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Exosomes secreted by placental stem cells selectively inhibit growth of aggressive prostate cancer cells

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Abstract

The current paradigm in the development of new cancer therapies is the ability to target tumor cells while avoiding harm to noncancerous cells. Furthermore, there is a need to develop novel therapeutic options against drug-resistant cancer cells. Herein, we characterized the placental-derived stem cell (PLSC) exosomes (PLSC^{Exo}) and evaluated their anti-cancer efficacy in prostate cancer (PCa) cell lines. Nanoparticle tracking analyses revealed the size distribution (average size 131.4±0.9 nm) and concentration of exosomes ($5.23 \times 10^{10} \pm 1.99 \times 10^9$ per ml) secreted by PLSC. PLSC^{Exo} treatment strongly inhibited the viability of enzalutamide-sensitive and -resistant PCa cell lines (C4-2B, CWR-R1, and LNCaP cells). Interestingly, PLSC^{Exo} treatment had no effect on the viability of a non-neoplastic human prostate cell line (PREC-1). Mass spectrometry (MS) analyses showed that PLSC^{Exo} are loaded with 241 proteins and mainly with saturated fatty acids. Further, Ingenuity Pathway Analysis analyses of proteins loaded in PLSC^{Exo} suggested the role of retinoic acid receptor/liver x receptor pathways in their biological effects. Together, these results suggest the novel selective anti-cancer effects of PLSC^{Exo} against aggressive PCa cells.

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Conflict of interest

The authors declare no competing financial interests.

Keywords

Placental stem cells; Exosomes; Prostate cancer; Mass Spectrometry; Retinoic acid receptor

1. Introduction

The current paradigm in the development of new cancer therapies is the ability to effectively target tumor cells while avoiding harm to the other healthy cells and organ systems of the body. Unfortunately, many of the drugs that are in clinical use today lack this specificity and lead to a number of untoward side effects. This is seen in all chemotherapeutic agents in use, as well as with the treatment of various immunomodulators, check-point inhibitors, and anti-angiogenic agents, even though toxic effects could vary [1, 2]. Therefore, it is important to develop therapies that specifically target cancer cells while sparing the healthy cells, thus reducing toxicity.

A new therapeutic approach currently being developed is the use of mesenchymal stem cells (MSCs). MSCs are multipotent stem cells with the ability to differentiate into a variety of cell types, including chondrocytes, osteoblasts, adipocytes, muscles, neurons, and stromal cells. As evidenced by their explosion as a therapy, MSCs are currently involved in more than 500 clinical trials around the world for tissue repair, hematopoietic stem cell transplantation, and autoimmune diseases [3]. Of the various forms of MSCs, mesenchymal-like placental stem cells (PLSC) offer multiple advantages as the stem cell of choice. It is believed that they contain more differentiation potential because they are derived from tissue that retained the properties of early embryonic cells [4, 5]. Furthermore, the placenta plays the role of the immunological interface between mother and fetus, and thus contributes to the feto-maternal tolerance. Therefore, it has been hypothesized that cells isolated from extra-embryonic tissues possess immune-regulatory properties [6]. Moreover, recent data have shown that due to their unique immunomodulatory properties extra-embryonic MSCs did not form the teratomas and terato-carcinomas after implantation in human body [5], and thus can be recognized as promising candidates for cell replacement therapies.

Exosomes are extracellular nanovesicles ~40–150 nm in size that are loaded with protein, nucleic acids and lipids, they signify a unique form of intercellular communication [7]. Recently, more research is focusing on the biological functions of exosomes released from multiple cell types, including MSCs [8]. This has led to not only a better understanding of the tumor biology, but it has brought about novel ideas in utilizing exosomes in treatment strategies [7, 9]. In the present study, we assessed the potential anti-cancer effects of exosomes secreted by PLSCs (PLSC^{Exo}) against prostate cancer (PCa) cells.

2. Materials and methods

2.1 Cells and reagents

PLSCs were generously donated by Dr. Anthony Atala, Wake Forest Institute of Regenerative Medicine (WFIRM). PLSCs were isolated from the chorionic tissue of the placenta via enzyme digestion [10]. Isolated placenta-derived cells were subsequently plated

in Alpha-MEM supplemented with AmnioMax media and Glutamax to obtain PLSCs. The cells were allowed to proliferate *in vitro* and were maintained in culture for 2–3 weeks. Cells were passaged with TrypLE when the cultures were 70% confluent until sufficient cells were obtained. C-kit antibody (CD117 PE cat# 130-099-672) from Miltenyi Biotec (Bergisch Gladbach, Germany) was used to label the cells, which were subsequently selected by BD FACS ARIA and maintained in culture until expanded. Cells were maintained in serum free media MSC NutriStem® XF Medium from Biologic Industries USA (Cromwell, CT) [10]. Human PCa enzalutamide-sensitive C4-2B-S cells and enzalutamide-resistant C4-2B-R cells (C4-2B-R) were obtained from Dr. Devasis Chatterjee lab at Brown University, Rhode Island. Human PCa enzalutamide-sensitive CWR-R1-S and enzalutamide-resistant CWR-R1-R cells as well as enzalutamide-sensitive LNCaP-S and enzalutamide-resistant LNCaP-R cells were obtained from the Vander Griend lab, at the University of Chicago [11]. PCa cell lines were grown in RPMI1640 medium supplemented with 10% FBS and 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate. Resistant PCa cell lines were cultured in 20 µM enzalutamide, dissolved in DMSO. PREC cells, non-neoplastic prostate epithelial cells, were cultured in PrEBM media (CC-4177, Lonza). All cell lines were cultured at 37°C in a 5% CO₂ humidified environment as adherent monolayer. Enzalutamide was purchased from Selleck Chemicals (Houston, TX), and stored at –80°C in DMSO. All other reagents were obtained in their commercially available highest purity grade.

2.2. Exosome isolation

PLSCs were cultured in complete media; thereafter, cells were trypsinized and replated in fresh basal media using MSC Attachment Media from Biologic Industries USA (Cromwell, CT). Cells were then cultured for 48 h. Subsequently, conditioned media was harvested and exosomes were isolated by serial centrifugation [12]. The collected cell culture media was centrifuged at 500 g at 4°C for 5 min to remove detached cells, then at 2,000 g for 5 min to remove cellular debris. The supernatant was collected and filtered through 0.22 µm filters to remove the apoptotic bodies and microvesicles. The filtrate was concentrated using Pierce concentrators (150K MWCO/20 ml) by centrifuging at 3,000 g for 15 min. The supernatants were then centrifuged at 100,000 g for 90 min (L-80 Ultracentrifuge, 70.1 Ti fixed angle rotor, Beckman Coulter, Indianapolis, IN). Finally, the pelleted exosomes (here onward mentioned as PLSC^{Exo}) were re-suspended in DPBS and stored at 4°C until further use.

2.3. 1D SDS-PAGE and mass spectrometry analysis of PLSC^{Exo}

Exosomes were isolated from PLSCs conditioned media by Exoquick™ reagent (System Bioscience, (Palo Alto, CA) following Vendor's instructions. Exosomes were lysed with RIPA lysis buffer and Halt Protease/Phosphatase inhibitor (ThermoFisher Scientific, Rockford, IL). Next, exosomal lysate (50 µg) was electrophoresed on a 10% 1D SDS-PAGE and the gel was stained with Coomassie brilliant blue and cut into 5 slices. Gel slices were digested using a protocol modified from Lee et al [13]. Proteins were processed by LC/MS/MS at Proteomics and Metabolomics Shared Resource at Wake Forest Baptist Medical Center. The annotated human protein database from UniProtKB (20,161 entries, date; 10-1-2014) was used within Proteome Discoverer analytical software (v1.4, Thermo Scientific, Rockford, IL, USA) applying the generic MASCOT search algorithm. There were at least 2 peptides per hit, and the protein subcellular localizations and functions were

determined from the Ingenuity Systems software (Redwood City, CA, USA; <http://www.ingenuity.com/index.html>). Pathway analysis and network constructions were assembled using the Ingenuity software.

2.4. Mass spectrometry analysis of lipids in PLSC^{Exo}

Lipids loaded in PLSC^{Exo} were characterized by the Mass spectrometry lipidomics core facility, University of Colorado Anschutz Medical Campus, Aurora, Colorado following published methods [14, 15].

2.5. Nanoparticle tracking analysis (NTA)

Quantification of the hydrodynamic diameter distribution and concentration of PLSC^{Exo} was performed using the Nanosight NS500 (Malvern Instruments, UK) equipped with a violet laser (405 nm) and running software version NTA3.2. The instrument was primed using phosphate buffered saline, pH 7.4 (PBS) and the temperature was maintained at 25°C. Accurate nanoparticle tracking was verified using 50 nm and 200 nm polystyrene nanoparticle standards (Malvern Instruments) prior to examination of the samples. Purified exosome samples were diluted in an appropriate volume of PBS such that there were 20–60 particles per field of view. Five measurements (60 seconds each) were obtained for each sample. Data are represented as the average result of these five measurements.

2.6. Transmission electron microscopy

Exosomes were suspended in glutaraldehyde, and applied to 400 mesh copper grids (formvar/carbon coated, glow-discharged) for 5 min, followed by negative staining with 2% uranyl acetate for 2 min. Grids were briefly washed in DI water, allowed to dry and viewed using FEI Tecnai Transmission electron microscope equipped with Gatan Ultrascan digital high-resolution camera (Pleasanton, CA).

2.7. MTT assay

Cells were plated at a density of 2,000 cells/well in 96-well plate under standard culture condition. The following day, the media was changed to include either control, enzalutamide, PLSC^{Exo}, or combined treatments. After 72 h, fresh media containing 20 µl of MTT (5 mg/ml stock) was added, and incubated for another 4 h at 37°C in a CO₂ incubator. At the end, media was removed and 100 µl of DMSO was added to each well. Color intensity was measured with absorbance at 560 nm. Background absorbance was measured at 650 nm. For each assay, we performed 6–14 technical replications. We duplicated MTT assays for all cell lines.

2.8. Statistics

Statistical analysis was performed using GraphPad prism and presented as mean±SD. A two-tailed t-test was performed and a p value of 0.05 was accepted as statistically significant. Statistics used for IPA (Ingenuity Pathway Analysis) can be found at the website <http://www.ingenuity.com/index.html>.

3. Results

3.1. Characterization of PLSC^{Exo}

PLSC^{Exo} were isolated from the conditioned media of PLSCs by serial centrifugation and analyzed for particle size distribution by nanoparticle tracking analysis (NTA). PLSC^{Exo} size and concentration distribution is shown in Figure 1A and average size of the PLSC^{Exo} was 131.4 ± 0.9 nm. NTA data also revealed that the PLSC^{Exo} concentration was $5.23 \times 10^{10} \pm 1.99 \times 10^9$ per ml. When normalized for cell number, it was determined that PLSCs secreted $\sim 2 \times 10^3$ exosomes per cell. Next, we characterized the PLSC^{Exo} by transmission electron microscopy and a representative image with exosome is shown in Figure 1B. As shown in the image, PLSC^{Exo} has double layer membrane with approximately 40 nm size. Measurements of cell culture derived exosomes using NTA typically have a modal size of 90–160 nm, which is in agreement with our data [16]. Although this is larger than what we and others observed using electron microscopy, the dehydrated, negatively stained samples used for TEM shrink during preparation, causing a “cup shaped” morphology, and thus the size differences between NTA and TEM measurements of exosomes are expected [16].

We next characterized the cargo (proteins and lipids) loaded in PLSC^{Exo} by mass spectrometry. PLSC^{Exo} were loaded with 241 proteins and some of the common biomarkers for exosomes (such as tetraspanins and annexins) were presented in PLSC^{Exo} (data not shown). Further, we identified that proteins loaded in PLSC^{Exo} were mainly of extracellular (40.1%), cytoplasmic (30.8%), plasma membrane (16%) and nuclear (10.5%) origin (Figure 1C).

Lipid analysis of the PLSC^{Exo} showed that long chain free fatty acids (FFA) palmitic and stearic were the most abundant FFA in the exosomes (> 200-fold compared to the rest of the detectable FFA) (Figure 2A). We also examined phospholipid (PL) species and found that the most relatively abundant PL species in the PLSC^{Exo} contained stearic (18:0), oleic (18:1) and palmitic (16:0) FFA species (Figure 2B). The diacylglycerol species (DAG) were also examined, as they represent a hub of multiple lipid metabolism pathways [17]. Interestingly, the 34 carbon DAG species (likely representing 16:0 and 18:0 FFA- containing molecules) were the most abundant in the PLSC^{Exo} samples (Figure 2C). Thus, our lipid results suggest that isolated PLSC^{Exo} mainly contain 16C and 18C-length FFA that make up their membranes and neutral lipids species.

3.2. PLSC^{Exo} inhibits growth of prostate cancer cells

New anti-androgen treatments such as enzalutamide have shown benefits for castration resistant metastatic prostate cancer, but treatment is non-curative and resistance ultimately occurs [18]. Therefore, we studied the effect of PLSC^{Exo} treatment on the viability of various enzalutamide-sensitive and –resistant prostate cancer cells. As shown in Figure 3A–C, we found decreased cell viability, as measured by the MTT assay, when both enzalutamide-sensitive (C4-2B-S, CWR-R1-S, and LNCaP-S) and enzalutamide-resistant (C4-2B-R, CWR-R1-R, and LNCaP-R) cell lines were treated with 10 µg of PLSC^{Exo} for 72 h. Relative to control, we found a 44% ($p < 0.0001$) decrease in cell viability when C4-2B-S

cells were treated with PLSC^{Exo} (Figure 3A, left panel). Similarly, we found 43% ($p<0.0001$) decrease in cell viability when C4-2B-R cells were treated with PLSC^{Exo} (Figure 3A, right panel). Upon treatment with PLSC^{Exo}, the LNCaP-S cell line demonstrated a 44% ($p<0.0001$) decrease in cell viability relative to control, and LNCaP-R cell line showed a 33% ($p<0.0001$) decrease in cell viability (Figure 3B). CWR-R1-S and CWR-R1-R were relatively less responsive to the growth inhibitory effects of the PLSC^{Exo} with a decrease of 31% ($p<0.0001$) and 30% ($p<0.0001$) in cell viability, respectively (Figure 3C).

3.3. PLSC^{Exo} do not significantly affect the growth of non-tumorigenic cells

In order to determine if the effect observed in the above cell lines was cancer-specific, we treated a non-neoplastic cell line, PREC (a human prostate epithelial cell line), with PLSC^{Exo}. We found that PLSC^{Exo} treatment had no significant effect on the cell viability of PREC ($p=0.060$) (Figure 3D).

3.4. Signaling pathways targeted by PLSC^{Exo}

After demonstrating that PLSC^{Exo} selectively inhibit the growth of cancer cells, we explored the mechanism that led to such an inhibitory effect. We began by using the IPA software to group the exosomal proteins identified by mass spectroscopy into networks of associated functions and canonical pathways. The top 5 networks/associated functions for PLSC^{Exo} proteins were “Cellular movement, Hematological system Development and function, Immune cell trafficking”, “Cancer, Connective tissue disorders, Organismal injury and abnormalities”, “Cellular movement, Cell-to-cell signaling and interaction, Cardiovascular system development and function”, “Infectious diseases, Inflammatory disease, Organismal injury and abnormalities”, and “Cellular movement, Cellular function and maintenance, Cell-to-cell signaling and interaction”.

In Figure 4, we have presented the top 15 canonical pathways associated with proteins loaded in PLSC^{Exo}. The top canonical pathways were Liver X receptor/Retinoid X receptor (LXR/RXR) and Farnesoid X receptor/Retinoid X receptor (FXR/RXR) activation.

4. Discussion

Results from present study are important as for the first time we characterize the exosomes secreted by placental stem cells in terms of their concentration, size distribution as well as proteins and lipids. Our results show the remarkable efficacy of PLSC^{Exo} against drug-sensitive and drug-resistant prostate cancer cells.

Targeting the retinoic acid pathway is known to be critically important within tumor biology. LXR and RXR are groups of nuclear receptors known to suppress tumor cell proliferation, tumorigenesis and metastasis [19, 20]. Furthermore, retinoids are known to suppress carcinogenesis in animal models for bladder, ovarian, and prostate cancer to name a few [19, 21–23]. Furthermore, RAR γ has been shown to regulate PTEN, a tumor suppressor that plays a critical role in the growth of human cancer cells. However, LNCaP and C4-2B cells have mutated/null PTEN, so this pathway might not be involved in the PLSC^{Exo} – mediated growth inhibition in these cells.

One of the noticeable observations in preset study was abundance of 16:0 and 18:0 fatty acids, both of which are saturated long-chain fatty acid chains (SLCFA), in PLSC^{Exo}. These specific fatty acids regulate retinoic acid signaling and suppress tumorigenesis [24]. One study found that these fatty acids, in particular, target fatty acid-binding protein 5 [24]. SLCFA displace retinoic acid from FABP5 and diverts it to retinoic acid receptor (RAR), consequently leading to its activation. Further, SLCFA inhibited the activation of PPAR β/δ , a protein known to have a pro-tumorigenic effect. Unsaturated LCFA (ULCFA), on the other hand activates both RAR and PPAR β/δ leading to carcinoma cell growth. These results suggest that changes in cellular lipid content could affect cell growth. The abundance of SLCFA, and relative absence of ULCFA, explains why the PLSC^{Exo} treatment result in inhibition of cancer cells growth rather than the reverse.

Another possible interpretation of our results is that the increased SLCFA delivery to the cells via the PLSC^{Exo} could results in a significant blockade of β -oxidation via the RA-PPAR signaling pathway [25], leading to accumulation of pro-apoptotic ceramides rich 16:0 and 18:0 species [25, 26]. Thus, it is possible that the excess SLCFA delivered by the PLSC^{Exo} to the recipient cancer cells is unable to be oxidized in the mitochondria and shunted towards synthesis of ROS or pro-apoptotic ceramides. Non-cancer cells, with less dependence on β -oxidation for survival, are likely able to deal with the excess of SLCFA by shunting it into triglycerides or lipid bodies. These mechanisms need to be explored further.

In this study, we also demonstrated the selective nature of PLSC^{Exo}, with no significant growth inhibition observed in normal prostate cell line. This could be the result of differences in the intrinsic ability of these exosomes to actually enter the normal and cancerous cells, respectively. In one study, researchers demonstrated extracellular reactive oxygen species penetrating model systems of the plasma membrane of cancerous cells, but not the plasma membrane of normal cells [27]. It is plausible that PLSC^{Exo}, loaded with SLCFA, could relatively easily penetrate the cancer cell membranes, leading to upregulation of RAR γ and the resultant cell growth inhibition.

Exosomes represent a novel therapeutic potential for oncologic disease. Because they are composed of a lipid bilayer, they can more efficiently be delivered into target cells. Furthermore, there is evidence to suggest that stem cells, when injected *in vivo* can track to the tumor site [28]. The present study has clearly demonstrated the selectivity of PLSC^{Exo} in targeting the growth of prostate cancer lines. It will be fascinating to examine whether these PLSC^{Exo} possess not only this selectivity in an *in vivo* model, but whether they will home to the tumor site upon injection. In future, molecular mechanism/s of PLSC^{Exo} action as well as their efficacy and biodistribution in *in vivo* tumor models will be studied further.

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Abbreviations

FXR	Farnesoid X receptor
IPA	Ingenuity pathway analysis
LC	Liquid chromatography
LXR	Liver X receptor
MS	Mass spectrometry
MSCs	Mesenchymal stem cells
NTA	Nanoparticle tracking analysis
PLSC^{Exo}	Placental-derived stem cell exosomes
RXR	Retinoid X receptor

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Highlights

1. Detailed characterization of exosomes secreted by human placental stem cells
2. Identified anti-cancer efficacy of exosomes secreted by human placental stem cells against aggressive prostate cancer cells

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Figure 1. Characterization of exosomes secreted by PLSCs

(A) PLSC^{Exo} were analyzed for particle size distribution and concentration by NTA. Representative particle size distribution for exosomes is presented. (B) PLSC^{Exo} were analyzed by transmission electron microscopy, and representative image is shown (magnification 98,000x). (C) Proteins loaded in PLSC^{Exo} were identified through mass spectroscopy. Subsequently, protein data was analyzed using IPA software, and proteins subcellular (or extracellular) localization is presented in pie diagram.

Figure 2. Characterization of lipids loaded in PLSC^{Exo}

PLSC^{Exo} were analyzed for fatty acids (FFA), phospholipid (PL) and diacylglycerol (DAG) by mass spectrometry. (A) Total amount of FFA in the exosomes normalized to protein content. (B) PL analysis of exosomes showing the most abundant species for each class. PS: phosphatidylserine, PI: phosphatidylinositol, PE: phosphatidylethanolamine, PC: phosphatidylcholine, PA: phosphatidic acid. (C) Major DAG species detected in PLSC^{Exo}. The first number represents the total number of carbons between the 2 fatty acid chains (likely 16C + 18C) and the second number indicates the total number of double bonds.

Figure 3. Treatment with PLSC^{Exo} reduces the cell viability of PCa cells but not in non-tumorigenic cells

(A–C) Enzalutamide-sensitive (S) and –resistant (R) PCa cells (C4-2B, LNCaP and CWR-R1) and (D) PREC cells were seeded at a density of 2×10^3 cells/well in 96-well plates. After 24 h of seeding, cells were treated with 10 μ g of PLSC^{Exo}. At the end of 72 h, MTT assay was performed to determine cell viability and presented as mean $\pm$ SD relative to control. ‡p < 0.0001

Figure 4. Signaling pathways targeted by PLSC^{Exo}

Proteins loaded in PLSC^{Exo} were analyzed using Ingenuity IPA software and top 15 canonical pathways are shown. Scores (total number of proteins in the sample versus total number of known proteins of that pathway) are plotted as $-\log$ value, which is derived from a p-value and indicates the likelihood of the Focus Proteins in a network being found together due to random chance. Threshold value was set at 1.25.