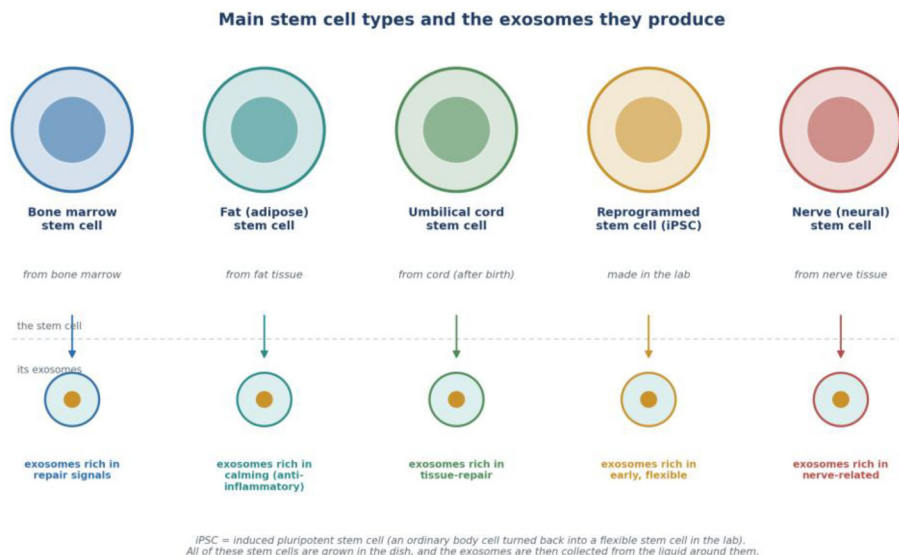


Fig. 3. Main stem cell types used to produce exosomes and the different strengths of their exosomes; iPSC – induced pluripotent stem cell [21; 39; 40]

The most frequently used source is the mesenchymal stromal/stem cell (MSC). The International Society for Cellular Therapy lays down three defining features: attachment to plastic in standard culture, a surface phenotype positive for CD73, CD90 and CD105 but lacking haematopoietic antigens, and the ability to give rise to bone, cartilage and fat cells in the laboratory [21]. Such cells are obtainable from bone marrow, adipose tissue, the umbilical cord and dental tissue, among other sites [7; 21]. A further group is the pluripotent stem cells – the embryonic stem cells and the induced pluripotent stem cells (iPSCs), the latter being ordinary somatic cells returned to a pluripotent condition, an achievement first realised in mouse [22] and later in adult human fibroblasts [23]. Other, more specialised sources are used as well, among them embryonic [24], neural [25; 26], endothelial-progenitor and cardiac progenitor cells [27; 28], each stamping its own molecular character on the exosomes it releases.

Obtaining exosomes from different stem cells

Two matters govern exosome production: where the parent cells come from and how the vesicles are recovered from the culture fluid; these are laid out in Table 3 and Figure 4, respectively. The tissues of origin are collected in different ways. Bone marrow MSCs are drawn from the iliac crest by aspiration [21]; adipose MSCs are gathered with comparatively little trauma from fat obtained by liposuction [29]; and umbilical cord MSCs are taken from cord tissue left over after birth, a source that is otherwise discarded and raises no ethical objection [7]. Similar vesicles are supplied by other tissues of the perinatal period, amniotic fluid in particular [30]. Dental stem cells can be retrieved from extracted or naturally shed teeth [31], whereas iPSCs and the cells derived from them can be grown almost without limit in culture [32; 33]; exosomes from iPSCs behave differently

Table 3 – Stem cell sources and their collection [7; 21; 29]

Stem cell source	How the cells are collected	Notes on the exosomes
Bone marrow stem cell	Needle draw from the hip bone (aspiration); collection is invasive	Rich in repair signals
Fat (adipose) stem cell	From fat removed by minor liposuction	Easy to obtain in large numbers; anti-inflammatory signals
Umbilical cord stem cell	From the cord after a normal birth; non-invasive, otherwise-discarded tissue	Tissue-repair signals
Dental stem cell	From extracted or naturally shed teeth	Repair and blood-vessel signals
Pluripotent (iPSC / embryonic)	Made and grown in the laboratory / are obtained from the inner cell mass of the embryo at the blastocyst stage	Early, flexible signals
Nerve / heart / blood-vessel cells	From the matching tissue	Signals matched to that tissue

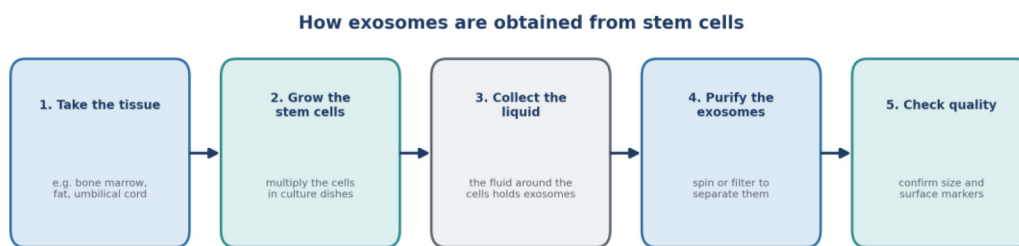


Fig. 4. Workflow for obtaining exosomes from stem cells [35; 38]

from those obtained from MSCs [34]. Since exosomes can neither divide nor become tumours, exosome preparations sidestep the main cancer-related risk that accompanies the grafting of pluripotent cells – although the purity and safety of each preparation must still be checked individually [32; 33].

Exosomes released into the culture fluid are collected by several long-standing techniques (Table 4). Differential ultracentrifugation, the classic method of reference, drives the vesicles into a pellet at roughly $100\,000\text{--}110\,000 \times g$, giving preparations of high purity but requiring lengthy processing and scaling up only with difficulty [35; 36]. Size-exclusion chromatography sorts the vesicles by their size as they pass through a porous column, keeps them intact and yields comparably pure material; a refined ultracentrifugation scheme has been described that clears away more than 95 % of the accompanying plasma proteins [36; 37]. Precipitation with polymers and filtration are quicker and more readily scaled but give material of lower purity [35]. Whatever the method, the size range of the recovered vesicles is usually checked against the

expected 30–150 nm window by nanoparticle tracking analysis [38]. Side-by-side proteomic comparisons confirm that the parent cell largely dictates what an exosome contains: vesicles from bone marrow, adipose and umbilical cord MSCs hold many proteins in common yet also show source-specific differences that leave their mark on the end product [39; 40].

Preconditioning to improve exosomes

Since the contents of an exosome track the condition of the cell that made it, culture conditions are often altered deliberately before the vesicles are collected – an approach termed preconditioning – in order to strengthen the properties of the resulting exosomes [41]; the main strategies are gathered in Table 5. The commonest is exposure to low oxygen (hypoxia), which stabilises hypoxia-inducible factor 1- α (HIF-1 α) and steers the exosomal contents toward mediators that promote blood-vessel formation [42]; the same HIF-1 α response has also been found to improve the survival of the producing MSCs themselves [43]. Treatment with inflammatory cytokines – chiefly tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) – redirects the contents toward

Table 4 – Methods of exosome isolation [35–37]

Isolation method	How it works	Real figures and notes
Ultracentrifugation	Very high-speed spinning that pellets the vesicles	About $100\,000\text{--}110\,000 \times g$ for ~70–120 min; clean but slow
Size-exclusion chromatography	Separates particles by size through a porous column	Gentle and high-purity; keeps vesicles intact
Optimised ultracentrifugation	Several careful spin cycles	Removed more than 95 % of contaminating blood proteins
Precipitation or filtration	Chemical precipitation, or filtering by size	Fast and simple; more material but lower purity

Table 5 – Preconditioning methods and their reported effects [41; 42; 44]

Preconditioning method	What is done to the cells	Reported effect on the exosomes
Low oxygen (hypoxia)	Grow the cells in a low-oxygen environment	More blood-vessel-forming signals (through the protein HIF-1 α)
Inflammatory signals (cytokines)	Add TNF- α , IFN- γ , or similar molecules	Better at calming inflammation; more repair-friendly immune signals
3D culture (spheroids)	Grow the cells as small three-dimensional balls	More exosomes produced; more anti-inflammatory signals
Drugs	e.g. melatonin or deferoxamine	Change how exosomes form, or mimic low oxygen

immune-modulating mediators and encourages a repair-oriented macrophage state [44; 45]; jointly applying TNF- α and IFN- γ raised the level of pro-angiogenic proteins in MSC vesicles and was accompanied by a tendency toward stronger responses in endothelial cells [46]. Growing the cells as three-dimensional spheroids rather than as a flat layer increases both the number of exosomes obtained and their content of anti-inflammatory mediators [47]. Drug-based preconditioning has been tried as well: melatonin affects how exosomes are formed, and deferoxamine mimics low oxygen chemically, although the evidence for the latter concerns the whole secreted fraction rather than isolated exosomes and should be weighed accordingly [48; 49]. Taken together, these levers – oxygen level, cytokine exposure, culture geometry and drugs – provide a flexible way of shaping what an exosome carries.

CONCLUSIONS. Exosomes are vesicles of endosomal origin that carry proteins, lipids and nucleic acids between cells, and they have risen to a leading position in cell-free regenerative medicine by reproducing the paracrine effects of stem cells while avoiding the drawbacks of transplanting the cells themselves. As set out above, an exosome consists of a lipid-bilayer membrane displaying its characteristic tetraspanins (CD9, CD63, CD81) and holding a purposefully selected cargo; confirming its identity calls for a set of markers rather than any one alone; and recovering it begins with the fluid in which stem cells have been cultured. The tissue from which the parent cell is taken, and the preconditioning applied to it – low oxygen, inflammatory cytokines, three-dimensional culture or drugs – strongly shape what the resulting exosomes contain and how they act.

Two reservations deserve emphasis. First, most samples called “exosomes” are really mixed populations of small vesicles together with co-purified particles, so that results depend heavily on how the vesicles were made, separated and examined. Second, much of the present evidence is still limited to laboratory or early clinical settings, and features such as purity, dose and safety have to be established for every individual preparation before firm clinical claims can be made. Further advance will rest

on standardising how exosomes are produced and characterised and on mapping out, for each cell source and each preconditioning method, exactly what the resulting vesicles carry – the groundwork required to turn stem cell-derived exosomes into dependable and clinically usable treatments.

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ЕКЗОСОМИ, ОТРИМАНІ ЗІ СТОВБУРОВИХ КЛІТИН: БІОГЕНЕЗ, ХАРАКТЕРИСТИКА, ВИДІЛЕННЯ ТА ПРЕКОНДИЦІОНУВАННЯ

Анотація

Вступ. Екзосоми – нанорозмірні позаклітинні везикули діаметром приблизно 30–150 нм, які секретуються практично всіма типами клітин і опосередковують міжклітинну комунікацію, переносячи білки, ліпіди та нуклеїнові кислоти. Екзосоми, отримані зі стовбурових клітин, значною мірою відтворюють паракринну активність материнської клітини, не потребуючи її трансплантації, тому розглядаються як перспективний засіб безклітинної регенеративної медицини.

Мета дослідження – узагальнити сучасні дані про будову, молекулярні маркери, клітинні джерела та методи виділення екзосом стовбурового походження, а також про стратегії прекондиціонування, які модулюють їхні властивості.

Методи дослідження. Здійснено огляд наукової літератури з баз даних “PubMed”, “Web of Science” і “Scopus”; пріоритет віддавали первинним експериментальним дослідженням, широко цитованим оглядам і міжнародним настановам щодо позаклітинних везикул.

Результати й обговорення. Розмежовано екзосоми, мікровезикули й апоптотичні тільця; охарактеризовано будову екзосом (ліпідний бішар, тетраспаніни CD9, CD63, CD81, білки ALIX і TSG101 та внутрішній вміст); викладено принципи ідентифікації за позитивними і негативними маркерами; окреслено основні типи стовбурових клітин (мезенхімальні з кісткового мозку, жирової тканини, пуповини та зубних тканин; плюрипотентні, зокрема, індуковані плюрипотентні; нейральні й інші) і способи їх забору; охарактеризовано методи виділення екзосом (ультрацентрифугування, ексклюзійна хроматографія, преципітація, фільтрація) із наведенням реальних показників. Показано, що склад екзосом залежить від типу клітини-джерела й умов культивування.

Висновки. Тип стовбурових клітин і режим прекондиціонування (гіпоксія, цитокіни, тривимірне культивування, фармакологічні агенти) значною мірою визначають властивості екзосом. Стандартизація методів отримання залишається ключовою умовою переходу екзосом від дослідницького об'єкта до клінічно застосовного безклітинного засобу.

КЛЮЧОВІ СЛОВА: екзосоми; позаклітинні везикули; мезенхімальні стовбурові клітини; біогенез екзосом; виділення екзосом; прекондиціонування; регенеративна медицина.

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