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WORD COUNT	3461	TIME SUBMITTED	29-MAR-2023 11:10AM
		PAPER ID	98114608

Name of Journal: *World Journal of Stem Cells*

Manuscript NO: 82702

Manuscript Type: MINIREVIEWS

Human pluripotent stem cells-derived extracellular vesicles: From now to the future

Matos BM *et al*. hPSC-EVs: From now to the future

Abstract

Extracellular vesicles (EVs) are nanometric particles that enclose cell-derived bioactive molecules in a lipid bilayer and serve as intercellular communication tools. Accordingly, in various biological contexts, EVs are reported to engage in immune modulation, senescence, cell proliferation, and differentiation. Therefore, EVs could be key elements for potential off-the-shelf cell-free therapy. Little has been studied regarding EVs derived from human pluripotent stem cells (hPSC-EVs), even though hPSCs offer good opportunities for induction of tissue regeneration and unlimited proliferative ability. In this review article, we provided an overview of studies using hPSC-EVs, focusing on identifying the conditions in which the cells were cultivated for the isolation of EVs, how they were characterized, and applications already demonstrated. The topics reported in this article highlight the incipient status of the studies in the field and the significance of hPSC-EVs' prospective applications as PSC-derived cell-free therapy products.

Key Words: Pluripotent stem cells; Extracellular vesicles; Exosome; Cell-free therapy

Matos BM, Stimamiglio MA, Correa A, Robert AW. Human pluripotent stem cells-derived extracellular vesicles: From now to the future. *World J Stem Cells* 2023; In press

Core Tip: The research on extracellular vesicles (EVs) derived from different cell types, such as adult stem cells, has shown potential in the treatment of various pathologies. However, little has been explored regarding EVs derived from human pluripotent stem cells (hPSC-EVs). In this review, we provided an overview of studies carried out on these EVs, highlighting methodologies used for the culture of hPSCs for isolating EVs, their characteristics, and potential applications. We noted the potential of hPSC-EVs as future acellular therapies. However, studies are in the infancy, and more research is needed to confirm their benefits.

INTRODUCTION

Extracellular vesicles (EVs) are nanometric particles that are enclosed by a lipid bilayer and released by all cell types. They lack a functional nucleus and are therefore unable to replicate[1]. EVs are composed of bioactive factors such as lipids, proteins, and nucleic acids, including mRNAs and non-coding RNAs[2]. EV is an umbrella term that encompasses a heterogeneous population of membrane vesicles generated through a variety of mechanisms. The two major EV subpopulations include microvesicles (MVs) and exosomes (EXOs). EXOs are intraluminal vesicles of endosomal origin released when multivesicular bodies fuse with the plasma membrane, whereas MVs or ectosomes are generated from the outer budding of the plasma membrane[3]. Due to their distinct biogenesis, MVs are generally larger (up to 1000 nm in diameter) than EXOs (less than 200 nm). However, these vesicle populations overlap not only in terms of size but also in composition[4]. Recently, other nomenclatures were described in the "Minimal information for studies of extracellular vesicles 2018" guidelines (MISEV2018) based on the physical characteristics of EVs, for example, size (< 200 nm, small EVs; > 200 nm, medium or large EVs) or density (low, middle, or high)[1].

Potential uses of EVs, such as for the diagnosis and treatment of pathologies or as potential drug carriers, have been investigated. In the field of regenerative medicine, the secretome of adult stem cells, primarily mesenchymal stem/stromal cells (MSCs), including their EVs, are of great interest as they have been shown to act mainly in a paracrine manner rather than their potential for differentiation[5]. An interesting list of advantages and disadvantages of the use of EVs instead of stem cells has been presented by Öztürk *et al*[6]. Among the advantages of using EVs cited by them and others are low immunogenicity and toxicity; minimal risk of malign transformation; minimal risk of getting trapped in the lung or causing vasculature obstruction; avoidance of contamination with undesired cell types; avoidance of uncontrolled cell division; the ability to manipulate EVs in order to obtain potential improvements; optimization of MSC culture to obtain a higher amount of EVs, and their ability to cross

the blood-brain barrier, among others[4,6]. In addition, EVs mimic the beneficial effects of MSCs in cell therapies in a wide range of animal models for different diseases[7-9].

MSC-derived EV (MSC-EV) has been extensively studied and has demonstrated several promising effects, as reviewed by Gowen *et al*[10], Tieu *et al*[11], Fuloria *et al*[12], Kou *et al*[13] and Yudintceva *et al*[14]. However, despite the high potential of MSC-EVs, several factors limit their use. Recently some reviews highlighted the difficulty of establishing criteria to define the specific characteristics of MSC-EV and discussed the great variation in the MSC-EV preparations[15,16]. Disadvantages of MSCs as a source for EVs include the variability between cells derived from different tissues, the variability between different donors, their limited ability to proliferate, the fact that they enter senescence, and genomic instability after a few passages[17]. This raises the question of whether pluripotent stem cell (PSC) derived EVs have a similar or better therapeutic potential than adult stem cell-derived EVs.

In this context, our objective is to show, using a non-systematic search, studies that use or characterize EVs derived from human PSC (hPSC-EVs) to understand the advances in the area. We also aimed to identify the conditions in which the cells were cultivated for the isolation of EVs, how these were characterized, and any demonstrated applications (*in vivo* or *in vitro*).

HPSC-EVS

Overview of hPSCs

hPSCs are characterized by unlimited proliferation and the potential to generate specialized cell lineages[18]. Human embryonic stem cells (hESC) were first isolated from human blastocysts in 1998 by Thomson *et al*[19], and to date, hundreds of hESC lineages have been established worldwide. hESC-based therapeutic technologies have applications in many diseases and conditions, such as spinal cord injuries, age-related tissue degeneration, and diabetes[20]. However, ethical issues related to using cells from embryos have hindered the application of hESC in research and treatment, leading to the development of the induced PSC (iPSC) technology developed by Takahashi and

Yamanaka[21] and Takahashi *et al*[22]. Since generation of the first iPSC, many research groups have developed human iPSC (hiPSC) lineages reprogrammed from different adult cells, and obtained lineages very similar to hESC in terms of morphology and differentiation potential[23]. For more information about hPSCs see Karagiannis *et al*[24], Liu *et al*[25] and Yamanaka[26].

Especially after the discovery of hiPSC, pluripotent cells represented a promising alternative for regenerative medicine, transplants, disease modeling, and many other research applications[27-29]. The possibility of generating pluripotent cells from patients and, from them, differentiated cells for tissue repair may mitigate common transplant issues, such as immunologic rejection. Nevertheless, the immunogenicity of pluripotent cells remains controversial[30], and the potential for tumorigenesis hinders the wide application of these cells in clinics. The risks of contaminating the differentiated cell populations with remaining pluripotent or proliferative cells, as well as the transmission of active pluripotency transcription factors or the acquisition of mutations by the pluripotent cells during *in vitro* culture[26,31], limits the acceptance of hPSC-based therapies. Therefore, cell-free therapeutic approaches, including EVs, offer promising possibilities for applying hPSC-derived products[32].

It seems that the role of the secretomes of these cells has only recently begun to be investigated, possibly due to the difficulties still encountered in using hPSC in the clinic. Some interesting studies show that EVs from ESC could help with embryo implantation[33] and maintaining ESC stemness[34], while others have investigated the biogenesis of ESC-EVs[35,36], although they used murine PSC. We will focus this review on studies with hPSCs due to their potential clinical applications.

HPSC-EVs: Isolation and characterization methodologies

The first investigation on the isolation of EVs from hPSC dates from 2015. In this initial approach, EVs were isolated from hiPSC cultured in Essential 8™ medium using differential centrifugation (DF)/ultracentrifugation (UC). It showed that the hiPSC-derived EVs (hiPSC-EV) contains a variety of microRNAs (miRNAs) (such as miR-382,

miR-611, and others) related to pathways such as focal adhesion, Wnt, PI3K-Akt, and MAPK signaling, as well as proteins related to processes involved in signal transduction, receptor binding, and others. In addition, the EVs positively affected the metabolism, proliferation, apoptosis rate, and differentiation capacity of cardiac MSC. Better results were obtained when cells were exposed for only 22 h to EVs[37]. This initial attempt demonstrated how hPSC-EV could be beneficial and of interest for future acellular therapy applications.

Despite the potential of hPSC-EVs, we observed that the number of publications in this area is still low, and most of the existing publications date from the last five years (Figure 1A, Table 1). Some studies evaluate EVs that were isolated during the differentiation process or from cells that differentiated from PSC, such as hiPSC-derived-keratinocytes[38]; hPSC-derived-cardiac progenitors or cardiomyocytes[39-41]; hPSC derived-MSC[42-44]; hiPSC derived-neurons[45-47]; and hESC derived-chondroprogenitor cells[48]. However, our review explores studies that isolated EVs from undifferentiated hPSC.

Using a non-systematic search, we found 36 studies that isolate EV-hPSC, mainly from the hiPSC lineages (Figure 1B). Table 1 summarizes these studies, highlighting the cell culture medium used to culture the PSC, time of conditioned medium collection, EV isolation method, and EV mean size. The most common culture media were commercial, with defined components (Figure 1D). The two most common media used were mTeSR™1 (StemCell Technologies) and Essential 8™ Medium (Thermo Fisher) (Table 1). A study published by Luo *et al*[49] aimed to optimize culture conditions for isolation of hiPSC EVs. Using DMEM with different concentrations of EV-depleted KnockOut™ Serum Replacement (ED-KSR), they observed that cells remained viable at 0.5% ED-KSR concentration and were able to isolate EVs from PSC cultured in this condition efficiently. However, after five days of culture, there was a reduction in the expression of some pluripotency markers. Thus, although it may be cheaper than commercial mediums, it is necessary to consider the additional step of centrifugation of

the KSR to remove particles, as well as the effects of the change in pluripotency-related parameters on the composition and potential of the EVs.

The biggest variations in EV isolation methods relate to the collection time of the conditioned medium: Many studies do not state the conditioning time. In most studies, however, the EVs were isolated after 24 h of cell culture or every 24 h for 3-5 consecutive days (Table 1), avoiding exceeding the 80%-90% cell confluence in the cell cultures. This collection time is possibly related to the nature of PSCs, as the culture medium must be changed daily, and cells must not reach 100% confluence to guarantee their viability and pluripotency.

Other relevant aspects of EVs are their size, morphology, and estimated particle concentrations. Most studies presented the information listed in MISEV2018, including positive and negative protein markers in EVs, usually using the western blot technique (31/36 articles) and performing a single EV analysis mainly using transmission electron microscopy (31/36 articles) to verify EV morphology and nanoparticle tracking analysis (20/36 articles) to verify its mean size and concentration (Figure 1C). The greatest number of studies used small EVs/EXOs, with sizes up to 200 nm (small EVs) (Table 1).

The most common method for hPSC-EV isolation is DF (here defined as the initial centrifugations to remove cellular debris and apoptotic bodies) followed by UC (Table 1). Although this is the most common method used, it is unsuitable for isolating EVs from large-scale experiments and clinical trials. Using a large-scale 2D culture, Andrade *et al*[69] isolated hPSC-EVs using tangential flow filtration (TFF) with or without subsequent UC (TFF + UC). The isolated EVs presented a size of approximately 100 nm, regardless of whether UC had been performed, with similar particle concentration, although TFF + UC resulted in a smaller number of proteins. The effect of different culture conditions (hypoxia - 1% O_2, physiological hypoxia - 5% O_2, and normoxia) on the therapeutic potential of hPSC-EVs was also investigated. The results showed that EVs derived from hPSC cultured in 1% O_2 (hypoxia) had greater angiogenic potential than those derived under other conditions and that better results were achieved when obtaining EVs using TFF[69].

Another highly discussed topic about PSC is the possible formation of teratomas, as well as the biodistribution of these cells when applied in *in vivo* models. These concerns also extend to PSC-EVs. To clarify these points, Gu *et al*[75] evaluated the safety and biodistribution of hiPSC-EVs. They used several approaches to show that PSC-EVs are safe, have no adverse effects on cells (*e.g.*, do not cause hemolysis), are not genotoxic, and can be administered by different routes (nasal, intramuscular, or intravenous) without generating adverse effects (*e.g.*, inflammation at the site or pathological changes in the organs of rats).

Potential therapeutic applications of hPSC-EV

Although few investigations have been carried out with hPSC-EVs, we noticed that almost all of them have already applied hPSC-EVs to different disease models, both *in vitro* and *in vivo*. PSC-EVs have been described as having: Protective effects in *in vitro* and *in vivo* models of ischemia-reperfusion kidney injury[64]; neural protective abilities[60]; the capacity to modulate neuroinflammation and protect against ischemic stroke through Treg cell expansion[73]; antifibrotic effects *in vivo* and in *in vivo* models of liver injury[61,72]; and reduced cartilage degradation in an osteoarthritis model[77]. They have shown improvements in wound closure, angiogenesis, and increased nerve fiber density in a wound-healing diabetic mouse model[54,79]; and improved recovery of ovarian function in a premature ovarian failure mouse model[67]. EVs were also associated with acellular nerve grafts demonstrating their potential to repair peripheral nerve defects[80].

It was also demonstrated that MVs, but not EXOs, retrodifferentiated Müller cells into retinal progenitor cells *in vitro*[71]. Other studies showed the ability of PSC-EVs to promote regeneration of diseased or damaged retinas[56] and to accelerate corneal epithelium defect healing *in vivo*[68]. Other potential uses cited for PSC-EV were: In antitumoral activity[51,63]; in angiogenesis stimulation[69]; as a gene delivery vector[50]; to increase the functional properties of cord blood-derived hematopoietic stem and

progenitor cells[70]; and to improve the number of beating EBs depending on the hiPSC origin[66].

One noteworthy effect shown in some studies is the capacity of PSC-EVs to "rejuvenate" different cell types, such as senescent endothelial cells[52,58], senescent human dermal fibroblasts[55], senescent chondrocytes[77] and others. Considering this potential, the hPSC-EVs, hESC-EVs and hiPSC-EVs, were investigated as therapeutic tools for age-related diseases. Regarding on neurological diseases, the hPSC-EVs showed potential in recovery of senescent hippocampal neural stem cells in rats with vascular dementia - partially through the transfer of miRNAs that inhibit mTORC1 activation - resulting in an improvement in disease status (*e.g.,* reverse cognitive impairment)[81]. Furthermore, using mice of varying ages, hPSC-EVs were found to rejuvenate hippocampal NSC partly through the transfer of SMAD proteins that activate myelin transcription factor 1 (MYT1), which is reduced in senescent cells, and activates a signaling cascade in the MYT1-Egln3-Sirt1 axis[81].

In an ischemic stroke model, hPSC-EV reduced the expression of inflammatory cytokines and, leukocyte infiltration and, increased the number of regulatory T cells and other immunomodulatory effects that alleviate neurological deficits[73], they also reduced blood-brain barrier damage in aged stroke mice through blood-brain barrier rejuvenation, partially through the transfer of AKT1 and CALM from EVs to endothelial cells leading to activation of the endothelial nitric oxide synthase-Sirt1 axis[78]. Therefore, hPSC-EV could be a promising cell-free therapy to treat age-related diseases associated with cellular senescence.

In order to evaluate the benefit of hPSC-EV compared to other EVs, one interesting study demonstrated that both hiPSC-EV and hMSC-EV, isolated through size exclusion chromatography (Table 1), could improve the proliferation of senescent MSCs and alleviate cellular aging in a replicative aging model, possibly modulating reactive oxygen species production with peroxiredoxins presented in EVs. However, despite the similar effects, EVs derived from iPSC enter target cells more efficiently, and the

production of hiPSC-EV was about 16-fold higher than MSC-EV (using the same culture medium)[59].

hPSC-EV composition

Even though many articles described the effects of hPSC-EV, few made deeper characterizations of, for example, the protein and miRNA content of these EVs. Some performed proteomic analysis to help explain some of the effects[59] or as a control (time 0) to study the differentiation process[39,46]. In one interesting approach using high-density lectin microarray, Saito *et al*[57] demonstrated that rBC2LCN, a specific lectin for hPSCs, bound to hiPSC-derived EVs but not to adipose-derived stem cell-, hemodiafiltration- or chondrocyte-derived EVs, which suggests a particular glycan-signature for hiPSC-EVs, resembling the glycome signature of the cell surface.

One recent study that provided a detailed description of the contents of hPSC-EVs was conducted by Bi *et al*[74]. The proteomics of hESC-, hiPSC-, and hMSC-EXOs showed that the main enriched proteins were related to distinct pathways between vesicles of pluripotent and multipotent cells. In hPSC, EXO content was more focused on development, metabolism, and anti-aging properties, and in hMSC, it was related to immune regulation. Another study of 2022 also indicates that hMSC-EVs content is strongly related to immune regulation while hPSC-EVs content do not present many of the proteins related to this function[76]. Actually, 79 proteins were found to be shared between hMSC- and hPSC-EVs, yet the main biological processes related to them were DNA regulation, signal transduction and cell communication[76]. Liu *et al*[59] also compared the protein content of hiPSC-EVs and hMSC-EVs and described more than 1100 proteins shared between the different EVs, allowing to identify proteins that could be responsible for the anti-senescent effect observed in the study.

Considering the protein content of hESC and hiPSC EXOs, Bi *et al*[74] suggests hESC-EXOs are more prone to regulate development and pluripotency pathways, and hiPSC-EXOs have stronger correlation with metabolism. Regarding the most enriched miRNAs for both hPSC-EVs it was shown that they were related to cell cycle and

metabolism regulation. Interestingly, miRNAs found in both hESC-EXOs and hiPSC-EXOs were involved in cell differentiation, development, and cell cycle, even though the hiPSC-EXOs set of miRNAs seemed to play a less significant role in these functions than the hESC-EXOs set[74].

In order to explore whether apoptosis-linked gene 2-interacting protein X (ALIX), a protein present in the endosomal sorting complex required for the transport and biogenesis of EXOs, could regulate the protein content of EV, Sun *et al*[62] isolated EVs from hiPSC overexpressing (using lentiviral transduction) or that were knockout (using CRISP-Cas9 system) for ALIX. EVs isolated from these cell lineages were of a similar size, although EVs generated from KO cells were slightly larger. The evaluation of protein content in EVs showed that those derived from KO cells had fewer proteins, while EVs from overexpressing cells presented a higher number of proteins. These differences could be related to the differences demonstrated in functional assays, *e.g.*, cell viability, apoptosis inhibition, and formation of capillary-like structures, where EVs from overexpressing cells had better effects. So, EVs with different protein profiles could have different therapeutic applications.

CONCLUSION

Although hPSC cultivation has been carried out for some time, the requirements for *in vitro* culture of these cells are very specific, as many factors are necessary to maintain them in their undifferentiated state. This, together with the cost, could be one of the reasons why secretomes and isolation of hPSC-EVs have not been extensively studied so far. Commercial media is now defined with a few components that are no longer as expensive as before, which may have contributed to the increase in publications in recent years.

An overview of the hPSC-EV studies is shown in Figure 2, which illustrates the potential use of these EVs for regenerative medicine. Regarding EV characterization, we observed in the publications that hPSC-EVs follow the basic requirements described in MISEV2018. However, despite the recent increase in research in this area, further

characterization of the content of these EVs needs to be carried out. In addition, studies with modified cells aimed to enriching the content of EVs with some specific protein or miRNA may be of great interest. One interesting approach requiring more extensive discussion is the possible use of hPSC-EVs in reprogramming adult cells into PSCs. A recent study used EVs derived from ESCs undergoing cardiac differentiation to transdifferentiate fibroblast to cardiomyocyte-like cells with relatively high efficiency[82].

Our review shows that hPSC-EVs have therapeutic potential, although no publications demonstrate that they are effectively better than other EVs, such as hMSC-EVs. The advantages of PSC-EVs could be related to a higher level of EV production since the cells have greater proliferative capacity, along with the fact that we can isolate EVs from a single source, possibly reducing the variability between batches. However, studies in this area are still needed as current results are highly variable. Alternatives to EVs include the use of cell-engineered nanovesicles generated by serial extrusion of hiPSCs, as described by Lee *et al*[83], which presented similar results to PSC-EV, but with higher production yield. However, more studies are needed to verify the viability of this method for future applications. Thus, challenges that remain are the large-scale production of EVs, which in the case of hPSC cultivation can be expensive, and the investment in efficient methodologies for EV isolation that could be used in good manufacturing practices for future acellular therapies.

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2	Anny Waloski Robert, Bruna Hilzendeger Marcon, Addeli Bez Batti Angulski, Sharon de Toledo Martins et al. "Selective Loading and Variations in the miRNA Profile of Extracellular Vesicles from Endothelial-like Cells Cultivated under Normoxia and Hypoxia", International Journal of Molecular Sciences, 2022 Crossref	18 words — < 1%
3	www.ncbi.nlm.nih.gov Internet	15 words — < 1%
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